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Research Article
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MORPHOMETRIC CHARACTERIZATION OF AFRICAN BONYTONGUE (*Heterotis niloticus* Cuvier, 1829) FOUND IN THE LOWER RIVER NIGER OF NIGERIA

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Abstract: This study was conducted to characterize the African bonytongue (*Heterotis niloticus*) found in the lower River Niger of Nigeria at the following locations; Onitsha (6.1329° N, 6.7924°E), Illah (6.4219° N, 6.6491°E) and Ilushi (6.6688° N, 6.6304°E) with the use of morphometric (measured distance) and random amplified polymorphic DNA (RAPD). A total of one hundred and fifty (150) samples were used for the study. Morphometric distances of body parts were subjected to the Principal Component Analysis on Unscramble@ X version 10.4 software. DNA samples were analyzed using muscular portion of the caudal region. Genomic molecular analysis was carried out for the RAPD band pattern. Denodogram was constructed using clustering analysis (UPGMA). PCR was confirmed using gel electrophoreses. Result shows that the Anal fin length at Illah point is statistically significant to Illushi but different from Onitsha, with F value of 3.326 and the P value of 0.039 and standard deviation of 0.1479, snout length of fish sampled at Onitsha is significantly different from those sampled in Illah and Illushi, having F value of 3.837 and P value of 0.024 and standard deviation of 0.0125, Dorsal fin length sampled in Illushi is statistically significant to Illah but different from Onitsha having F value of 4.164, P value 0.017 with standard deviation of 0.0145. A total of 46% of original grouped cases were correctly classified, while 45.3% of cross-validated grouped cases were also classified with overall diversity of 0.67 to 1.00. This gives respective similarity coefficients of 0.45, 0.37 and 0.31.

Keywords: Morphometric, Characterization, Electrophoreses, Molecular, Clustering

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1. Introduction

The African bonytongue (*Heterotis niloticus*) are primarily freshwater fish, they are commonly found in tropical rivers and freshwater lakes in western and central Africa (Leveque et al., 1990). They are characterized by certain distinct feature such as short head with terminal mouth and thick lips, large sensory pith, lateral line extending from operculum to the middle of the caudal peduncle, spineless dorsal and anal fins, a small rounded tail which is relative to body size, lastly the fish possesses scales of a cycloid appearance on the body except the head (Reed et al., 1967; Idodo–Umeh, 2003). The fish can be cultured commercially and readily accepts compounded feeds coupled with its exceptional growth it is of economic interest in inland fisheries (Moreau, 1982; Adite et al., 2006; Odo et al., 2009; Ezekiel and Abowei, 2013). Investigations have been carried out on H. *niloticus* in River Niger at the Pategi area of Kwara State by Adekeye (1989) with the view of establishing its potential in commercial aquaculture as well as providing a basis for its take off.

Studies done by Hockaday et al. (2000) and Nwachi and Irabor (2022) infer that morphometric methods are simple and direct method of assigning species to their population and also could be used for actual species identification. The measuring of body distances morphometric remain a significant method that is used in species differentiation. Hassanien et al. (2011) is of the opinion that morphometric traits are an important trait that can have invariable affects in reproduction, similarly estimate of morphology and genetic variability is essential in other to predict response to selection, during breeding plans and predict breeding values. It is of note that external morphology (body shape) might also serve as economic importance and marketing point of view. Although the extent placed on the use of morphometric traits to determine both the genetic and environmental variation remain poorly understood.

Schwanck and Rana (1996), is of the opinion that mode of inheritance or morphology will contribute positively to the express traits similarly, Gunawickrama (2007), Hassanien et al. (2011), Edema and Osagiede (2011), Kuton and Adeniyi (2014), infer that morphometric could be used in proper identification. However, fish taxonomists used morphology to classify the various types of fish into their respective genre, families and

species, i.e. they made use of more or less morphological features and characteristics of fishes as tools to identify fishes for their systematic identification, hence such information with respect to fish taxonomic identifications were accumulated. In a similar manner molecular analysis with the aid of researched or optimized primer could also serve as an important tool in understanding traits of strains. Normala et al. (2021) reported the use of Random Amplified Polymorphic DNA Technique (RAPD) in evaluating the traits that could be used in separating triploid from diploid African catfish (*Clarias gariepinus*). Bakar et al. (2018), opined on the use of molecular method in managing wild fish especially those with little or no morphological variations due to technological advancements in the field of genetics, the use of DNA based markers have increasingly being used for identification and unrevealing previously unknown genetic variations in fish species (Isabel et al., 1999). It has been advocated that the RAPD-PCR technique is a simple and straightforward method where the work is based on the amplification of discrete regions of genome by using arbitrary primers to evaluate genetic variability and structure of a variety of species Almeida et al. (2001), Leuzzi et al. (2004) and Matoso et al. (2004). Molecular markers could also be applied for use in identifying different types of populations of fish such as species, hybrid identification, phylogeny and many others (Almeida and Sodre, 2002). Other workers have also used Random Amplified Polymorphic DNA (RAPD) technique for their investigations such as Welsh and McClelland (1990), Hassanien et al. (2004) and Toth et al. (2005). African bonytongue (*Heterotis niloticus*) is an economic fish found in River Niger, recent observation shows an increasing interest for domestication and mass production of fish in controlled environment and fishes from the wild is the major source of most genetic improvement program. To domesticate this fish proper identification is required using both the morphometric and molecular analysis.

2. Materials and Methods

This study was carried out at the teaching and research laboratory of Delta State University Abraka and African Bioscience Laboratory Ibadan Oyo state Nigeria. The fish species used for this research where obtained from three different locations along the river Niger; the stations were marked as A (Onitsha) 6.1329° N, 6.7924° E, B (Illah) 6.4219° N, 6.6491° E and C (Ilushi) 6.6688° N, 6.6304° E as shown on the map in Figures 1 and 2. River Niger is regarded as the principal river of West Africa extending about 4,180 km (2,600 mi).

Figure 1. Map of Nigeria.

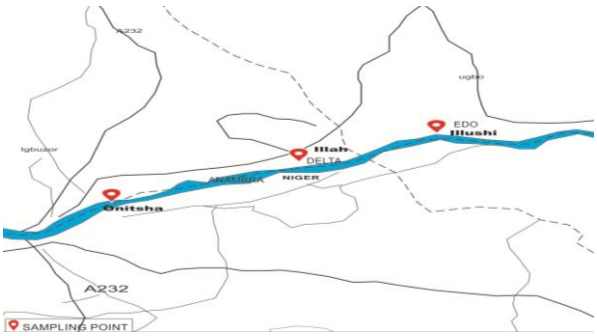

Figure 2. Sampling stations of Onitsha, Illushi and Illah.

Its drainage basin is 2,117,700 km² (817,600 sq mi) in area. The river Niger originates from Guinea highlands in the southeastern Guinea. It runs in a crescent through Mali, Niger, on the border with Benin and then through Nigeria, discharging through a massive delta, known as the Niger Delta into the Gulf of Guinea in the Atlantic Ocean (Gleick, 2000).

The water flowing in the river is relatively clear with little silt compared to the river Nile. The river Niger just like the Nile River floods yearly; this begins at September, peaks in November, and finishes by May. During the period of flood, the water is not as clear due to debris washed into the river by runoff water from rains and drainages. The gradient of the river decreases drastically and forms the Inner Niger Delta, the decrease gradient causes the flow of the river to slow down resulting in numerous marches, lakes, and streams. In this area about two thirds of the rivers potential flow is lost, leaking into lakes and marches. When the region floods, during the rainy season, excellent conditions for fishing and farming are created.

The River Niger is important in the livelihood of locals such as Igbuzo, Okpanam, Oko, Okwe, Ugbolu, Illah Imbaka, Odegune, and Umudora Anam. Aside domestic importance which include fishing, irrigation, transportation and agriculture, it is also used for hydropower generation in the Kainji and Jebba dams.

Samples were collected at stations A, B and C as shown on the map, Figure 1 and 2. A total of 25 samples were collected from each station on sampling days totaling 75 samples. Accurate morphometric measurements were done using buss truss protocols as presented in Table 1.

Table 1. Measured distances (Morphometric distances)

Parameters	Distance
Total length (TL)	Distance between the tip of the snout (upper jaw) to the tip of the tail.
Standard length (SL)	Distance between the tip of the snout (upper jaw) to the tip of the anal fin.
Pelvic fin length (PL)	Measured distance from the base to the tip of the pelvic fin.
Head length (HL)	Distance between the tip of the snout to the upper operculum.
Body distance (BD)	Distance between the base of the dorsal fin to the pectoral fin.
Snout length (SL)	Distance between the tip of the upper jaw to the base of the eyes
Pre-dorsal fin length (Pre-DL)	Distance between the tip of the snout to the origin of the dorsal fin.
Dorsal fin length (DL)	Distance between the base of the first dorsal to the last dorsal fin.
Anal spine length (ASL)	Distance from the top of the anal fin to the last of the anal fin ray.
Pectoral fin length (PFL)	Distance from the tip of the pectoral to the base caudal pundicle
Pre-pectoral length (Pre-PL)	Distance from the front of the pectoral base to the snout.

2.1. Total DNA Extraction

Extracting DNA from the fish samples was done by dissecting part of the strong muscular portion of the caudal region, at an earlier conservation workstation to maintain a strategic distance from cross contamination (Nugroho, 2011). Each sample was tagged for easy identification and also to avoid mix up during the DNA extraction process. Preservation of the samples was done using 95% ethanol and stored in temperatures below -20 degree freezer. The genomic DNA of fish tissues was isolated using CTAB method (Stewart and Laura, 1993). Genomic DNA was extracted utilizing (gSYNC) extraction kit. Each sample was dissected into smaller pieces of weight 0.25 mg using a scalpel and placed into a 1.5 mg micro centrifuge tubes which already had corresponding tags of the samples. The samples were then individually removed from the microcentrifuge into mortars, 200 µl of GST Buffer was added to each sample in the mortar and macerated evenly. 20 µl of proteinase K was added to the macerated samples and then returned to their respective microcentrifuge. Vortex for 10 seconds to allow proper mixing to yield a homologous solution and then incubated at 60 ^{o}C for 1 hour. Insoluble material which remained after incubation was centrifuged for 2 min at 15000xg. Carefully the supernatant was transferred to a new 1.5 ml microcentrifuge tube 200 µl of GST Buffer was added and vortexed for 10 sec. Here 200 µl of absolute ethanol was added to the sample and vortexed immediately for 10 sec. Placing a GS column in a collecting tube and transfer all the mixture to the GS column, centrifuge for 1mimute then discard the 2 ml collecting tube containing the flow through. Transfer the GS column to a new 2 ml collecting tube. A total of 400 µl of WI buffer was added to the GS column and centrifuge for 30 sec at 15000xg. The flow through was discarded and placing the GS column back in the 2ml collecting tube, wash buffer was added to the GS column and centrifuged again for 30 sec at 15000xg. The process was repeated by placing the GS column back in the 2 ml collecting tube and again centrifuging for 3 min at 15000xg to dry the column matrix. The GS column was then transferred to a dry 1.5 ml microcentrifuge tube, 100µl of pre heated elution buffer was added into the center of the column matrix and left to stand for 20 min. Centrifuge was done at 15000xg for 30 sec to elude purified DNA. The ReliaPrepTM ^{Tm} Binding column was disposed while the genomic DNA was stored in -20 ^{o}C for further analysis. The resulting sample was diluted to 100 ng while the resulting genomic DNA was stored in temperatures below -20 ^{0}C until further analysis. Test Genomic DNA using a nanodrop to ascertain DNA concentration and viewed in gel electrophoresis under ultraviolet light using Gel doc XR system Pc and Mac form USA to ascertained DNA quality.

2.2. Screening of Primers and PCR Amplification

A total of 15 arbitrary primers of OPA series (Operon Technologies Ltd. USA) with random sequence was used to screen using the following protocol of Azrita et al. (2014) and Kusmini et al. (2011). The PCR amplication was screened using Veriti 96 thermal cycler at the laboratory of African Bioscience Ibadan. At a total reaction volume of 25 µI containing 50 ng genomic DNA, 10X PCR buffer (10 mM Tris-HCL, pH 9.0, 50 mM KCL and 0.01% gelatin), 2.5 mM of each dNTP, 5 pmol; of primer followed by 25 cycles at 94 ^{0}C, for 1 min, 40 ^{0}C for 1min and 72 ^{0}C for 2min with a final extension at 72 ^{0}C for 10 min.

2.3. Agarose Gel Electrophoresis and Visualization of Bands

Preparation of the agarose gel will be carried out by mixing 1 xTBE buffer at a ratio of 1.1 (1%). A total of 5µl of µl of GelRed™ (Nucleic Acid Gel Stain). The harden gel will be placed in such a way that wells will be created. The PCR products will be load inside the wells. The molecular weight of each band will be estimated using a standard marker. The PCR products will be viewed Gel doc XR system Pc and Mac form USA.

2.4. Statistical Analysis

Body part values was analyzed using multivariate, specifically Principal Component Analysis (PCA). PCA was chosen due to its ability to handle high-dimensional data, quantify differences among observed components, and reveal innate relationships between data points. The Unscramble@ X version 10.4 software aided in this process. To eliminate variation resulting from allometric growth, the researchers standardized and normalized the

morphology data. Next, molecular analysis was conducted using RAPD (Random Amplified Polymorphic DNA) band patterns from sample test fish across various stations. The most prominent primer was used for molecular characterization of the *H. niloticus* population in the sample station. The RAPD band patterns were visualized and scored from photographs. Only distinct and well-separated bands were selected for comparative analysis. Gene assignment was determined by scoring "1" for presence and "0" for absence, excluding weak and unresolved bands. Finally, a dendrogram was constructed using genetic distance values through clustering analysis (UPGMA) to reveal the relationship between different *H. niloticus* populations.

3. Results

The Anal fin length of *H. niloticus* that was sampled in rable 2 at Illah point is statistically significant to Illushi but different from Onitsha, which has the F value of 3.326 and the P value of 0.039 and standard deviation of 0.1479902, similarly the snout length of fish sampled at Onitsha is significantly different from those sampled in Illah and Illushi, although it has F value of 3.837 and P value of 0.024 and standard deviation of 0.0125637, however dorsal fin length sampled in Illushi is statistically significant to Illah but different from Onitsha and the F value is 4.164, the P value is 0.017 with standard deviation of 0.0145544 the anal fin length of Illushi and Illah is statistically significant but different from Onitsha, which has the F value of 4.719 and P value of 0.010. Illushi and Illah are also statistically significant in terms of pectoral fin but different from Onitsha with the F value of 3.099, P value of 0.48 and standard deviation of 0.0122627.

Table 2. Morphometric characteristics of the Predictors

Parameters	Onitsha (NI)	Illah (LA)	Illushi (IL)	Std. deviation	Std. error	F value	P value
Total length	1.4559	1.4700	1.4775	0.0425	0.0035	1.650	0.195
Pelvic length	0.0303	0.0873	0.0255	0.2481	0.0201	0.967	0.383
Head length	0.6725	0.5993	0.6725	0.3427	0.0278	0.904	0.407
Body length	0.7520	0.7573	0.7684	0.1869	0.0152	0.102	0.903
Snout length	0.1652	0.1511	0.1698	0.2056	0.0167	0.112	0.894
Dorsal fin length	0.0645[b]	0.1640[a]	0.0853[b]	0.1579	0.0128	5.904	0.003
Anal fin length	0.0137[b]	0.0476[a]	0.0084[a]	0.2590	0.0210	0.337	0.714
Pectoral fin length	0.3995	0.3755	0.3114	0.1941	0.0157	2.889	0.059
Caudal fin length	0.2862	0.2822	0.2736	0.1408	0.0114	0.106	0.899
Lower jaw length	1.1791[a,b]	0.2015[a]	0.0421[b]	0.2803	0.0227	4.365	0.014
Standard length	1.4191[a,b]	1.4360[a]	1.4129[b]	0.0448	0.0036	3.746	0.026
Pre-dorsal length	1.2309	1.2286	1.2208	0.0738	0.0060	0.262	0.770
Pre-anal length	1.1399	1.1928	1.1775	0.1328	0.0108	2.136	0.122
Pre--pectoral fin length	0.3521[a,b]	0.3706[a]	0.2660[b]	0.1966	0.0159	4.318	0.015
Pre-dorsal fin length	0.7043	0.7288	0.7405	0.1231	0.0010	1.140	0.323

Means without superscript on the same row indicates no statistical difference by the Duncan test P≤0.05 for the water quality parameter.

The pre-caudal fin length of the samples at Illah and Illushi point is statistically significant but different from Onitsha which has the F value of 3.230 and P value of 0.042, similarly the lower jaw length at Onitsha is significantly different from Illushi and Illah although it has F value of 3.730 and P value of 0.026 and standard deviation of 0.0192956, however, pre-dorsal fin length sample at Illushi is statistically significant to Illah but different from Onitsha with F value of 3.843, P value of 0.024 and standard deviation of 0.017662.

Table 3. Structure matrix

Parameters	Functions	
	1	2
Dorsal fin length	0.721*	0.693
Head length	0.222*	0.187
Pectoral length	0.255	0.967*
Standard length	0.284	0.710*
Total length	0.309	0.704*
Caudal fin length	0.220	0.671*
Body length	0.024	0.208*
Pelvic fin length	0.327	0.493*
Snout length	0.320	0.480*
Anal fin length	0.131	0.473*
Lower jaw length	0.106	0.453*
Pre-dorsal fin length	0.226	0.379*
Pre-anal fin length	0.123	0.338*

In Table 3 the structure matrix indicates the loadings at two functions; dorsal fin length loaded at 0.721 and 0.693, the head length load at 0.222 and 0.187 respectively. The caudal fin length is at 0.220 and 0.671 while the snout length loads at 0.320 and 0.493. In the function evaluated the pre-dorsal fin length was at 0.226 and 0.379, total length at 0.309 and 0.704 although the pre-anal fin length is at 0.226 and 0.379. The standard length recorded a loading of 0.284 and 0.710. A total of 46% of original grouped cases were correctly classified, the cross-validation is done only for those cases in the analysis, in cross-validation each case is classified by the functions derived from all cases other than that case. A total of 45.3% of cross-validated grouped cases were correctly classified in Table 4.

Table 4. Predicted group membership

	Site	SHA	LAH	ILU	TOTAL
Original count	SHA	21	17	12	50
	LAH	16	23	11	50
	ILU	8	17	25	50
%	SHA	42	34	24	100
	LAH	32	46	22	100
	ILU	16	34	50	100
Cross-validated count	SHA	21	46	12	50
	LAH	17	34	11	50
	ILU	8	17	25	50
%	SHA	42	34	24	100
	LAH	34	44	22	100
	ILU	16	34	50	100

Function analyses of predictors from the sampling station of Illah and Illushi, for the Eigen values and the Wilk's Lamda at Table 5, that was carried out to evaluate the origin of the sampled population showed no significant difference. In Table 6 while the Wilk's Lamba diversity within Illah and Illushi was at 0.27303 with significance value of 0,009 at 0.94 degree of variation. The differential function between Onitsha and Illah infer that the Eigenvalues and Wilk's Lamba diversity gives a value of 29958, while on analysis between Onitsha and Illushi a value of .35622 with significance value of .107 at 0.94 degree of variation in Table 7. The Correlations of predictors loading indicate that head length load negatively (-077) likewise body length (-022) on the plot and could not be used for proper identification of strain. However, anal fin length, Pectoral fin length and lower jaw length has a positive loading of 034, 027 and 032 respectively on Figure 3. It is of note that in Figure 4 the result of the *H. niloticus* shows overall diversity of 0.67 to 1.00. This gives respective similarity coefficients of 0.45, 0.37 and 0.31. These values were the calculated Nei's genetic similarity matric of the data scoring for the electrophoresis gel.

Table 5. Eigenvalues and canonical correlations for Illah and Illushi

Root	Eigenvalues	Canon. cor.	% of variance
1	1.40784	0.76465	58%
2	0.31352	0.48856	24%

Table 6. Eigenvalues and canonical correlations for Onitsha and Illah

Root	Eigenvalues	Canon. cor.	% of variance
1	1.56352	0.78097	60%
2	0.15303	0.36431	13%

Table 7. Eigenvalues and canonical correlations for Onitsha and illushi

Root	Eigenvalues	Canon. cor.	% variation
1	0.89476	0.68719	47%
2	0.24438	0.44316	19%

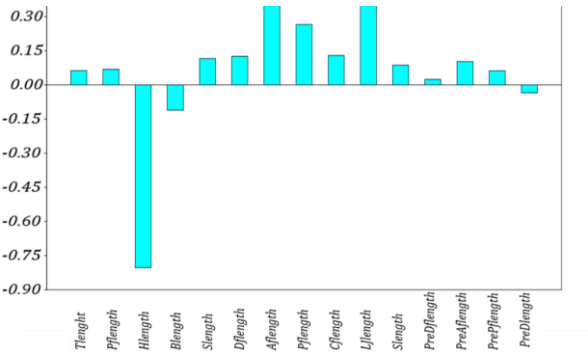

Figure 3. Correlations of predictors loading plot. Tlenght= total length; Pflength= pectoral fin length; Hlength= head length; Blength= body length; Slength= snout length; Dflength= dosal fin length; Aflength= anal fin length; Pflength= pectoral fin length; Cflength= caudal fin length; LJlength= lower jaw length; Slength= standard length; PreDflength= pre-dorsal length; PreAflength= pre-Anal fin length; PrePflength= pre-Pectoral fin length; PreDlength= pre-Dorsal fin length.

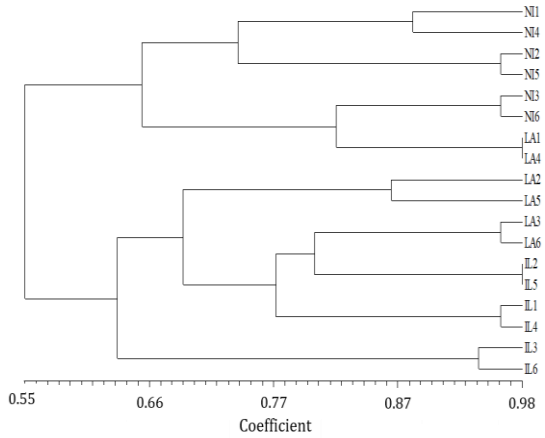

Figure 4. Dendogram for fish samples. NI1-NI3– *H. niloticus* in Onitsha; LA1-LA3 – *H. niloticus* in Illah; IL1 – IL3 – *H. niloticus* in Illushi.

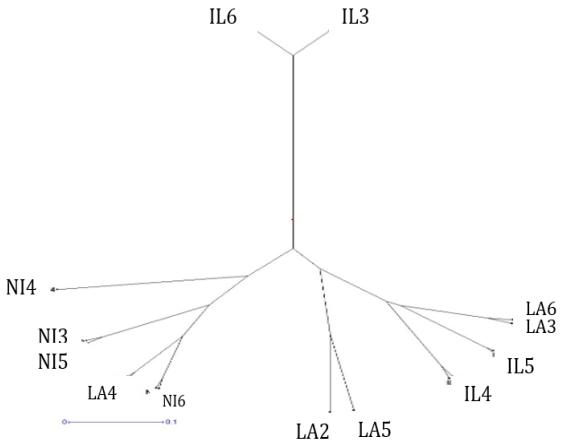

Figure 5. Darwin's hierarchy of the RAPD analysis of sampling station.

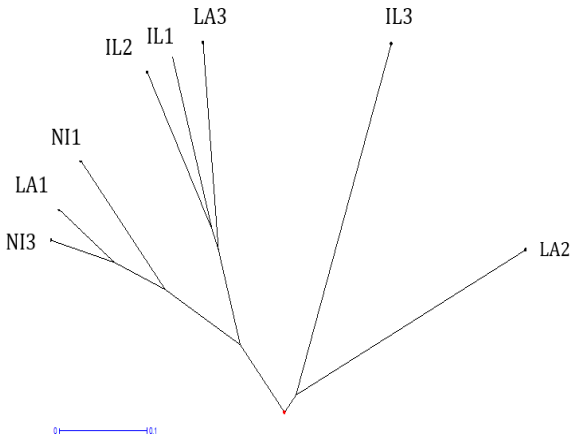

Figure 6. Darwin's radii graph of the sampling stations.

The relationship between the test fish as shown in Figures 5 and 6 Darwin's hierarchy and radii graph infer that all the fish sampled could come from the same family as shown in the graph.

4. Discussion

Differences have been recorded between species and strains of fish because of their ability to respond to environmental changes. Water quality parameters such as temperature and available food could affect both the growth and over all well-being of fish. Morphology is useful in distinguishing differences among fishes of same strain but obtained from different sampling stations. In this study discriminant analysis was used to evaluate differences that could be used in assigning the correct strain to the sample population of *H. niloticus* that is sampled from Onitsha, Illah and Ilushin point of the River Niger.

Observation from studies indicated that *H. niloticus* found in the three locations of (Onitsha, Illah and Illushi) show higher levels of similarities, but samples at Illushi and Illah exhibited the most similarities across the strains. However, in the analyses done to know if the samples were sufficiently different, the Eigenvalues and Wilk's Lambda shows no significant differences at 0.95. This indicated that the genus may be descended from a single ancestor. On further examination of the morphological trait of *H. niloticus* in Table 4 there is variation on the values from measured distances of; head length, snout length, dorsal fin length, anal fin length, pectoral fin length, pre-caudal fin length, lower jaw length and pre-dorsal fin length among fish sampled at Illushi and Illah.

They were also statistically different to samples from Onitsha despite the fact that they were from the same strain. This could be attribu Table to the fact that some of them had the same parent stock, which demonstrates why they are so similar. Senay et al. (2017), evaluated the morphological differentiation in northern pike and found out that elongated heads, deeper bodies and caudal peduncles and dorsal fin could be used for proper identification of same strain from the sampling stations. Hassanien et al. (2011), Pathak et al. (2013), reported that measured distances (morphometric) could be used in actual differentiation of strain. Similarly, total length, carapace length, standard length and abdominal lengths were used by Amin et al. (2010), to evaluate variation among the species of genus acetes.

In the study variation was recorded from the measured distances of the sampled stations, some of the measured distances seem similar to each other while element of dissimilarities also takes place between the sampled stations. Statistical significant were recorded among the sampled stations with variable p value. Wilk's Lamba analysis infers that all the test fish were from same strain although variation was recorded in the sampling stations. In Figure 3 the Correlations loading of predictors in the study could be used to explain the fact that the anal fin length, pectoral fin length and lower jaw length could be used to place the strain to their base population indicating that one can actually make positive identification of strain based on their location by examining these predictors. In general, structure matrix in Table 3 showed the loadings of predictors in two

functions, the dorsal fin length; pectoral length anal fin length and lower jaw length load positively in either function which is an indication the sampled fish could be link to their sampled station with the aid of these predictors. In the study although a total of 46% of the sampled strains were correctly assigned to their population with the aid of the predictors, the cross-validation gives 45.3% indicating that predictors could actually serve as a mean of identification.

Standard characterization and evaluation could be carried out by using different methods including traditional practices for more information (Barletta et al. 2015), hence molecular analysis was carried out for the study with the aid of Random Polymorphic Primers (RAPD) Characterization' is the description of a character or quality of an individual it could also be referred to as the distinguishing factor. In genetic terms, characterization is referred as the detection of variation that could result from differences in modification (Novroski et al., 2015). A total of 15 arbitrary primers of OPA series (Operon Technology LtD USA) was used in screening sampled fish from the landing points of Onitsha, Illah and Ilushi. The hierarchical clustering of the sampled fish gives a diversity of 0.67 to 1.00, although similarity coefficients of 0.45, 0.37 and 0.31for Onitsha, Illah and Ilushi were observed

In Onitsha, Illah, and Illushi, genetic diversity among species was minimal, with matrix index of 1. According to Sousa et al. (2020), genetic variability in a population is important for biodiversity because without variability if a population's ability to adapt to environmental changes becomes more complicated, it becomes more vulnerable to extinction. Species in Onitsha shows more variability difference from species in Illah and Illushi. The distribution of species or populations and their genetic structure depends not only on biological and environmental but also on historical factors (Agnèse et al., 1997), these climatic variations could explain the genetic structure of some fish populations in West Africa (Giddelo et al., 2002).

The study discovered a low degree of genetic diversity at the sampled station, analysis among the species found along the Niger River in Onitsha, Illah, and Illushi. In Figures 5 and 6 Darwin's hierarchy indicate the relationship between the sampled station and the strain, the relationship explained the relatedness of the strain if the stations were used as base line. Sousa et al. (2020), opined that genetic diversity in a population is critical for biodiversity because without it, a population's ability to adapt to environmental changes is compromised, making it more vulnerable to extinction. Morphometric and genetic analysis is an efficient and accurate method of evaluating fish populations.

5. Conclusion

The study evaluated the use of morphometric traits (measured distances) and Random Amplified Polymorphic DNA (RAPD) in characterizing African bonytongue fish that was sampled in three stations of Onitsha, Illah and Ilush along the lower region of River Niger. Anal fin length, pectoral fin length and lower jaw length were found to be traits that could be used in assigning the fish to their base population. The uniqueness of sampled fish based on gene analysis was also examined with the aid of polymerized chain reaction and the products evaluated with gel electrophoresis. Results infer that despite the similarities as indicated by Darwin's hierarchy each strain from the sampled location has its own unique traits.

Author Contributions

The percentages of the author' contributions are presented below. The author reviewed and approved the final version of the manuscript.

	O.F.N.
C	100
D	100
S	100
DCP	100
DAI	100
L	100
W	100
CR	100
SR	100
PM	100

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management.

Conflict of Interest

The author declared that there is no conflict of interest.

Ethical Consideration

The experimental procedures were approved by the local ethics committee of Faculty of Agriculture Delta State University (approval date: 07 May, 2020, protocol code: S179/037).

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Research Article
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EVALUATION OF ASSOCIATIVE EFFECTS OF *Gliricidia sepium* AND *Megathyrsus maximus* COMBINATIONS ON FIBRE FRACTIONS, *IN-VITRO* CARBON DIOXIDE AND METHANE PRODUCTION

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Abstract: The voluntary feed intake and digestibility of forages are contingent upon the quality of the pasture, which in turn has an impact on ruminant productivity. There is limited research on the proximate/chemical composition, in-vitro gas production, and degradability of mixtures containing *Megathyrsus maximus* (MM) and *Gliricidia sepium* (GS). The study investigated the impact of different combinations of MM and GS on in-vitro gas production, degradability, and chemical composition. *Megathyrsus maximus* (MM) and *Gliricidia sepium* (GS) were combined as follows: T_1- 100%MM + 0%GS, T_2- 75%MM + 25%GS, T_3- 50%MM + 50%GS, T_4- 25%MM + 75%GS, and T_5- 0%MM + 100%GS. The treatments were assayed using standard procedures. The proximate composition of the treatments was also determined using standard procedures. Data were analyzed using descriptive statistics and ANOVA at $\alpha_{0.05}$. Initial gas produced in T_1 and T_2 and net gas volume (NGV) observed in T_1 and T_2 were significantly lower compared to other treatments. In addition, the CO_2 gas produced in T_3, T_4, and T_5 was significantly higher than that produced in T_1 and T_2. However, T_1 had the lowest CO_2 gas production. The treatment with 100% legume (T_5) had the highest methane (CH_4) production, followed by T_4 and T_3. Treatments containing 100% (T_1) and 75% (T_2) grass had the lowest CH_4 production. In terms of degradability, it was observed that T_5 had significantly higher organic matter degradability (OMD) compared to other dietary treatments. The crude protein observed in T_5 was also significantly higher than other treatments. In conclusion, mixtures with a high content of soluble carbohydrates presented the lowest gas production. It was determined that a mixture of 75% *Megathyrsus maximus* and 25% *Gliricidia sepium* has increased carbohydrate, ash, lower moisture content, and in vitro gas production and can be utilized by ruminant farmers as a cheap and readily available source of nutrition for their animals.

Keywords: *In-vitro* gas production, Chemical composition, Organic matter degradability, Ruminant

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1. Introduction

Ruminant production in developing countries, including Nigeria, faces significant constraints due to the scarcity and fluctuating quality of year-round feed, particularly forages. This challenge is exacerbated by the tropical climate, where forage growth and quality closely mirror the seasonal distribution of rainfall, leading to acute feed shortages during the dry season (Preston, 1995; Babayemi and Bamikole, 2006). Compounding the issue is the high population density in Nigeria, which intensifies land-use competition between pasture for livestock and food crop cultivation. Addressing these challenges to ensure adequate, high-quality feed for ruminants is critical for sustaining livestock productivity and remains a focal concern for agricultural scientists and policymakers (Anim-Jnr et al., 2023).

Forage is herbaceous plants or herbaceous plant parts made available for animal consumption. Forages form the basal diet of ruminants, encompassing a wide array of plant materials consumed by grazing livestock, including grasses, legumes, and crop residues. It can also be defined as 'edible parts of plants, apart from separated grain, that can provide feed for grazing animals or that can be harvested for feeding' (Forage and Grazing Terminology Committee, 1991; Barnes and Baylor, 1995). Grasses, primarily from the *Poaceae* family, are essential forage components, with species such as *Megathyrsus maximus* (syn. *Panicum maximum*, commonly known as guinea

grass) demonstrating high adaptability and production potential under local conditions (Ajayi and Babayemi, 2008). Guinea grass is versatile, suitable for cutting, silage, or grazing, and offers higher protein yield and dry matter content compared to other tropical grasses like elephant grass (*Pennisetum purpureum*) (Man and Wiktorsson, 2003). Ajayi and Babayemi (2008) stated that *P. maximum* is one of the most common grasses in the savanna region of Nigeria. Under adequate conditions, its nutritional value is high, having up to 12.5% crude protein, Total Digestible Nutrients (TDN) of 10.20 %, and some minerals such as calcium, phosphorus, and magnesium. Other researchers (Aderinola et al., 2014) affirmed that *P. maximum* has been classified among the best forage grasses due to its high nutritive value. Also, *P. maximum* produces a high yield of palatable fodder and is suited for grazing, but rapidly declines in nutritive value with age and could also die off if continually grazed close to the ground.

Odeyinka (2001) mentioned that ruminants cannot meet their maintenance needs on grass alone. Bamikole and Babayemi (2004) stated that although ruminants relish *P. maximum*, this grass becomes scarce during the dry season, and this causes nomads to travel long distances with their livestock in search of greener pastures and in the process, they cause damage to farmlands, lose their animals to snakebites and exposure to extremes of weather, and destroy lives and properties of farmers. These findings led to the search for leguminous forages, which are more palatable and well-accepted by ruminants all year round (Odeyinka, 2001). One such shrub in Nigeria is *Gliricidia sepium*, which is a medium-sized, semi-deciduous tree that grows up to between 10 and 15 m high. *Gliricidia sepium*, which contains an average of 22.3% crude protein, is described as a suitable feed for ruminants (Bawala et al., 2006). Meanwhile, the integration of mixed agricultural systems such as agro-pastoralism and silvo-pastoralism in providing suitable tropical forages for ruminants has been an effective strategy in combating feed shortages whilst reducing environmental footprint (Arango et al., 2020).

Furthermore, the combined use of grasses and legumes, for instance, guinea grass and *Glyricidia sepium*, in feeding ruminants is beneficial because the legume fixes nitrogen to the soil, which can be utilized by grasses and thereby increase their crude protein content (Cook et al., 2020). It also provides fermentable nitrogen, other nutrients for rumen microbes, readily fermentable cellulose, and bypass protein. In addition, *Glyricidia sepium* is commonly used as fencing material (Mbah et al., 2024), and it grows all year round; this makes it a legume of great choice for ruminant farmers.

There are many ways in which feed/diets can be evaluated. It can be carried out by proximate or chemical analysis (AOAC, 1990), *in vitro* method (Menke et al., 1979 and Babayemi, 2007), and *in vivo* method, in which the diet is introduced to animals whilst considering feed intake. Although there are other methods used to carry out *in vitro* fermentation assessment, the gas method is mostly used.

Altering the mix of dietary components is frequently suggested as a tactic that farmers could utilize to diminish the amount of energy expended by animals through eructated gasses (methane (CH_4), carbon dioxide (CO_2), nitrous oxide (N_2O)) and to enhance feed and energy efficiency. Methane is one of the ruminal fermentation end-products synthesized by methanogenic archaebacteria from CO_2 and H_2 derived from the fermentation of carbon-containing substrates. It is now widely understood that methane is a potential greenhouse gas capable of causing even more global warming than carbon dioxide over time (IPCC, 2013). In ruminants, the production of enteric gas is mostly determined by nutrition and feed intake. Usually, several factors such as forage processing, type of carbohydrate, additives, fat source, degradability, and amount of H_2 produced can influence gas production (Janssen, 2010). In vitro approaches can yield substantial insights into the mechanisms of digestive interactions among different feed sources. Furthermore, evaluating forages individually and in association should yield insights into a plant's capacity to influence nutrient utilization from another plant (Zhang et al., 2017).

Despite these developments, limited research exists on the associative effects of *M. maximus* and *G. sepium* mixtures on proximate composition, in vitro gas production, and nutrient degradability. This study aims to fill this knowledge gap by investigating the impact of varying grass-to-legume ratios on fermentation dynamics, nutrient digestibility, and greenhouse gas emissions. The findings will provide critical insights into optimizing forage mixtures for sustainable ruminant production systems, especially in low-income countries.

2. Materials and Methods

The experiment was carried out in 2018 at the Teaching and Research farm of the University of Ibadan, Oyo State, Nigeria. It is situated in the derived savanna vegetation belt (Latitude 7º 27' N and 3º 45'E) and at an altitude between 200m and 300m above sea level; mean temperature of 25-29ºC with an average annual rainfall of about 1250mm. The soil is well-drained and belongs to the alfisol (Babayemi et al., 2003). The analyses were, however, carried out in the Laboratories of the Department of Animal Science, University of Ibadan.

2.1. Experimental Design and Substrates

The samples used for the experiment were derived from plant materials at the Teaching and Research farm and surroundings of the University of Ibadan, Oyo State, Nigeria. Fresh blades of guinea grass and tender stems and leaves of gliricidia were cut from matured plants. Gliricidia contains approximately 25% DM, and guinea grass contains 23% DM levels. The grass regrowth was cut at 15 cm height above the ground, while the legume was cut at 30 cm from the branch tip. Each fresh sample consisting of leaves and tender stem harvested was sun

dried for a week and ground. MM and GS were combined as follows: T_1- 100%MM + 0%GS, T_2- 75%MM + 25%GS, T_3- 50%MM + 50%GS, T_4- 25%MM + 75%GS, and T_5- 0%MM + 100%GS.

2.2. Chemical Composition

The ground samples were oven-dried to constant weight at 105 °C. Further proximate analysis (ash, ether extract, crude protein, and crude fiber determination) of all the samples was carried out according to the procedures laid down by the Association of Official Analytical Chemists (AOAC, 2005). Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were assayed by the method of Van Soest et al. (1991).

2.3. In Vitro Fermentation Procedure and Measurement of Gas Production

The rumen fluid was collected prior to early morning feeding. *In vitro* gas production analyses were performed based on the procedure described by Menke and Steingass (1988). The rumen fluid was collected through the suction method from three West African Dwarf goats under the same feeding regime and condition with the use of a suction tube as described by Babayemi and Bamikole (2006). The animals were fed with 40% concentrate feed (40% corn, 10% wheat offal, 10% palm kernel cake, 20% groundnut cake, 5% soybean meal, 10% dried brewers grain, 1% common salt, 3.75% oyster shell, and 0.25% fish meal) and 60% browse plants. The fluid was then filtered through a four-layered cheesecloth into a warm flask, flushed with carbon dioxide (CO_2) gas, and kept in a water bath previously heated to 39 °C. The mixture was intermittently stirred using an automatic Two hundred milligrams (200mg) DM of each dried and ground sample was carefully weighed into 100 ml calibrated syringes with pistons lubricated with Vaseline and thereafter, the syringes were filled with 30ml of medium consisting of 10ml of rumen fluid and 20ml of buffered mineral solution (NaHCO3+3Na2HPO4+KCl+NaCl+MgSO4.7H2O+CaCl2.2H2O) and each sample was replicated three (3) times. The syringes were tightly capped and carefully arranged in an incubator maintained at 39±1^0 C along with three (3) blank syringes containing 30ml of medium (inoculums and buffer) only as control. The gas production was recorded at 3, 6, 9, 12, 15, 18, 21, and 24 hours. The gas produced was read by measuring the space formed between the top of the piston and the liquid in the syringe. The net gas produced was recorded as the gas produced (in ml) at 24 hours of incubation. After every reading (every 3 hours), the content of the syringes was shaken properly to allow for proper mixing of the substrate and the liquid. After incubation time, 4ml of 10M NaOH solution was introduced to estimate methane (CH_4) production according to Fievez et al. (2005). Mixing of the contents with NaOH allowed absorption of CO_2, with the gas volume remaining in the syringe considered to be CH_4 (Demeyer et al., 1988). Graphs of the volume of gas produced every 3-hour interval of the three replicates of each sample were plotted against the incubation time.

From the graph, the degradation characteristics were estimated as defined in the equation 1:

Y= a+ b (1-ect) (Orskov and Mc Donald, 1979) where
Y= degradability at time (t)
a= intercept (or initial gas produced)
b= potentially degradable of b
c= rate of degradation of b
t= incubation time (1)

The asymptote represents (a+b) of the potential degradability. The intercept of the curve is represented by a and given the DMD value at a time (zero hours). The b value was calculated as the difference between the asymptotic DMD and the intercept a, i.e. (a+ b) – a. To get a good estimate of c, Y was selected (DMD% at the time) when the curve changed rapidly. The gas produced on incubation, together with the levels of other chemical constituents, was used to predict the digestibility of organic matter (equations 2 and 3).

OMD (percent) =14.88 + 0.889Gp + 0.45CP + 0.0651XA, R2=0.92 (2)

Partition Factor (PF) = OMD/Gp (3)

Where,
DM dry matter,
OMD organic matter digestibility.
CP, crude protein in percent.
XA, ash in percent.
Gp, the net gas production in ml from 200 mg dry sample after 24 h of incubation and after correction for the day-to-day variation in the activation of rumen liquor using the Hohenheim standard.

2.4. Statistical Analysis

Data obtained was subjected to a one-way Analysis of Variance using SAS software (1990), and the significant differences among the means were separated using the Duncan Multiple Range Test.

3. Results

3.1. In vitro Gas Production

The in vitro gas production characteristics of forage mixtures are presented in Table 1. A graph showing methane gas production as affected by different proportions of grass-legume mixtures is also presented in Figure 1.

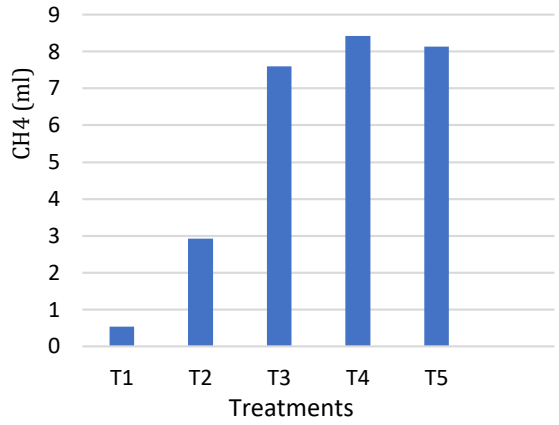

Figure 1. Methane gas production as affected by different proportions of forage mixtures. T_1= 100% Megathyrsus maximus +0% Gliricidia; T_2= 75% Megathyrsus maximus +25% Gliricidia; T_3= 50% Megathyrsus maximus +50% Gliricidia; T_4= 25% Megathyrsus maximus +75% Gliricidia; T_5= 0% Megathyrsus maximus +100% Gliricidia

It was observed that initial gas (a) produced in T_1 (3.00) and T_2 (2.50) were similar and significantly (P<0.05) lower compared to other treatments. The potentially degradable fraction (b) observed in T_3 (7.67) and T_5 (10.33) were significantly (P<0.05) higher compared to other treatments. However, the lowest (P<0.05) potentially degradable fraction was observed in T_1 (1.67). Higher (P<0.05) potential gas production (a+b) was observed in T_3 (17.00), T_4 (16.33), and T_5 (17.67) compared to other treatments. The lowest (P<0.05) potential gas production was observed in T_1 (1.33). The T_4 (0.13) had a significantly (P<0.05) higher rate of degradation compared to other treatments. However, T_1 (0.00) and T_3 (0.01) were significantly (P<0.05) lower compared to other treatments. Higher (P<0.05) incubation time (t) was observed in T_2 (16.50) compared to T_1 (7.50) and T_3 (10.00) but did not differ significantly (P<0.05) from T_4 (11.00) and T_5 (15.00). The degradability at t (Y) was significantly (P<0.05) affected by different proportions of forage mixtures. It was observed that degradability in T_4 (12.66) and T_5 (15.22) was significantly (P<0.05) higher compared to T_1 (4.67) and T_2 (4.17).

Table 1. *In vitro* Gas production characteristics of the forage mixtures

Parameters	T1	T2	T3	T4	T5	SEM	P value
inital gas produced	3.00^{b}	2.50^{b}	9.33^{a}	11.00^{a}	11.00^{a}	2.80	0.02
potentially degradable fraction	1.67^{c}	3.33^{bc}	7.67^{a}	5.35^{b}	10.33^{a}	1.50	0.04
potential gas production	1.33^{c}	5.83^{b}	17.00^{a}	16.33^{a}	17.67^{a}	0.83	0.01
rate of degradation	0.00^{c}	0.04^{bc}	0.01^{c}	0.13^{a}	0.07^{b}	0.02	0.02
incubation time	7.50^{b}	16.50^{a}	10.00^{b}	11.00^{ab}	15.00^{ab}	3.03	0.04
degradability at time (t)	4.67^{b}	4.17^{b}	10.22^{ab}	12.66^{a}	15.22^{a}	2.71	0.04

^{abc} Means for treatments along x axis with different superscripts differed significantly (P<0.05)

T_1= 100% Megathyrsus maximus +0% Gliricidia; T_2= 75% Megathyrsus maximus +25% Gliricidia; T_3= 50% Megathyrsus maximus +50% Gliricidia; T_4= 25% Megathyrsus maximus +75% Gliricidia; T_5= 0% Megathyrsus maximus +100% Gliricidia.

3.2. In- vitro Fermentation Parameters

Table 2 shows the in vitro fermentation parameters of grass-legume mixtures. The net gas volume (NGV) observed in T_1 (1.33) and T_2 (5.83) was significantly (P<0.05) lower compared to other treatments. The CH_4 gas produced in T_1 (0.53) was the lowest when compared to other treatments and was not significantly different from T_2 (2.93). However, T4 (8.43) had the highest (p < 0.05) CH_4 gas production. Different proportions of forage mixtures did not significantly (P<0.05) affect net methane (NM) to net gas (NG) ratio and ranged from 0.40 (T_1) to 0.53 (T_4). The CO_2 to organic matter digestibility (OMD) ratios observed in T_3 (0.25), T_4 (0.28), and T_5 (0.26) were similar and significantly (P<0.05) higher compared to other treatments. The T_1 (0.02) had the lowest (P<0.05) CO_2/OMD.

Table 2. *In vitro* fermentation parameters of the grass and legume mixtures

Parameters	T1	T2	T3	T4	T5	SEM	P value
NGV	1.33^{b}	5.83^{b}	17.00^{a}	16.33^{a}	17.67^{a}	2.54	0.02
CH_4	0.53^{b}	2.93^{b}	7.60^{a}	8.43^{a}	8.13^{a}	1.29	0.04
NM/NG	0.40	0.51	0.45	0.53	0.47	0.04	0.36
OMD	30.61^{b}	29.99^{d}	29.91^{d}	30.49^{c}	30.79^{a}	0.03	<0.0001
CO_2	0.80^{c}	2.90^{c}	9.40^{a}	7.90^{a}	9.53^{a}	0.80	0.02
CO_2/OMD	0.02^{c}	0.10^{b}	0.25^{a}	0.28^{a}	0.26^{a}	0.03	0.02
CH_4/OMD	0.03^{b}	0.09^{b}	0.31^{a}	0.26^{a}	0.31^{a}	0.03	0.03
PF	0.07^{a}	0.02^{b}	0.01^{c}	0.01^{c}	0.01^{c}	0.003	0.004

^{abcd} Means of treatments along a row with different superscript differed significantly (P<0.05), NGV= net gas volume, CH_4= methane, NM/NG- Net methane: Net gas. T_1= 100% *Megathyrsus maximus* +0% *Gliricidia;* T_2= 75% *Megathyrsus maximus* +25% *Gliricidia;* T_3= 50% *Megathyrsus maximus* +50% *Gliricidia;* T_4= 25% *Megathyrsus maximus* +75% *Gliricidia;* T_5= 0% *Megathyrsus maximus* +100% *Gliricidia.*

The T_1 (0.03) and T_2 (0.09) had significantly (P<0.05) lower CH_4 to OMD ratios compared to treatments. The CO_2 gas produced in T_3 (9.40), T_4 (7.90), and T_5 (9.53) were significantly (P<0.05) higher compared to other treatments. However, T_1 (0.80) had the lowest (P<0.05) CO_2 gas production. The net CO_2 to net gas ratio was not significantly (P<0.05) affected by different proportions of forage mixtures and ranged from 0.47 (T_4) to 0.60 (T_1).

3.3. Nutritional Composition of Grass-legume Mixtures

Table 3 shows the proximate composition of dietary treatments as influenced by different proportions of forage mixtures. Higher (P<0.05) Moisture Content (MC) was observed in T_5 (5.95%) compared to other dietary treatments. The T_2 (4.37%) had the lowest (P<0.05) MC. The Crude Protein (CP) observed in T_5 (21.48) was significantly (P<0.05) higher compared to other treatments. However, the lowest (P<0.05) CP was observed in T_1 (13.16). Higher (P<0.05) ether extract (EE) was observed in T_1 (3.29) compared to other treatments. The T_5 (1.98) had the lowest (P<0.05) EE but was similar to T_3 (2.01). The crude fiber (CF) observed in T_1 (25.99) was significantly (P<0.05) higher compared to other dietary treatments. Similar (P<0.05) CF values were observed in T_3 (22.60) and T_4 (22.59). However, T_3 (20.20) had the lowest (P<0.05) CF. Ash content observed in T_1 (9.13) was significantly (P<0.05) higher compared to other treatments. The lowest (P<0.05) ash content was observed in T_3 (5.55). The NFE observed in T_2 (71.46) was significantly (P<0.05) higher compared to other dietary treatments. The T_1 (69.71) and T_3 (69.72) had similar (P<0.05) NFE values. However, T_5 (65.04) had the lowest (P<0.05) NFE value. Higher (P<0.05) partitioning factor (PF) was observed in T1 (0.07) compared to other dietary treatments.

Table 3. Proximate composition of grass and legume mixtures

Parameters	T1	T2	T3	T4	T5	SEM	P value
Moisture content	4.70^{d}	4.37^{c}	5.42^{b}	4.84^{c}	5.95^{a}	0.02	<0.0001
CP	13.16^{c}	13.99^{d}	15.38^{c}	18.39^{b}	21.48^{a}	0.03	<0.0001
EE	3.29^{a}	2.01^{c}	2.01^{d}	2.09^{b}	1.98^{d}	0.01	<0.0001
CF	25.99^{a}	24.01^{b}	22.60^{c}	22.59^{c}	20.20^{d}	0.01	<0.0001
Ash	9.13^{a}	8.16^{b}	7.47^{c}	6.59^{d}	5.55^{c}	0.03	<0.0001
NFE	69.71^{b}	71.46^{a}	69.72^{b}	68.08^{c}	65.04^{d}	0.05	<0.0001

^{abcd} Means of treatment along a row with different superscript differed significantly CP= crude protein, EE- (P<0.05) ether extract, CF= crude fiber, NFE-Nitrogen free extract. T_1= 100% Megathyrsus maximus +0% Gliricidia; T_2= 75% Megathyrsus maximus +25% Gliricidia; T_3= 50% Megathyrsus maximus +50% Gliricidia; T_4= 25% Megathyrsus maximus +75% Gliricidia; T_5= 0% Megathyrsus maximus +100% Gliricidia.

3.4. Fiber Fractions of Dietary Treatments

The fiber fractions of dietary treatments as affected by different proportions of forage mixtures are shown in Table 4. İt was observed that the Neutral Detergent Fiber (NDF) was not significantly (P<0.05) affected by different proportions of forage mixtures and ranged from 58.90 (T_2) to 62.76 (T_4). Higher (P<0.05) acid detergent fiber (ADF) values were observed in T_4 (31.90) and T_5 (30.60) compared to other dietary treatments. The T_2 (27.41) had the lowest (P<0.05) ADF value compared to other dietary treatments. It was observed that the T_3 (16.60) had significantly (P<0.05) higher acid detergent lignin (ADL) value compared to other dietary treatments. Similarly (P<0.05) ADL values were observed in T_4 (15.80) and T_5 (15.53) and were higher (P<0.05) than in T_1 (14.60) and T_2 (14.20). However, the lowest (P<0.05) ADL value was observed in T_2 (14.20).

Table 4. Fiber fractions of dietary treatments as affected by different proportions of forage mixtures

Parameters	T1	T2	T3	T4	T5	SEM	P value
NDF	60.06	58.90	60.02	62.76	60.39	1.45	0.47
ADF	28.60^{c}	27.41^{d}	31.06^{a}	30.90^{b}	30.60^{b}	0.08	<0.0001
ADL	14.60^{c}	14.20^{d}	16.60^{a}	15.80^{b}	15.53^{b}	0.12	<0.0001

^{abcd} Means of treatments along a row with different superscript differed significantly (P<0.05). NDF= neutral detergent fiber, ADF= acid detergent fiber, ADL= acid detergent lignin. T_1= 100% Megathyrsus maximus +0% Gliricidia; T_2= 75% Megathyrsus maximus +25% Gliricidia; T_3= 50% Megathyrsus maximus +50% Gliricidia; T_4= 25% Megathyrsus maximus +75% Gliricidia; T_5= 0% Megathyrsus maximus +100% Gliricidia.

4. Discussion

Megathyrsus maximus is one of the most common grasses in the savanna region of Nigeria. Under adequate conditions, its nutritional value is high, having up to 12.5% crude protein, Total Digestible Nutrients (TDN) of 10.20%, and some minerals such as calcium, phosphorus, and magnesium (Ajayi and Babayemi, 2008). Aderinola et al. (2014), affirmed that *Megathyrsus maximus* has been classified among the best forage grasses due to its high nutritive value and produces high yield of palatable fodder suitable for grazing, but rapidly declines in nutritive value with age and could also die off if continually grazed close

to the ground. These reports are consistent with the findings of the current study, as observed in T_1 with a crude protein content of 13.16%.

The result of the current study confirms the report of Bawala et al. (2006), who described *Gliricidia sepium* as a suitable feed for ruminants and that the combined use of grasses and legumes, for instance, guinea grass and *Gliricidia sepium* in feeding ruminants is beneficial because the legume fixes nitrogen to the soil which can be utilized by the grass and thereby increase its crude protein. It also provides fermentable nitrogen, other nutrients for the rumen microbes, readily fermentable cellulose, and bypass protein.

Kearny (2005), noted that fiber plays a fundamental role in ruminant nutrition, and it is the component in a feed that is not digested by mammalian enzymes. Some of these components are soluble under mild extraction procedures and thus result in 'soluble' and 'insoluble' fiber. Most constituents of soluble fiber (pectin, fructans, and β-glucans) are readily fermented in the rumen and may even be readily fermented in the large intestine of monogastric animals (Righi et al., 2008). From the current study, the NDF was not significantly affected by different proportions of forage mixtures and ranged from 58.90 (T_2) to 62.76 (T_4). Higher ADF values were observed in T_3 (31.06) and T_4 (30.90) compared to other dietary treatments. The T_2 (27.41) had the lowest ADF value. Merten (1997), stressed that the chemical definition of dietary fiber, such as neutral detergent fiber (NDF) or acid detergent fiber (ADF) content, was an inadequate description of the fiber content of a diet. The ADF fraction of feedstuffs includes cellulose and lignin as the primary components. Concentrations of ADF and lignin are correlated more with digestibility than with intake. It is an indicator of digestibility; as the ADF increases, digestibility decreases. NDF is a measure of cellulose, hemicelluloses ADF, and lignin fractions of feeds. NDF is more highly correlated with feed volume and chewing activity than ADF or crude fiber (CF) (Oba et al., 1999).

Some of the NDFs are highly digestible, but forage NDF is the best indicator of an animal's voluntary feed intake (VFI). As the NDF content of forage increases, the VFI decreases. Also, it has been shown several times that the digestibility of plant material in the rumen is related to the proportion and lignification of plant cell walls (NDF). Forage with high lignin contents is often of low digestibility (Norton, 2010). Merry et al. (2006), reported that when animals are fed a grass and legume mixture, digestive interactions can occur in the rumen between substrates contained in the different plants, and the response of the animal to the combination of forages can differ from the balanced median values of their components considered individually. These interactions, named associative effects, can modify the metabolic processes in the digestive tract, particularly in the rumen, so that the response of an animal to a combination of forages can differ from the balanced median values of its components considered individually. This kind of response can be synergistic or antagonistic with a possible impact in terms of nutrient use by the animal, N excretion, and methane emissions. Dhiman and Satter (1997) noted that the nutritional complementarities of plant species could contribute to integrating productivity and environmental requirements, as observed from the current study. The net gas volume produced was greatest in the mixtures ranging from 50%-100% Gliricidia. This may be due to two factors. One is the higher crude protein content in these combinations, as the greater availability of crude protein in the diet allows for greater microbial activity as it is not limiting in the diet.

Johnson et al. (1995) reported that the production of methane represents a loss of between 2 and 15% of the gross energy in the feed. The authors increased feed efficiency by production and inhibition of rumen methanogenesis because methane plays a role in the global warming phenomenon and the destruction of the ozone layer. Chynoweth (1996) reported that compared to other greenhouse gases, methane is an excellent candidate to reduce global warming in the near term. However, Houghton (1997) reported that because of the shorter lifetime of methane in the atmosphere (about 12 years compared with 100-200 years for CO^2), only a relatively small reduction in the anthropogenic emission of CH4, about 8% would be required to stabilize its concentration at the current level. Leng (1993) concluded that enteric methane emission is one of the few global sources of methane that can be reduced relatively. The author further stated that it is easier to manipulate, for instance, methane produced from marshes or in rice production. Furthermore, methane reduction strategies from livestock will directly benefit the farmers by improving animal productivity. The result of the current study on methane production has shown that 100% *Megathyrsus maximus* or a combination of 75% *Megathyrsus maximus* and 25% *Gliricidia sepium* had the least methane production.

Furthermore, Beauchemin et al. (2009) reported that improving forage quality (i.e., increasing dietary starch content) through the supplementation of alternative forages, such as leguminous and non-leguminous shrubs, has the potential to reduce CH_4 emissions per kg animal products as a result of increased diet digestibility and a shortened duration of feeding. The authors further stated that dietary strategies such as this have been successful in manipulating methanogenesis, at least in the short term, through either direct inhibition of methanogens, reducing the production of hydrogen in the rumen, or providing alternative sinks for the disposal of hydrogen. Carulla et al. (2005) observed a similar report and stated that secondary plant compounds (e.g., condensed tannins and saponins) have been shown to reduce enteric CH_4 emissions through the direct inhibition of methanogens. Similarly, including high starch feedstuffs favors propionate production and reduces ruminal pH, thus inhibiting methanogen and protozoal growth (Boadi et al., 2004). From the current study, the degradability of forage

mixtures was affected by different proportions of inclusion. The T_5 (30.79) had significantly higher OMD compared to other dietary treatments. Similar OMD values were observed in T_2 (29.99) and T_3 (29.91) and were significantly lower compared to other dietary treatments. The result of the present findings corroborates the report of Swan et al. (2006), who noted that the degradation of feed ingredients also depends on the distribution of starch granules within the substance. Qin et al. (2012) also added that starch granules of wheat endosperms seem to be floury and have a relatively small particle size. Consequently, the smaller starch granules have a larger surface area available for microbial and enzymatic starch hydrolysis, which results in rapid degradation. Bonhomme (1990) reported that increased methane emission can be observed as a result of the optimum symbiotic relationship between bacteria and protozoans and the efficient exchange of hydrogen between these microorganisms. Furthermore, methane production is affected by the type of carbohydrate fed to the animals (Moe and Tyrrell, 1979). Qin et al. (2012) reported that wheat had relatively higher effective degradability of dry matter (EDDM), which was more rapidly fermented by ruminal microbes. Thus, having a higher methane production of wheat may be attributed to EDDM. Moreover, low methane production of other feed ingredients might also be attributed to low EDDM and, thus, slow fermentation of ruminal microbes. Low EDDM might be due to the thickness of the protein matrix, which coats starch granules, and this matrix is relatively difficult to hydrolyze with water and enzymes (McAllister et al., 1996). The result of the present study contradicts the findings of Shibata (1992), who noted that providing ruminants with feed containing carbohydrates and high protein levels had a negative effect on methane emission while providing a diet rich in fiber resulted in an elevated volume of methane being produced. It was observed that T_5 has the highest methane production, followed by T_4 and T_3. T_1 and T_2 had the lowest methane production. T_1 had the lowest degradability, whereas T_5 had the highest degradability.

5. Conclusion

The study demonstrated that varying proportions of *Megathyrsus maximus* and *Gliricidia sepium* significantly influence nutrient composition, in vitro gas production, and organic matter degradability. Treatments with higher proportions of *Gliricidia sepium* exhibited elevated crude protein levels, methane, and CO_2 production, with T_5 (100% G. sepium) achieving the highest crude protein content (21.48%) and methane production (8.13 mL). Conversely, treatments with higher *M. maximus* content, such as T_1 (100% *M. maximus*), had higher crude fiber (25.99%) but lower methane production (0.53 mL), indicating reduced fermentability.

The degradability of organic matter significantly improved with increasing *G. sepium* inclusion, with T_5 recording the highest value (30.79%). However, methane emissions also increased with higher legume proportions, highlighting a trade-off between enhanced digestibility and environmental impact. The study suggests that a 75% *M. maximus* and 25% *G. sepium* mixture offers an optimal balance between nutrient availability, low methane emissions, and adequate degradability, making it a suitable option for sustainable ruminant feeding systems. This proportion minimizes greenhouse gas emissions while ensuring efficient nutrient utilization, supporting productivity in tropical livestock systems.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	G.A.I.	O.O.	U.Ş.
C	30	70	
D	30	70	
S		100	
DCP	70	30	
DAI	50	50	
L	90	10	
W	80		20
CR	30	40	30
SR	30	40	30
PM	50	50	
FA	100		

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because there was no study on animals or humans.

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ASSESSMENT OF ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF ETHANOLIC FLOWER EXTRACTS FROM *Rosa damascene* AGAINST PATHOGENIC MICRO-ORGANISMS

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Abstract: This study investigates the antimicrobial potential of *Rosa damascena* flower extract, with a focus on its antibacterial and antifungal properties. The primary objective was to assess the inhibitory activity of the ethanolic extract against a spectrum of bacterial and fungal pathogens. Using the agar disc diffusion method, the extract was evaluated at a concentration of 100 µg/ml against two Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*), one Gram-negative bacterium (*Escherichia coli*), and four fungal strains (*Aspergillus fumigatus AF293*, *Aspergillus niger ATCC 16404*, *Candida albicans SC5314*, and *Monascus purpureus ATCC 1008*). The zones of inhibition produced by the extract were compared to those of standard antibiotics: *ciprofloxacin* for antibacterial activity and *fluconazole* for antifungal activity. The findings revealed significant antibacterial effects, particularly against Gram-positive bacteria, with clear zones of inhibition, suggesting that *Rosa damascena* harbors a diverse array of bioactive secondary metabolites, the extract demonstrated notable antifungal activity, with inhibition observed across several fungal strains. These results underscore the extract's promising antimicrobial potential, highlighting its efficacy as a source of bioactive compounds, the study suggests that *Rosa damascena* could serve as a valuable resource for the development of novel antimicrobial agents, particularly in light of increasing resistance to conventional antibiotics. Further investigation into the specific mechanisms of action, toxicity, and the isolation of active compounds is warranted to advance its potential as a therapeutic agent in the pharmaceutical industry. The synergistic effects with other natural compounds could enhance its therapeutic efficacy and expand its potential applications in clinical settings.

Keywords: *Rosa damascene,* Ethanolic extract, Antimicrobial activity, Minimum inhibitory concentration (MIC)

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1. Introduction

Rosa damascena (Damask rose) is an important species of the flowering plant genus because of the fragrant smell and uses in medicine and cosmetics as well as in preparation of fragrances (Majedi et al., 2024). It has been cultivated for thousands of years beginning from the Middle East as mentioned by (Bari et al., 2024) It has a unique composition that consists of more than two hundred phytochemicals that include essential oils, flavonoids, and a range of phenolic compounds these components provides the extraordinary fragrance and some other therapeutic value associated with this species. *Rosa damascena* has also been employed in traditional healthcare for its capacity to treat a broad spectrum of ailments due to its impacts on inflammation, oxidation, and antimicrobial profile (Niazi et al., 2023; Wang, 2024).

There has been a rising interest in research related to *Rosa damascena* due to its highly potent antimicrobial activity and its efficiency against different pathogenic microorganisms (Poonia et al., 2024) However, in recent years, the use of plant derived antimicrobial has become increasingly important because of the emergence of antibiotic resistance bacteria, which has led to further research into the agents. *Rosa damascena* is among the few plants which contain a diverse profile of bioactive (DpBC) compounds, and as such has been identified as a potential candidate in this regard (Antoniadou et al., 2024).

Again, antibiotic resistant remains a significant threat to public health for it is gradually making infections that were hitherto easily treatable with standard antibiotics to become more complex to control (Brüssow, 2024). The overly use of antibiotics both in hospitals and in farming has led to the emergence of resistant strains, and therefore, there is growing call for natural antimicrobial compounds that should be used alongside standard

antibiotics or can perhaps be used as a replacement In this regard, plant secondary metabolites have received extensive attention for their antimicrobial activity, and, some investigations have revealed that *Rosa damascena* flower ethanolic extracts exhibited remarkable antibacterial and antifungal activities, These observations collectively indicate that the *Rosa damascena* extracts can be an effective option to control of pathogenic microbes (Antoniadou et al., 2024; Dini et al., 2024).

Furthermore; Plants, including *Rosa damascena*, produce a wide range of secondary metabolites organic compounds that, while not essential for the basic growth and development of the plant, play critical roles in defense mechanisms and reproductive processes, these secondary metabolites are structurally diverse and hold great promise for pharmaceutical applications due to their bioactive properties (Laftouhi et al., 2024; Rahimi et al., 2024). Historically, natural products derived from plants have been instrumental in the development of various medicines, improving public health and contributing to the creation of cost-effective treatments worldwide (Azizi et al., 2023; Chaughule and Barve, 2024) many of these medicines, particularly those used to treat microbial infections, have their origins in the secondary metabolites produced by plants (Niazi and Monib, 2024).

The antimicrobial and antiviral properties of secondary metabolites are well documented, with specific compounds like alkaloids, phenolics, polyphenols, flavonoids, quinones, tannins, coumarins, terpenes, lectins, polypeptides, and saponins showing particular promise (Saini et al., 2024), each of these groups exhibits unique biological activities that can be harnessed for medicinal purposes. For instance, polyphenols have recently been identified to possess antimicrobial effects and have widely applied in the food processing sectors owing to their ability to minimize the usage of chemical preservatives (Kakar et al., 2024; Islam et al., 2025). In the same way, the pharmaceutical industry is also considering polyphenols and other secondary metabolites as possible treatments for infections that result from antibiotic-resistant bacteria (Niazi, 2024). Specifically, the flowers possess important antimicrobial activity, especially *Rosa damascena* which has been used worldwide as an ethnomedical remedy for various ailments and conditions (Oargă et al., 2024). Studies done in the current years have been directed towards the isolation of bioactive compounds from the plant flowers; notably, the attention has been given to their antimicrobial activity (Bhadange et al., 2024). Among these extracts, the ethanolic ones have been reported to be the most active against various pathogenic bacteria and fungi; these results support the traditional uses of *Rosa damascena* and its possible use as a source of new antimicrobial compounds (Abdel-Nasser et al., 2024; Abdel-Malek et al., 2024). Study on *Rosa damascena* determine the antibacterial and antifungal properties of ethanolic flower extracts against important and relevant bacterial and fungal species (Fayaz et al., 2024). The antibacterial potential of *Rosa damascena* as a natural agent is examined using microbiological techniques, such as agar disc diffusion and determination of the extracts' minimum inhibitory concentrations (MIC). (El-Shouny et al., 2016; Trendafilova et al., 2023). Disc diffusion (DD) is one of the methods used in assessing the antibacterial property of plant extracts by measuring the zone of inhibition, which is an aspect of the extract's ability to inhibit the growth of microbes (Golus et al., 2016; Sarwari et al., 2024). The MIC (Minimum Inhibitory Concentration) on the other hand offers a more quantitative approach in determining the lowest concentration of the extract that would help inhibit the growth of a given microorganism (Hafidh et al., 2011).

Consequently, ought the properties of *Rosa damascena* extracts against microbes as stated be proved, the world could envision novel therapies for eradicating microbial infections (Akram et al., 2020; Gupta et al., 2024; Trivedi et al., 2025). The *Rosa damascena* extracts could replace synthetic antibiotics, particularly for microbial infections that are resistant to conventional treatment (Lee et al., 2023). The information about bioactive compounds in *Rosa damascena* may prompt more research. Into other plant species, taking the range of such natural solutions to the global challenge of resistance to antibiotics (Minteguiaga et al., 2023).

Rosa damascena is not only appreciated for its olfactory and ornamental properties, but for its healthy phytochemical content that takes promising potential for the therapeutic usage of the species (Simin et al., 2024), especially in the modern world, which faces the problem of antibiotic resistance (Farooq et al., 2020). The extensive use this plant in the traditional medicine is gradually being supplemented by scientific confirmation and its potential as a source of natural, effective remedies for microbial infections increases while the research on the antimicrobial activity of *Rosa damascena* goes on and while microbial resistance poses one of the major challenges to modern medicine, (Reisi-Vanani et al., 2024). This study aims at assessing the antimicrobial activity of ethanolic *Rosa damascena* flowers extracts against a number of pathogenic microorganisms, mainly bacteria and fungi. Using agar disc diffusion method, the inhibitory ability of the extract against these microorganisms; *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Aspergillus niger* and *Candida albicans* were determined. The diameter of the inhibition zones was carefully reported and compared with normal antibiotics in order to explain the mild anticancer property in the plant. These microorganisms can cause various diseases and damage to *Rosa damascena* plants, they not only reduce the quality and yield of this valuable plant but also pose a threat to its cultivation in agricultural and horticultural settings, below is an overview of how these microorganisms can impact *Rosa damascena*.

Bacillus subtilis

While *Bacillus subtilis* is often regarded as a beneficial bacterium for plants due to its role in biocontrol and plant growth promotion, some strains may become opportunistic pathogens under specific conditions, these pathogenic strains can cause soft rot or necrosis in plant tissues by producing enzymes that degrade plant cell walls. Such infections can weaken *Rosa damascena* plants, reducing their vitality and flower production (El-Saadony et al., 2022; Niazi, 2024).

Staphylococcus aureus

Staphylococcus aureus is primarily known as a human pathogen, but its presence on *Rosa damascena* may result from contamination during cultivation, harvest, or storage. While it does not directly cause diseases in the plant, it can form biofilms on plant surfaces, potentially facilitating the growth of other pathogens and reducing the plant's market value, especially in rose-based products like essential oils (Verešová et al., 2024).

Escherichia coli

Escherichia coli contamination in *Rosa damascena* is typically linked to irrigation with contaminated water or improper handling during harvesting and processing. Although it does not directly cause plant diseases, *E. coli* poses a significant threat to the safety of rose-based products, such as oils and extracts that are intended for human use, contaminated products can lead to gastrointestinal infections in humans, making strict hygiene essential in *Rosa damascena* cultivation (Samad and Saeed, 2024).

Aspergillus niger

Aspergillus niger is a common fungal pathogen that can infect *Rosa damascena* plants, especially under conditions of high humidity and poor air circulation. It causes black mold on plant surfaces, particularly on flowers and stems, this fungal infection can lead to reduced flower quality, discoloration, and economic losses. *A. niger* produces mycotoxins that can contaminate rose products, rendering them unsafe for human use (Bi et al., 2024; Wadhwa et al., 2024).

Candida albicans

Candida albicans is primarily a human pathogen and is not a natural plant pathogen, its presence on *Rosa damascena* may result from contamination during processing or handling, especially in moist environments. Though it does not cause direct harm to the plant, it can compromise the microbiological safety of rose-based products and contribute to spoilage during storage (Ghavam, 2024).

Microbial contamination and infection pose a significant challenge in *Rosa damascena* cultivation and processing. While some microorganisms like *Aspergillus niger* directly damage the plant, others such as *Escherichia coli* and *Candida albicans* mainly affect product safety. Implementing proper agricultural practices, hygienic handling, and effective microbial control measures are essential to ensure the quality and safety of *Rosa damascena* products (Ignatov et al., 2024).

2. Materials and Methods

2.1. Preparation of Ethanolic Extract

2.1.1. Collection of plant materials

Volatile oils and bioactive compounds used in this study were extracted from fresh *Rosa damascena* flowers that were harvested during February at their optimal bloom period, at a trusted source: The flowers were harvested early in the morning to ensure they were at their optimal blooming stage, which is widely believed to be the time when the plant has the most potent concentration of volatile oils and other valuable phytochemicals.

2.1.2. Rinsing of samples

The flowers were collected as soon as possible and were brought to the laboratory to prevent exposure to factors that might affect the degradation of the compounds, before extraction or microbial testing, the *Rosa damascena* flowers that are newly harvested were washed under clean distilled water to eliminate dust, soil or any contaminants that may bear on the extraction process. Special attention was paid unto the flowers during washing to avoid mechanical abrasion that might physically injure the petals and cause leaching of volatile compounds (Monib et al, 2024).

2.1.3. Drying process

Following the rinse step, the flowers were well arranged to form a single layer on clean drying trays and the drying of the flowers was done under shade since direct sunlight may affect the quality of the phenolics and essential oils. These flower samples were kept in a cool, dry, well-ventilated room with regulated airflow and temperature to minimize degradation of the compounds, the trays used to dry the flowers were kept away from contaminants or any source of moisture to prevent mold/fungal growth during the drying period, the temperature of the room was maintained at a range of 25 to 28°C (Room temperature) because this allowed flowers to dry naturally without exposure to artificial heat and affects its chemical composition.

2.1.4. Duration of drying

The flowers were allowed to dry for a total period of 12 days, this extended drying time ensured complete removal of moisture content while maintaining the integrity of the plant's bioactive compounds, care was taken to prevent over drying, which could result in the loss of volatile oils and other thermo labile constituents, at the end of the drying period, the flowers were visually inspected to ensure they were completely dry, brittle to the touch, and free from any residual moisture, any flowers that did not meet these criteria were removed from the sample batch to avoid compromising the quality of the subsequent extract (Zhao et al., 2019).

2.1.5. Powder preparation

Once thoroughly dried, the *Rosa damascena* flowers were manually chopped into small pieces using clean, sterilized scissors, this step facilitated easier grinding and ensured uniform particle size in the final powder form, the chopped flower material was then ground into a fine powder using a mechanical grinder. The grinding

was performed in small batches to avoid overheating of the material, as excessive heat during grinding can lead to the degradation of thermo labile compounds, particularly essential oils and certain phenolics, the grinding process was continued until a homogeneous fine powder was obtained, with particle sizes small enough to maximize surface area for efficient solvent extraction during the subsequent steps (Fathima and Murthy, 2019).

2.1.6. Storage of powdered material

The ground flower powder was immediately transferred into sterile, air-tight glass containers to protect it from environmental factors such as moisture, light, and oxygen, these containers were clearly labeled with the date of preparation and stored in a cool, dry, and dark location to further preserve the integrity of the bioactive compounds, the storage temperature was maintained at approximately 4°C to slow down any potential oxidation or degradation processes, the powdered flower material was stored in this condition until further use in the extraction process (Siriamornpun et al., 2012).

2.2. Preparation of Plant Extract Using Soxhlet Extraction

A 25-gram sample of powdered flowers was combined with 250-ml of ethanol and extracted using a Soxhlet extractor for 72 hours, keeping the temperature below the boiling point of the solvent, the extracts were then incubated at room temperature for 48 hours before being used for further analysis (López-Bascón and De Castro, 2020).

2.2.1. Weighing the plant material

A precisely measured 25-gram sample of the previously prepared *Rosa damascena* flower powder (as described in the previous section) was used for the extraction process, the powdered plant material was carefully weighed using an analytical balance to ensure accuracy in the extraction protocol, the powder was stored in a clean, sterile container to avoid contamination until the extraction process was initiated (Woldemichael, 2022).

2.2.2. Preparation of the solvent

Ethanol (analytical grade, 95%) was chosen as the extraction solvent due to its efficiency in extracting a wide range of polar and non-polar compounds, particularly bioactive components such as phenolics, flavonoids, and essential oils present in the *Rosa damascena* flowers. A volume of 250 mL of ethanol was measured accurately using a graduated cylinder, the solvent was kept in a tightly sealed container to minimize evaporation or contamination before use (Anokwuru et al., 2011).

2.2.3. Soxhlet extraction procedure

The extraction of the plant material was performed using a Soxhlet extractor, a widely accepted and efficient method for continuous extraction of plant constituents, the 25-gram sample of powdered *Rosa damascena* flowers was carefully packed into a cellulose extraction thimble, the thimble was placed inside the main chamber of the Soxhlet apparatus (Qader et al., 2022).

2.2.4. Assembly of the soxhlet apparatus

The Soxhlet apparatus was assembled, with the extraction thimble placed in the extractor body and the condenser set up at the top of the apparatus, around-bottom flask containing 250 mL of ethanol was attached to the lower part of the apparatus, the flask served as the solvent reservoir and was placed on a heating mantle or water bath (Rajesh et al., 2023).

2.2.5. Extraction conditions

The Soxhlet extraction was allowed to proceed for a continuous duration of 72 hours to ensure thorough extraction of the bioactive components from the *Rosa damascena* flower powder, throughout the extraction, the temperature was carefully monitored and kept below the boiling point of ethanol (78.37°C) to prevent degradation of heat-sensitive compounds, the heating mantle was adjusted to maintain a gentle reflux of ethanol, allowing the solvent to continuously dissolve and extract the compounds from the plant material, which were then deposited back into the boiling flask upon condensation, during the extraction process, the solvent became increasingly colored as it absorbed the flower's bioactive constituents (Carvalho, 2016).

2.3. Post-Extraction Processing

2.3.1. Filtration of extract

After 72 hours, the extraction process was stopped, and the Soxhlet apparatus was dismantled. The ethanol extract, now containing dissolved plant compounds, was carefully removed from the round-bottom flask, to eliminate any remaining plant particles or debris, the liquid extract was passed through Whatman No. 1 filter paper into a clean container.

2.3.2. Incubation

The filtered ethanolic extract was incubated at room temperature for 48 hours in a sterile, sealed container, this step allowed the extract to stabilize and helped ensure uniform dissolution of any partially soluble compounds within the solvent (Martín et al., 2022).

2.4. Evaporation of Solvent Using Rotary Evaporator

2.4.1. Evaporation and rotary setup

The solvent is evaporated using a rotary evaporator to obtain a concentrated ethanolic extract, to obtain a concentrated ethanolic extract, the ethanol solvent needed to be removed from the extract, this was accomplished using a rotary evaporator (rotavap), a laboratory device commonly used for efficient and gentle evaporation of solvents under reduced pressure, the filtered extract was transferred to the round-bottom flask of the rotary evaporator, the apparatus was assembled by attaching the flask to the rotavap's rotating arm, and the setup was placed in a warm water bath to facilitate gentle heating (Cheng, 2003).

2.4.2. Temperature control

The water bath temperature was set to approximately 40°C, which is below the boiling point of ethanol (78.37°C) to prevent degradation of thermolabile bioactive compounds (TBC) in the extract, this low-temperature evaporation process also minimized the risk

of altering the chemical composition of the plant compounds (Amirullah et al., 2021).

2.4.3. Vacuum application

A vacuum was applied to the rotary evaporator system, lowering the atmospheric pressure inside the apparatus and allowing ethanol to evaporate at a lower temperature, the flask was continuously rotated to increase the surface area of the liquid extract, thereby enhancing the rate of evaporation.

2.4.4. Solvent removal

Over time, the ethanol vapor was condensed by the condenser and collected in a separate receiving flask, the process continued until most of the ethanol was removed, leaving a concentrated residue of the ethanolic extract in the round-bottom flask, the evaporation process was carefully monitored, ensuring that no excessive heating or prolonged exposure to the rotavap occurred, which could damage the bioactive compounds (Hrubesh et al., 2021).

2.4.5. Collection of concentrated extract (CCE)

Once the majority of the ethanol had been removed, the rotary evaporator was stopped, and the concentrated extract was collected. The extract, now in a thick, viscous form, was transferred into a sterile, air-tight glass container using a sterile spatula, the concentrated ethanolic extract was stored at 4°C in a dark, cool environment until further use in subsequent antimicrobial and antifungal activity assays. The final concentrated extract was weighed, and the extraction yield was calculated as a percentage of the initial 25-gram plant sample, the extract was labeled with the date of extraction, concentration, and batch number for proper tracking during subsequent analysis, this detailed preparation method ensured the careful extraction of bioactive compounds from *Rosa damascena* flowers, maintaining their integrity and maximizing their potential efficacy in antibacterial and antifungal activity testing (Acharya and Hare, 2022).

2.5. Statistical Analysis

The study likely utilized a combination of descriptive and inferential statistical methods to evaluate the effectiveness of *Rosa damascena* ethanolic extract against bacterial and fungal strains (Zhang et al., 2024).

2.5.1. Measurement of zone of inhibition

The diameters of the zones of inhibition around the discs were measured and recorded.

Descriptive statistics;

Mean: The average diameter of inhibition zones (calculated from two perpendicular measurements for each plate) was reported for each treatment group (extract, control antibiotics like *Ciprofloxacin*, and antifungals like *Fluconazole*).

Standard Deviation (SD): Likely calculated to quantify the variability of the inhibition zones across replicate tests.

Range: Minimum and maximum zone diameters may have been reported for completeness (Mailu et al., 2021).

2.5.2. Comparative analysis of inhibition zones

To compare the antimicrobial efficacy of *Rosa damascena* extracts with standard drugs (*Ciprofloxacin* and *Fluconazole*):

One-Way Analysis of Variance (ANOVA): If multiple concentrations of the extract were tested, ANOVA could be used to determine if there were statistically significant differences in the inhibition zones among the treatments.

Post-hoc Tests (Tukey's Test): If ANOVA identified significant differences, post-hoc tests would specify which pairs of treatments were significantly different.

t-Test: A paired or independent t-test may have been used to compare the inhibition zones of the extract with standard drugs *Ciprofloxacin* and *Fluconazole* (Seidel et al., 2008).

2.5.3. Minimum inhibitory concentration (MIC)

The MIC determination involved a serial dilution technique to find the lowest concentration of the extract that prevented visible microbial growth (Mazzola et al., 2009).

Regression Analysis: A regression model (linear) have been used to analyze the relationship between extract concentration and microbial growth (measured by optical density at 600 nm or visual turbidity).

MIC Value Analysis: The MIC values were reported for each tested microorganism.

Descriptive comparisons of MIC values were made against standard antimicrobial agents (*Fluconazole* and *Ciprofloxacin*) to assess relative potency.

2.5.4. Data visualization

Bar Charts or Boxplots: Graphical representation of inhibition zones or MIC values for each strain, comparing extract efficacy with standard drugs.

Line Graphs: Used to depict the concentration-dependent effects of the extract on microbial growth during MIC determination (Colclough et al., 2019).

Software and Tools: The statistical analyses have been performed using software such as SPSS, GraphPad, and Excel.

Spectrophotometric data analysis for optical density at 600 nm during MIC determination was likely supported by statistical tools for precision (Tavoosi et al., 2024).

3. Results and Discussion

3.1. Microorganisms Tested

3.1.1. Preparation of inoculum

3.1.1.1. Source of microbial cultures

The bacterial and fungal strains were obtained from laboratory-preserved slant cultures stored at 4°C. These slants contain actively growing microbial cells maintained under nutrient-limiting conditions to preserve their viability.

3.1.1.2. Preparation of inoculum

A loopful of bacterial or fungal culture was aseptically transferred from the slant into a 10 mL test tube containing fresh nutrient broth for bacterial strains and Sabouraud Dextrose Broth for fungal strains, the broth cultures were incubated at 37°C for 24 hours for bacterial strains and 28°C for fungal strains, respectively, to allow optimal growth. After incubation, the bacterial

and fungal suspensions were standardized to achieve the required turbidity using McFarland standards (usually 0.5 McFarland standard equivalent to (1.5 \times 10^8\) CFU/mL), to standardize the inoculum, sterile saline solution or broth was added to dilute the microbial suspensions until the turbidity matched the McFarland standard visually by comparison against a white background (Andrews, 2001; Budiman, 2016).

3.1.1.3. Sterile swabs and sterilization

Sterile cotton swabs were prepared by winding clean, dry cotton wool onto wooden or plastic applicators. These swabs were packaged in either culture tubes or sterile paper wrappers. For plastic swabs, autoclaving was used (121°C, 15 psi for 15-20 minutes), while wooden swabs could also be sterilized by dry heat (160-170°C for 2 hours) in a suitable oven, sterilized swabs were stored in sterile containers until further use to ensure that they remained uncontaminated. Sterile forceps were essential for placing discs onto the inoculated agar plates. Forceps were sterilized using the flame-sterilization technique: The forceps were dipped in 70% ethanol. After dipping, the ethanol was ignited to burn off and sterilize the forceps, this method ensured that the forceps were free from any microbial contaminants before use (Catalfomo and Schultz, 1966).

3.1.1.4. Inoculation of agar plates

Sterile Muller-Hinton agar plates (for bacterial testing) and Sabouraud Dextrose agar plates (for fungal testing) were used as the medium for microbial growth. Each plate was labeled with the corresponding microorganism to be tested (Chroho et al., 2022).

3.1.1.5. Application of inoculum

A sterile cotton swab was dipped into the standardized inoculum and excess inoculum was removed by gently pressing the swab against the side of the culture tube to eliminate drips and ensure uniform application, the agar plates were inoculated using the streaking method, the swab was streaked across the entire surface of the agar plate three times, each time rotating the plate by approximately 60° to ensure even distribution of the inoculum. After streaking, the swab was passed along the edge of the plate to cover the entire surface, the inoculated plates were allowed to sit undisturbed with the lid closed for approximately (10-15 minutes) at room temperature to allow the inoculum to absorb into the agar surface before the application of discs (Curtis et al., 2004).

3.1.1.6. Disc preparation and application

Filter paper discs (6 mm in diameter) were sterilized by autoclaving, each sterile disc was then soaked overnight in a solution containing (100 µg of the ethanolic extract of *Rosa damascene,* each inoculated agar plate was divided into two sections:

In one section, a sterile disc soaked in (100 µg of the ethanolic extract was placed. In the second section, a standard antibiotic disc (containing 10 µg of *Ciprofloxacin* as a positive control for antibacterial tests) was placed using sterile forceps, for antifungal tests, a similar procedure was followed, using *Fluconazole* as the standard antifungal disc.

3.1.1.7. Pre-incubation diffusion

Once the discs were placed on the agar plates, the plates were kept at 4°C or room temperature for 1 hour to allow for diffusion of the extracts and antibiotics from the discs into the agar, this pre-incubation step ensured that the compounds from the discs began to interact with the microbial cells before active growth occurred. After the pre-diffusion step, the plates were transferred to an incubator set to 37°C for 24 hours for bacterial strains and 28°C for 48-72 hours for fungal strains, the closed plates were incubated in an inverted position (agar side up) to prevent condensation on the lid, which could interfere with microbial growth. After incubation, the zones of inhibition (clear areas where microbial growth was prevented around the discs) were measured. A ruler, divider, or Vernier caliper was used to measure the diameter of the inhibition zone in millimeters. For accuracy, two perpendicular measurements of the zone diameter were taken, and the average was recorded, the average diameters of the inhibition zones were recorded for both the ethanolic extract and the standard control disc (Deattu et al., 2012).

3.1.2. Assessment of antifungal and antibacterial activates of rosa damascene ethanolic extracts

3.1.2.1. Source of microbial cultures

Fungal strains were obtained from preserved laboratory slant cultures, stored at 4°C to ensure long-term viability, these slants contained actively growing fungal cells, maintained in nutrient-limiting conditions.

3.1.2.2. Preparation of inoculum

In this experiment, the efficacy of the *Rosa damascena* ethanolic extract on fungal strains was determined by conducting in vitro antifungal susceptibility testing on five different fungal strains (*Candida albicans* SC5314, *Aspergillus niger* ATCC 16404 and *Trichophyton* ATCC 9533). A loopful of the preserved fungal slant culture was streaked or inoculated into a test tube containing a fresh nutrient enriched *Sabouraud Dextrose Broth (SDB)*, suitable for fungal growth (Francis et al., 2024).

3.1.2.4. Incubation and standardization

The broth culture was further grown at 25-30°C for 24-48 hs so that the fungi develop well enough for identification. After reaching log phase *(Optimal Growth Phase)*, the relative density of the suspension was measured visually and the fungal inoculum was standardized to the 0.5 McFarland turbidity, which is equivalent to approximately (1\times 10^8\) colony-forming units per millilitre). If turbidity of the sample was too high in there, the suspension was diluted by adding sterile saline solution or fresh broth. On the other hand, if the turbidity was very low, more incubation was kept in the water to allow the bacteria to produce turbidity (Gavra et al., 2022).

3.1.2.5. Assessment of antibacterial activity of *rosa damascena* extracts

In this initial description of the antibacterial potential of *Rosa damascena* ethanolic extracts, inhibition zones were determined around both the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli*. Preparation of Sterile Swabs: Sterile cotton wool swabs were prepared by tightly winding clean cotton onto sterile wooden or plastic applicators. For plastic swabs, the swabs were sterilized using autoclaving (121°C, 15 psi, for 20 minutes). For wooden swabs, dry heat sterilization was used (160°C for 2 hours). The wooden swabs were placed in sterile culture tubes or sterile paper wrapping to maintain their sterility, the swabs were stored in sterile containers until use to avoid contamination, and the forceps used for handling the discs were sterilized by immersing them in 70% ethanol, followed by immediate flaming to burn off the ethanol. This method ensured aseptic conditions during disc placement Figure 1 (Moussaoui and Alaoui, 2016; Hassand et al., 2024).

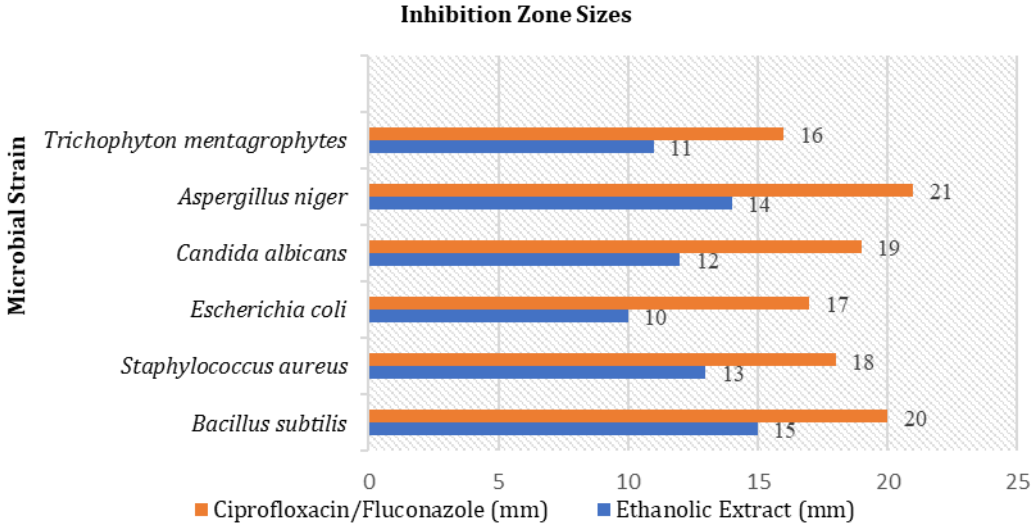

Figure 1. It illustrates the antifungal and antibacterial activity results using a bar chart. Each bar represents the diameter of inhibition zones for different microorganisms, comparing the *ethanolic* extract to the standard control disc (antibiotic and antifungal agent) across various bacterial and fungal strains, enabling a direct comparison of effectiveness.

3.1.2.6. Selection of fungal strains for antifungal activity testing

The following fungal strains were selected for the antifungal evaluation: *Candida albicans (SC5314)* - a common yeast responsible for candidiasis, *Aspergillus niger* (*ATCC 16404)* - a filamentous fungus that causes aspergillosis, and *Trichophyton mentagrophytes (ATCC 9533*) - responsible for *dermatophytosis*, these fungi were chosen for their clinical relevance and common pathogenicity.

3.1.2.7. Inoculating the plates, agar medium preparation and drying the inoculated plates

Sterile Sabouraud Dextrose Agar (SDA) plates were prepared and allowed to cool. SDA provides a nutrient-rich environment conducive to fungal growth, a sterile cotton swab was dipped into the standardized fungal inoculum. Excess inoculum was removed by pressing and rotating the swab against the inner side of the culture tube above the liquid level, the swab was streaked across the surface of the SDA plates in a systematic manner, the streaking was done in three directions, rotating the plate by 60 degrees after each streak, ensuring even distribution of the inoculum, the swab was passed around the edge of the plate for complete coverage of the agar surface, the inoculated plates were left at room temperature for 10-15 minutes with their lids closed, allowing the fungal inoculum to absorb into the agar surface Figure 2 (Saghafi et al., 2021).

3.1.2.8. Procedure for antifungal and antibacterial testing

The standardized fungal and bacterial inoculums were aseptically introduced onto the prepared agar plates using the method described above, ensuring even distribution across the surface, each Petri dish was divided into two sections: In one section, a sterile paper disc (6 mm in diameter) soaked in 100 mg of the *Rosa damascena* ethanolic extract was placed. The discs had been prepared by soaking overnight in the extract solution to enhance potency Figure 3 (Shi et al., 2024).

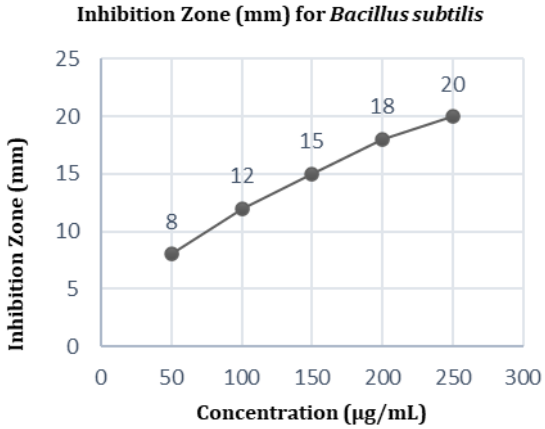

Figure 2. The graphical representation illustrates the relationship between the concentration of the extract (µg/mL) and the inhibition zone (mm) for *Bacillus subtilis*. The X-axis represents the concentration of the extract, while the Y-axis shows the corresponding inhibition zone in millimeters. The trend indicates that as the concentration of the extract increases, the inhibition zone also expands, suggesting a dose-dependent antibacterial effect.

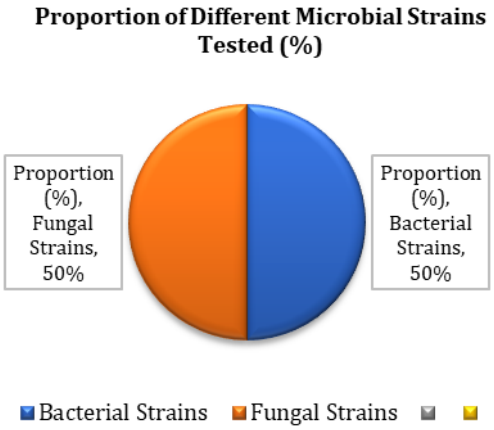

Figure 3. It illustrates the distribution of microbial strains tested for antifungal and antibacterial activity through a pie chart. The chart displays the proportions of strains such as *Candida albicans*, *Aspergillus niger*, and *Trichophyton mentagrophytes*, providing a clear visual of the microbial diversity in the experimental sample.

In the second section a control disc of *Fluconazole* (20 µg) was incorporated during the antifungal susceptibility testing. In the antibacterial testing, *Ciprofloxacin* (10 µg) was used as the control antibiotic disc. Both extract and control discs were applied onto the plates with the aid of the sterile forceps to prevent contamination. After the discs were placed on the inoculated agar plates, the plates were either: Kept in the refrigerator at 4°C for an hour with slow diffusion or left at room temperature to permit the extract and the standard drugs to diffuse into the surrounding medium. After the diffusion period, the plates were incubated at appropriate temperatures:

For bacterial cultures, plates were incubated at 37°C for 24 hours. For fungal cultures, plates were incubated at 25-30°C for 24-48 hours. The plates were placed in an inverted position (agar side up) during incubation to prevent condensation from dripping onto the agar surface, which could interfere with microbial growth.

3.1.2.9. Observation and measurement and data recording

After incubation, the plates were carefully examined for zones of inhibition (clear zones around the discs where microbial growth was prevented by the extract or control agent), the diameter of each zone of inhibition was measured in millimeters (mm) using a Vernier caliper, ruler, or divider, two perpendicular diameters were recorded for each zone, and the average diameter was calculated, the zones of inhibition were recorded for both the ethanolic extract of *Rosa damascena* and the standard antimicrobial agents (*Fluconazole* and *Ciprofloxacin*), the results were compared to assess the efficacy of the extract in inhibiting the growth of the selected bacterial and fungal strains.

3.1.2.10. Minimum inhibitory concentration (mic) determination for *rosa damascena* ethanolic extract

The Minimum Inhibitory Concentration (MIC) is a critical measure used to determine the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism after incubation. This method involves precise preparation of both the test antimicrobial extract and the microbial inoculum, followed by a series of dilutions to assess the effectiveness of the extract at varying concentrations.

3.1.3. Preparation of test drug (extract) for mic testing

3.1.3.1. Stock solution preparation

The ethanolic extract of *Rosa damascena* was first prepared by dissolving the dried extract in Dimethyl Sulfoxide (DMSO) or sterile distilled water, this served as the stock solution, the concentration of the stock solution was carefully adjusted to be 1,000 µg/mL by dissolving a precise amount of the extract in the solvent. For instance, to prepare a 1,000 µg/mL stock solution, 100 mg of extract was dissolved in 100 mL of solvent, to establish a range of concentrations for MIC determination, serial two-fold dilutions of the stock solution were performed using Muller-Hinton Broth (MHB) as the diluent, a series of 10 to 15 dilutions were prepared to cover a concentration range from 100 µg/mL to 1.56 µg/mL, this range ensures a broad spectrum to evaluate the antimicrobial potential of the extract.

3.1.3.2. Stepwise serial dilution

One milliliter of Muller-Hinton Broth was introduced into each of 10 sterile test tubes with numbers labelled. Tube 1 contained 1 mL from the 1,000 µg/mL stock solution diluted to yield 100 µg/mL in the final solution, 1 mL of the solution in tube 1 was then transferred to tube 2 resulting in another dilution to 50 µg/mL; the process

continued to the final dilution of 1.56 µg/mL in tube 10; in each step capping and shaking the tubes (Shohayeb et al., 2014).

3.1.3.3. Preparation of inoculum for MIC testing

From the study the target bacterial strains were *Escherichia coli (K-12), Staphylococcus aureus (ATCC 25923), Pseudomonas aeruginosa (PAO1)* and *Bacillus subtilis (168).* Inoculation of each bacterial strain was done in Muller-Hinton Agar (MHA) plates and incubated at 37°C for 24 hours to obtain fresh, pure colonies of the bacteria. In the overnight culture, well isolated colony was picked up and enriched in Muller-Hinton Broth (MHB) at 37°C for 18-24h. After incubation, the bacterial suspension was standardized to match 0.5 McFarland standard, which corresponds to a bacterial density of approximately 1-2 × 10^8 CFU/mL, to achieve this, the overnight culture was diluted using sterile Muller-Hinton Broth to create a 1:1% dilution or 10 ^ -2 molar concentration. This dilution makes the bacterial inoculum in the right concentration for the MIC assay as stated by (Talib and Mahasneh, 2010).

3.1.3.4. MIC test setup

Eight sterile test tubes were labeled from 1 to 8. Tubes 1 to 6 were designated for the serial dilutions of the extract. Tube 7 was used for the lowest extract concentration (0.5 mL), and tube 8 served as the negative control. 1 mL of Muller-Hinton Broth was added to each of the first five tubes. Tubes 6 and 7 each received 0.1 mL of the broth.

3.1.3.5. Inoculation of tubes with extract

In the first tube (Tube 1), 1 mL of the stock extract solution was added and thoroughly mixed to achieve the highest concentration of the antimicrobial agent (100 µg/mL), From Tube 1, 1 mL of the solution was transferred to Tube 2, mixed thoroughly, and this process was continued for Tube 3 through Tube 6, in Tube 7, 0.5 mL of the diluted extract solution from Tube 6 was added to achieve the lowest concentration for testing, tube 8 was left as a control, containing only the Muller-Hinton broth and inoculum, but no extract. Following the serial dilutions of the test extract, 0.1 mL of the standardized inoculum (10^-2 bacterial suspension) was added to each of the test tubes (Tubes 1 through 7), Tube 8 was treated similarly by adding 0.1 mL of inoculum but served as the growth control, ensuring no antimicrobial agent was present (Waksman and Reilly, 1945).

3.1.3.6. Incubation, measurement and conditions

All test tubes were incubated at 37°C for 18-24 hours in a shaking incubator to ensure thorough mixing and optimal growth conditions for the microorganisms, the incubation period allowed sufficient time for the bacterial cells to interact with the different concentrations of the *Rosa damascena* extract and grow in the absence of inhibitory concentrations. After the incubation period, each tube was visually inspected for turbidity, which indicates microbial growth, clear tubes suggest inhibition, while cloudy tubes signify microbial proliferation, for more accurate determination of MIC values, spectrophotometric readings were taken using a spectrophotometer set at an optical density (OD) of 600 nm, the OD value for each tube was recorded to quantify the microbial growth, lower OD values indicate less microbial growth due to the inhibitory effects of the extract, while higher values indicate substantial growth, the MIC is defined as the lowest concentration of the extract that resulted in no visible turbidity (clear solution) or no significant increase in OD compared to the control tube (Tube 8), for example, if Tube 5 (containing 6.25 µg/mL of the extract) is the first tube that appears clear (or shows no significant increase in OD compared to the control), then the MIC of the *Rosa damascena* extract against the tested bacterial strain would be reported as 6.25 µg/mL, the MIC values for each tested microorganism are compared to standard MIC values for known antimicrobial agents (*Fluconazole* and, *Ciprofloxacin*), lower MIC values suggest that the *Rosa damascena* ethanolic extract has strong antimicrobial properties, while higher MIC values indicate lower efficacy (White, 1965).

The ethanolic flower extract of *Rosa damascena* exhibits both antibacterial and antifungal properties, with greater potency against gram-positive bacteria and certain fungal strains, this could be attributed to the presence of bioactive compounds such as flavonoids, terpenes, and phenolic acids in the extract, the antimicrobial properties of these compounds disrupt the cell walls and membranes of pathogens, leading to cell death. For the antibacterial activity screening of the ethanolic flower extract of *Rosa damascena*, bacterial strains including *Bacillus subtilis 168, Staphylococcus aureus ATCC 25923*, and *Escherichia* coli *K-12* were utilized, in the antifungal screening, the following fungi were tested: *Aspergillus niger ATCC 16404, Aspergillus fumigatus AF293, Monascus purpureus ATCC 1008,* and *Candida albicans SC5314*.

Table 1. Antibacterial and Antifungal Properties of the Ethanolic Flower Extract of *Rosa damascena*

Zone of Inhibition (mm)									
Microorganism (strain tested)	Type	Inhibition by Extract	Mechanism of Action	*Rosa damascena* flower Extract (100µg)	Standard *ciprofloxacin* (10µg)	Observation and Measurement	MIC for *Rosa damascena* Extract (µg/mL)	Preparation and Incubation Conditions	Effectiveness
Bacteria									
Staphylococcus aureus	Gram-positive cocci	Significant	-	10 mm	18 mm	Zones of inhibition measured with Vernier caliper; average diameter calculated from two measurements	6.25	Inoculated on Muller-Hinton Agar; incubated at 37°C for 24 hours; standardized inoculum (0.5 McFarland) used for MIC testing	Significant inhibition against Gram-positive bacteria; effective at moderate concentrations
Escherichia coli	Gram-negative rod	Moderate	Outer membrane barriers	8 mm	15 mm	Clear zones around discs measured; lower susceptibility observed for Gram-negative bacteria	12.5	Prepared through serial two-fold dilutions from 1000 µg/mL to 1.56 µg/mL; incubated at 37°C with shaking for 18-24 hours	Moderate inhibition due to outer membrane barriers
Bacillus subtilis	Gram-positive rod	Highly susceptible	-	9 mm	12 mm	Zones accurately recorded and averaged; substantial inhibition at low MIC values	3.12	Stock solution prepared in DMSO; incubated at 37°C for 24 hours; bacterial suspension standardized to 1-2 × 10^8 CFU/mL	Good antibacterial action; effective at low MIC values
Fungi									
Aspergillus fumigatus	Filamentous fungus	Moderate	Increased cell permeability, oxidative damage	13 mm	22 mm	Zones measured, averaged, and compared to *fluconazol*e for assessment of antifungal efficacy	25	Serial dilutions prepared; incubated at 37°C for 18-24 hours in shaking incubator; turbidity visually inspected for MIC	Moderate susceptibility; antifungal effects at higher concentrations
Monascus purpureus	Mold	Mild	Inhibition of metabolic pathways	11 mm	20 mm	Inhibition zones recorded and compared; mild inhibition observed at standard extract concentrations	50	MIC test performed with diluted stock solution; incubated at 37°C; measurements taken at 600 nm OD for accuracy	Lower effectiveness; resistance observed, requiring higher concentrations
Candida albicans	Yeast	Significant	Disruption of cell membrane, inhibition of enzymes	11 mm	13 mm	Measurement of inhibition zones using standard techniques; consistent inhibition pattern observed	12.5	Inoculated on selective agar; incubated at 37°C; MIC readings recorded for OD consistency	Good antifungal activity; effective in inhibiting common pathogenic yeast
Aspergillus niger	Filamentous fungus	Moderate	Oxidative stress, cell wall disruption	10 mm	11 mm	Zones of inhibition observed; moderate inhibition indicating extract's limited potency against filamentous fungi	37.5	Prepared using 1:10 dilutions; inoculated and incubated with appropriate controls; OD measured post-incubation	Moderate inhibition; higher concentrations needed compared to yeast-like fungi

Table 1. Illustrate The *Rosa damascena* ethanolic flower extract generally exhibits better antibacterial activity against Gram-positive bacteria compared to Gram-negative bacteria. Gram-negative bacteria tend to have lower susceptibility due to the presence of an outer membrane that acts as a barrier to many antibacterial agents. The extract shows significant inhibition against bacterial strains such as *Staphylococcus aureus ATCC 25923*, *Bacillus subtilis 168,* and *Listeria monocytogenes EGD-e*, even at lower MIC values. Resistant strains are also somewhat susceptible to the extract, which suggests its potential in combating drug-resistant bacterial infections. In terms of antifungal activity, the extract's effectiveness is detailed in Table 1, where the micro-organisms listed include, pathogenic fungal species used in the study. The inhibition by the ethanolic extract is categorized as mild, moderate, or significant, depending on the observed level of antifungal activity. The mechanism of action varies, with the extract acting through mechanisms such as increasing cell permeability, causing oxidative damage, or inhibiting metabolic pathways. The zone of inhibition (mm) is measured to

assess the antifungal potency, with larger zones indicating more effective inhibition. The extract showed moderate to significant antifungal activity against the tested strains, with varying mechanisms of action. The standard antifungal agent, *fluconazole*, generally produced larger inhibition zones, indicating its higher potency compared to the plant extract.

The study revealed that the ethanolic flower extract of *Rosa damascena* exhibited the highest antibacterial activity against *Staphylococcus aureus*, with a recorded zone of inhibition measuring 12 mm, indicating a significant capacity to impede the growth of this organism. In contrast, the antibacterial activity against the other bacterial strains, *Bacillus subtilis 168* and *Escherichia coli K-12*, was observed to be moderate, as reflected in the zone of inhibition of 10 mm for both strains during the disc diffusion assay. Notably, the extract produced a relatively similar zone of inhibition across all tested bacterial strains, suggesting consistent antibacterial properties.

This investigation clearly indicates that the antimicrobial activity of the flower extract may vary slightly depending on the bacterial strain being tested. Typically, plant extracts tend to show greater efficacy against Gram-positive bacteria compared to Gram-negative bacteria. In this context, the present study screened the antibacterial effects of *Rosa damascena* flowers, and the results demonstrated moderate activity against all tested bacterial strains when compared to the standard antibiotic, *ciprofloxacin*, this investigation confirms that the flower extract of *Rosa damascena* possesses antibacterial properties against both Gram-positive and Gram-negative bacterial strains, highlighting its potential as a natural antimicrobial agent.

3.1.3.7. Minimum inhibitory concentration in ethanolic flower extract of *rosa damascena*

The minimum inhibitory concentrations (MIC) of the ethanolic flower extract of *Rosa damascena* were evaluated against three bacterias and four funguses, including *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Candida albicans, Aspergillus niger, Monascus purpureus*, and *Aspergillus fumigatus*, the results of this study are presented in the accompanying in table 3, which details the MIC values for each of the tested microbial strains.

In this investigation, the minimum concentration at which there was an absence of microbial growth was systematically determined. This critical parameter provides valuable insight into the effectiveness of the ethanolic extract in inhibiting the growth of various pathogenic organisms, by identifying the MIC for each microorganism, the study contributes to understanding the potential of *Rosa damascena* as a natural antimicrobial agent, highlighting its relevance in the development of alternative treatments for infections caused by these bacteria and fungi.

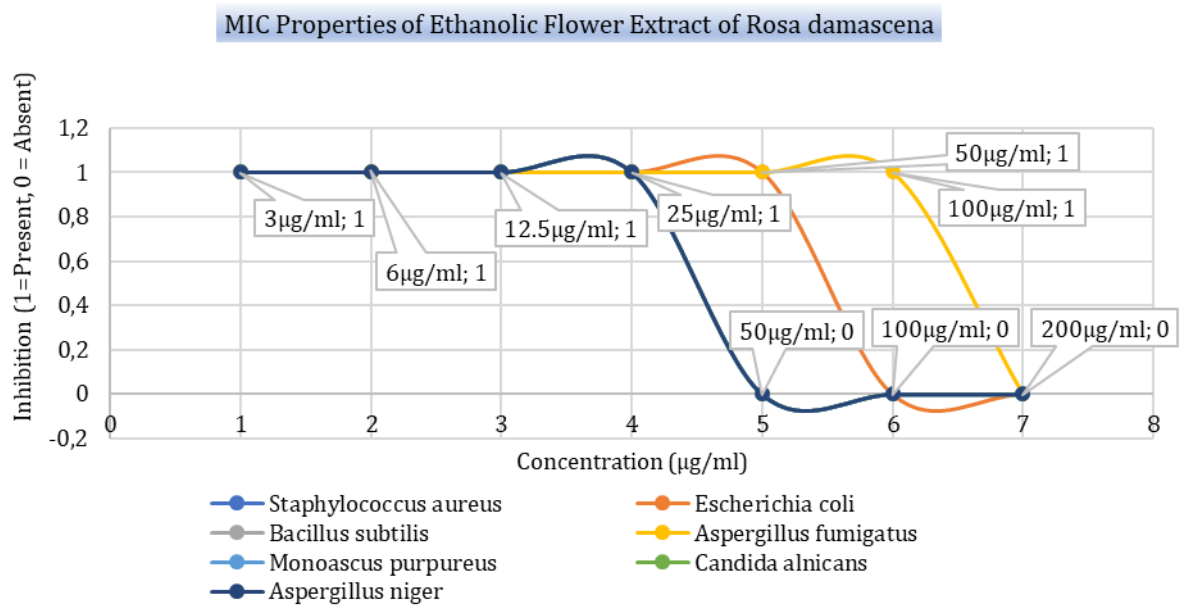

Figure 4. MIC properties of the ethanolic flower extract of *Rosa damascene* against following micro-organism (Pathogen data shown at different concentrations of the substance (in 3-200µg/ml).

Detailed Explanation of Figure 4. Pathogen: Lists the bacterial and fungal species used in the study to assess the MIC of *Rosa damascena* ethanolic extract. Concentrations (µg/ml): Ranges of concentrations tested, from 3 µg/ml to 200 µg/ml. Symbols (+/-): (+) indicates microbial growth, meaning that the concentration of the extract was not sufficient to inhibit growth at that level. (-) indicates no microbial growth, showing that the extract was effective in inhibiting the growth at that concentration. Findings: For *Staphylococcus aureus ATCC 25923* and *Bacillus subtilis 168*, inhibition started at concentrations between 25 µg/ml and 50 µg/ml. *Escherichia coli K-12* required a higher concentration (50 µg/ml) for inhibition. *Aspergillus fumigatus AF293* and

Aspergillus niger ATCC 16404 exhibited inhibition starting at 50 µg/ml. Fungal pathogens *Monascus purpureus ATCC 1008* and *Candida albicans SC5314* showed no growth at 50 µg/ml or higher. This data provides an overview of the antimicrobial efficacy of *Rosa damascena* extracts, demonstrating higher concentrations are needed to inhibit Gram-negative bacteria and certain fungi, while Gram-positive bacteria show susceptibility at lower concentrations).

The present study revealed that antimicrobial properties of ethanolic extract of *Rosa damascena* flowers are effective against a number of different bacteria. Among all the tested microorganisms, *S. aureus, C. albicans, A. niger, Monoscus purpures*, and *Aspergillus fumigates* were found to be highly sensitive to the flower extract. As illustrated in Table 3, their development was nearly suppressed at the concentration of 50µg/ml of the extract. This suggests that at a molecular level, the *Rosa damascena* extract possesses the capability to inhibit these particular bacterial and fungal strains robustly at very low concentrations.

Escherichia coli K-12 and *Bacillus subtilis 168* had the higher tolerance towards the extract, and above this concentration their growth was significantly affected, thus the minimum inhibitory concentration (MIC) of extract against these two bacterial strains was observed to be 100µg/ml. Nevertheless, it is significant to understand that *Rosa damascena* was still capable of exhibiting stronger antimicrobial effect in companionship with these microorganisms but with more concentrated extract.

In the present study, the percentage growth inhibition of the extract at the concentration of 100µg/ml was high both in case of antifungal and antibacterial activities for all the tested microorganisms. Therefore, based on these findings, it can be concluded that the extract derived from *Rosa damascena* flowers displays versatile antimicrobial activity against both bacteria and fungi at moderate concentrations. These results therefore provide credence to the application of this flower extract in the formulation of natural antimicrobial agents especially in addressing infections by both the Gram-positive and Gram-negative bacteria in addition to different species of fungal forms. Focusing more exploration on the properties of the bioactive compounds present in *Rosa damascena* could lead to the discovery of even more effective natural substitutes for stan dard antimicrobial treatments. Thus, by purifying these compounds, the authors could determine particular molecules that would be responsible for the plant's antibacterial activity and be useful in developing effective and eco-friendly methods for dealing with bacteria and mold, which would be especially important given the increase in antibiotic resistance and the need to find new forms of treatment that would not harm the environment.

Table 2. Some of the distinguishing features between MIC, antifungal and antibacterial properties of *Rosa damascena* extract are stated below

Property	MIC Properties	Antifungal Properties	Antibacterial Properties	Comparison
Concentration Range	3 - 200 µg/ml	100 µg/ml	100 µg/ml	MIC concentration for antifungal and antibacterial action varies.
Tested Microorganisms	Bacteria & Fungi	Fungi	Bacteria	Includes both Gram-positive, Gram-negative bacteria, and fungi.
Pathogens	- *Staphylococcus aureus* - *Escherichia coli* - *Bacillus subtilis* - *Aspergillus fumigatus* - *Monascus purpureus* - *Candida albicans* - *Aspergillus niger*	- *Aspergillus fumigatus* - *Monascus purpureus* - *Candida albicans* - *Aspergillus niger*	- *Staphylococcus aureus* - *Escherichia coli* - *Bacillus subtilis*	Specific pathogens tested across both antimicrobial activities.
Mechanism of Action	General inhibition of growth at varying concentrations	- Cell membrane permeability - Oxidative damage - Inhibition of metabolic pathways	Not specified for antibacterial action	Mechanisms for antifungal action are more specific.
Effectiveness by Organism	- Effective at 50 µg/ml against *Staphylococcus aureus, Bacillus subtilis, and Candida albicans* - Higher concentration (100 µg/ml) required for *Escherichia coli* and fungi like *Aspergillus fumigatus and Aspergillus niger*	- Most effective against *Candida albicans* (11 mm zone of inhibition) - Moderate effectiveness against filamentous fungi like *Aspergillus fumigatus and Aspergillus niger*	- Significant inhibition against Gram-positive *bacteria, e.g., Staphylococcus aureus* (12 mm zone of inhibition) - Moderate inhibition for *Gram-negative bacteria, e.g., Escherichia coli*	The effectiveness varies based on microorganism type and concentration used.
Zone of Inhibition	Not measured in MIC studies	Ranged from 10-13 mm for fungi	Ranged from 10-12 mm for bacteria	Zone of inhibition varies by pathogen and extract concentration.
Reference Standards	No reference drug used	Compared to *fluconazole* (20 µg/ml)	Compared to *ciprofloxacin* (10 µg/ml)	No reference drug for MIC properties; antifungal and antibacterial efficacy compared to standard drugs.

3.2. Future perspectives and challenges

3.2.1. Another area of interest is the synthesis, isolation and identification of new bioactive compounds

Thus, the exploration of antibacterial and antifungal properties of *Rosa damascena* flower extracts ushers the world to further research in the extraction of new bioactive compounds. As such, there is infinite scope for the discovery of fresh phytochemical species that may demonstrate new modes of antibacterial activity or may have a different range and spectrum of efficacy. Further studies could include the isolation of the molecules that cause the observed effects to be able to come up with more selective treatments, more variable research could be done on how these compounds work with microbial cells to provide a broader understanding on how these can be used in developing more localized treatments.

3.2.2. Interactions with other antibiotics

Because of the growing resistance rate to routine antibiotics, a future research direction is to examine the potential interaction between *Rosa damascena* extracts and conventional antibiotics. Together with plant extracts, chemical antimicrobial drugs could be made more effective, used in smaller concentrations, and have fewer side effects. Future research should try to implement different combinations in clinical trials in order to see if they have the ability to decrease or even halt the further development of antibiotic resistance.

3.2.3. Three applications: development of non-pharmacological treatments and natural conservants

The outcome of such studies could help to formulate plant derived antimicrobial agents and therapies compared to synthetic antibiotics, especially for resistant strains. Furthermore, more so, the extracts obtained from *Rosa damascena* can also be used as preservatives in food processing and cosmetics since they contain natural antimicrobial properties which consumers are leaning towards in the present world than using chemicals. Other researchers can aim at fine-tuning the extraction techniques and increasing the yield to suit the industrial requirements, and assessing long-term effects of these products.

3.2.4. Standardization and quality control have been mentioned as some of the challenges encountered in the development of learning objects

Pronostic and perspectives: Among the numerous problems that must be solved in the future, the issue of establishing an international standard for the extracts of *Rosa damascena* appears to be one of the most significant. Different growing conditions, plant cultivars, harvesting times, and extraction methods can cause differences in the bioactive compound content; therefore, guidelines for how to obtain and measure the effective compounds need to be established. Setting high quality control measures that will be accepted by the regulatory authorities will be paramount for using these extracts in therapeutic and commercial purposes.

3.2.5. Clinical trials and toxicological studies

Often times, when initial studies are conducted on tissue cultures, the results could be very encouraging, but the biggest challenge comes when it has to be developed for human use. Well-controlled clinical trials and meta-analyses are required before asserting the safety, effectiveness, and adverse effects of *Rosa damascene* extracts in clinical populations. These are crucial in the determination of safe dosages, and also the examination of any health risks associated in the periodic use of the product under test. Further studies should also focus on translating the findings from the laboratory to the real-world practices.

3.2.6. Regulatory and commercialization hurdles

There is a challenge in the commercialization of *Rosa damascena* extracts as they function as antimicrobial agents that comes with legal constraints. It may take time and go through lengthy and complicated procedures to get approval from the regulatory body for new plant-based medicines on discoveries of naturally occurring substances, there might be issues relating to intellectual property rights. These barriers will need the collaboration of researchers, pharmaceutical companies, and governmental regulatory bodies to tackle after defining the roles of each for the simplification of the process involved all in the appraisal of new drugs without compromising the drug's quality and effectiveness.

3.2.7. Environmental and economic sustainability

As the demand for natural antimicrobials increases in the future, the supply of *Rosa damascena* for its production will play a critical role. Excessive fishing and farming practices also have negative impacts on the environment, for example, through soil erosion and loss of biological diversity. Due to this, it is important that subsequent attempts are aimed at producing crops in a manner that is friendly to the environment in order to feed the ever expanding population. Further, studies that will focus on the possibility of large-scale plantations and extraction of *Rosa damascena* will be critical to guarantee the products' market pricing Figure 5.

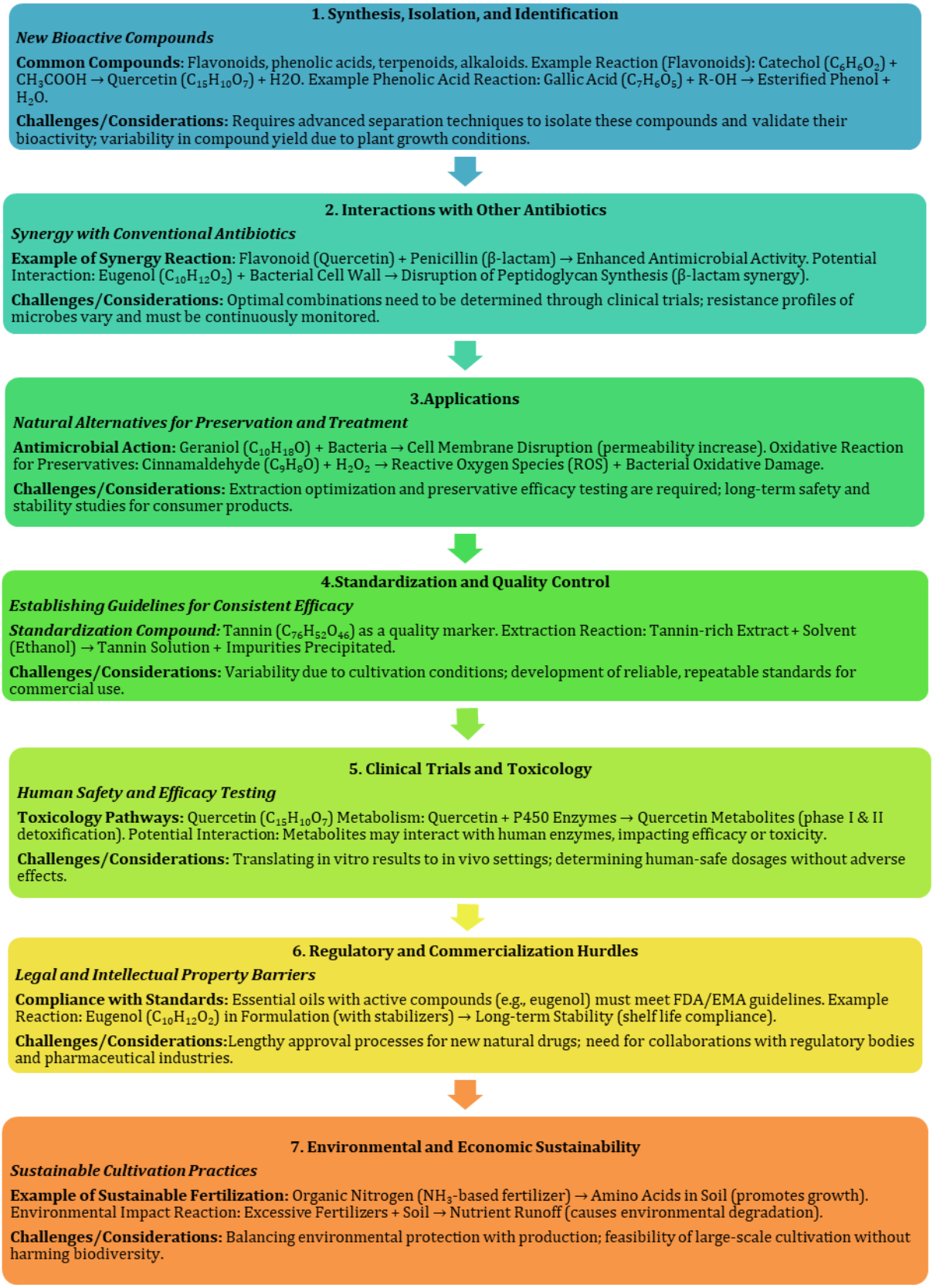

Figure 5. Future prospects for *Rosa damascena* extracts emphasize isolating potent bioactives for innovative antimicrobial applications. Synergizing these extracts with antibiotics could amplify efficacy and mitigate resistance, while their natural preservative properties show promise for food and cosmetic sectors. Rigorous standardization and clinical validations are imperative to ensure therapeutic safety, yet regulatory and commercialization obstacles necessitate strategic industry collaboration. Additionally, sustainable cultivation practices are crucial to align increased production with ecological stewardship.

4. Discussion

The findings of this study confirm the notable antimicrobial potential of the ethanolic extract from *Rosa damascena* flowers, supporting its traditional application in treating microbial infections. The extract demonstrated a broad-spectrum activity against both Gram-positive and Gram-negative bacterial strains, as well as multiple fungal species, which is especially relevant given the current global challenge of antimicrobial resistance. Observing effective inhibition zones in the disc diffusion method and obtaining minimum inhibitory concentration (MIC) values that quantify the extract's potency provide a reliable basis for recognizing *Rosa damascena as* a natural source of antimicrobial agents. This result aligns well with prior studies that have highlighted the medicinal properties of this plant, particularly in folk medicine.

The inhibitory effects of *Rosa damascena* were comparable to standard antibiotics such as *ciprofloxacin* (for antibacterial activity) and *fluconazole* (for antifungal activity). This suggests that the extract contains bioactive compounds capable of exerting effects similar to conventional antibiotics, albeit through potentially different mechanisms of action. The presence of secondary metabolites, as observed in the drastic decrease in bacterial and fungal growth, may account for these effects. Phenolic compounds, flavonoids, and other phytochemicals commonly found in rose extracts are known to disrupt microbial cell walls, inhibit protein synthesis, and impact nucleic acid functions, which could explain the antimicrobial properties observed in this study.

The extract's success in inhibiting growth across diverse microbial species emphasizes its potential as a broad-spectrum antimicrobial agent. Importantly, the extract showed effectiveness against *Staphylococcus aureus ATCC 25923* and *Escherichia coli K-12*, which are common pathogens responsible for various human infections, as well as against *Candida albicans SC5314*, a prevalent fungal pathogen. This wide range of activity could have significant implications for clinical applications, particularly in light of the rise in resistant microbial strains and the limitations of synthetic antimicrobials. Additionally, the findings raise the possibility of developing *Rosa damascena* extracts into topical antimicrobial products, especially for skin infections caused by bacterial and fungal pathogens.

The study also underscores the importance of further investigating the specific bioactive components responsible for the extract's antimicrobial properties. Identifying these compounds could lead to the isolation of new, plant-based antimicrobial agents, with potential applications in both pharmaceutical and cosmetic industries, the study has laid a foundation by demonstrating the antimicrobial efficacy of *Rosa damascena*, future research should focus on the chemical characterization of its active components, the mechanisms of antimicrobial action, and the evaluation of its safety and efficacy in vivo. The synergistic effects of *Rosa damascena* flower extracts with conventional antibiotics could enhance their effectiveness, offering a valuable addition to the fight against resistant pathogens. The observed antimicrobial activity of these extracts shows significant potential for addressing microbial infections and reducing dependence on synthetic antibiotics. Building on the promising findings of this preliminary study, comprehensive in vitro and in vivo investigations are essential to confirm the extract's therapeutic potential. This work adds to the growing body of research supporting the use of medicinal plants as sustainable and effective alternatives in antimicrobial therapy, addressing the urgent global challenge of antimicrobial resistance.

5. Conclusion

The ethanolic extract of *Rosa damascena* flowers demonstrated significant antimicrobial activity against a variety of clinically isolated bacteria and fungi, showing effectiveness comparable to standard antimicrobial drugs, this supports the traditional use of *Rosa damascena* in treating infections caused by microorganisms and highlights its broad-spectrum potential. Both Gram-positive and Gram-negative bacteria, as well as several fungal species, were inhibited by the extract, indicating its applicability as a natural antimicrobial agent. While the findings provide a solid foundation for further exploration of the plant's bioactive compounds, more in-depth research is needed to fully assess the extract's antimicrobial efficacy, ongoing studies are focused on isolating and identifying the specific components responsible for its antibacterial and antifungal properties, which could lead to the discovery of new plant-based antimicrobial agents, the study highlights the extract's potential applications in the pharmaceutical and cosmetic industries for developing natural antimicrobial products, the ethanolic extracts of *Rosa damascena* flowers are expected to exhibit inhibitory effects against the tested bacterial and fungal pathogens, indicating their potential as antimicrobial agents, the MIC and zone of inhibition data will help quantify this activity, the disc diffusion method provided clear zones of inhibition that were measurable and allowed for a comparative assessment of the efficacy of the extract against both bacterial and fungal pathogens, the MIC determination method is essential for evaluating the antimicrobial potency of natural extracts such as *Rosa damascene*, by preparing a series of dilutions and assessing the growth inhibition at different concentrations, the lowest effective concentration can be identified, providing insight into the extract's potential as a therapeutic antimicrobial agent, this process is crucial for both antibacterial and antifungal activity assessments, the assessment of the antibacterial and antifungal activities of ethanolic flower extracts from *Rosa damascena* holds great promise for addressing the growing issue of antimicrobial resistance.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	P.N.	O.A.	A.B.H.
C	80	10	10
D		50	50
S	100		
DCP	100		
DAI	100		
L	60	20	20
W	60	30	10
CR	60	20	20
SR	50	50	
PM		100	
FA		100	

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declare that the research was conducted without any financial and personal relationships with other people or organization that could inappropriately influence or bias the work.

Ethical Consideration

Ethics committee approval was not required for this study because there was no study on animals or humans.

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The author, Parwiz Niazi, responsible for Conceptualization, Data curation, Formal analysis, Writing-original draft and Supervision. Obaidullah Alimyar responsible for funding acquisition, Software, Validation and Writing-review and editing. Abdul Bari Hejran responsible for Investigation, Methodology, Resources, Software, Validation, Visualization, Writing - review and editing.

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PERCEPTIONS AND STRATEGIES FOR SUSTAINABLE AGRIBUSINESS ENTREPRENEURSHIP DEVELOPMENT IN NIGERIA: INSIGHTS FROM STAKEHOLDERS

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Abstract: Despite Nigeria's significant potential for agribusiness entrepreneurship, its agricultural productivity remains below regional and global averages, hindered by limited entrepreneurial competence, infrastructural deficits, and inconsistent policy frameworks. These challenges necessitate a focused investigation into stakeholder perceptions and strategies for overcoming internal and external barriers to agribusiness development and fostering sustainable practices that engage youth and drive economic growth. This study aims to assess the perceptions of stakeholders regarding the development of agribusiness entrepreneurship in Nigeria. The research involved two phases: first, identifying the internal strengths and weaknesses, as well as external opportunities and threats, perceived by stakeholders as major obstacles to agribusiness entrepreneurship development in Nigeria; second, gathering and analyzing stakeholders' opinions on the effectiveness of sustainable agribusiness development strategies. Using a qualitative approach, data were collected through 28 in-depth interviews, four focus group discussions, and interviews with key informants across four states: Kano, Benue, Cross River, and Oyo. Selected crops included rice, maize, yam, oranges, cassava, and palm oil. Major actors in agribusiness were engaged to provide insights into the sector's role in nutritional diets and income generation, particularly among youth. The study found that creating a favorable macropolicy environment, ensuring food safety and market supply consistency, enhancing human capacity, improving access to finance, and strengthening research capacity are crucial for sustainable agribusiness development. These strategies align with institutional economics and market failure theory, emphasizing the importance of supportive policies and quality control. The findings underscore the need for collaborative efforts between the public and private sectors to create an enabling environment for agribusiness entrepreneurship, aiming to boost productivity, competitiveness, and profitability while addressing economic and societal challenges.

Keywords: Sustainable agribusiness; Entrepreneurship; SWOT; Agrotourism; Rural infrastructure, Qualitative analysis

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1. Introduction

The challenge of addressing global food security is paramount, especially in low-income countries facing rapid population growth. By 2050, the world's population is projected to reach 9.7 billion, with a significant portion experiencing food insecurity. Sub-Saharan Africa, in particular, has severe food access issues, especially among children. Despite global food production being adequate, inefficiencies in value chain systems, notably in low-income nations, obstruct food distribution, contributing to hunger (Xie et al., 2021). In this context, agribusiness entrepreneurs play a crucial role in identifying opportunities within agriculture to achieve sustainable food production and distribution, particularly in sub-Saharan Africa.

Agribusiness entrepreneurship has garnered increasing attention due to population growth and limited alternative employment opportunities in the region. However, it faces substantial challenges such as limited access to capital, inadequate infrastructure, and inconsistent policy frameworks. Scholarly research has increasingly focused on evaluating the current landscape, identifying gaps, and offering insights for policymakers (Payumo et al., 2017; Olaoye, 2014). Nigeria, a significant player in agricultural income, struggles with lower productivity compared to regional and global averages, attributed to various factors such as natural conditions and a lack of agribusiness entrepreneurial competence (USDA-Economic Research Services, 2022).

Scholars have identified key determinants influencing agricultural productivity, including technology, physical and natural resources, and human capital. Human capital, in particular, plays a pivotal role in transforming these resources into actionable forms that drive business and economic growth (Ndour, 2017). Agribusiness entrepreneurship integrates this concept, focusing on leveraging innovative technological capital to optimize production while minimizing environmental impact. This managerial competency is vital for boosting productivity, especially in countries with above-average agricultural

performance (Escalante et al., 2006).

The adoption of entrepreneurial concepts along agricultural value chains is linked to increased productivity and growth imperatives for small agribusiness enterprises. This necessitates the adoption of disruptive innovation frameworks and youth engagement in agriculture (Markides, 2013; Christensen et al., 2015). In the context of Nigeria, where soil quality varies, agribusiness entrepreneurship has significant economic growth potential (Regmi and Naharki, 2020). However, challenges persist, including elderly farmers' resistance to new technologies and climate change impacts (Ikuemonisan et al., 2024).

Agribusiness entrepreneurship research is driven by the need for cutting-edge innovations to improve and sustain agricultural business (Regmi and Naharki, 2020; Bairwa et al., 2014). Managerial capacity, encompassing traits such as drive, ambition, creativity, and problem solving, is crucial for launching and running successful farm and agribusiness enterprises (Bairwa et al., 2014).

1.2. Objective

This study aims to assess the perceptions of stakeholders regarding the development of agribusiness entrepreneurship in Nigeria. The research process consisted of two phases. The first phase involves identifying the internal strengths and weaknesses, as well as external opportunities and threats, perceived by stakeholders as major obstacles to agribusiness entrepreneurship development in Nigeria. This information will be valuable in formulating future strategies for engaging young people in this field. The second phase involves gathering and analysing stakeholders' opinions on the effectiveness of sustainable agribusiness development strategies in Nigeria. The research questions for this study are as follows: 1) What are the primary concerns of stakeholders regarding the internal and external factors that impact the development of agribusiness entrepreneurship in Nigeria? 2) What do stakeholders in the agricultural sector think about the feasibility of implementing global agribusiness practices and strategies for fostering sustainable agribusiness development in Nigeria?

Table 1 provides an explanation of the research questions listed to achieve the objectives of the study.

Table 1. Explanation of research questions*

S/N	Leverage Points	Research Questions
1	Strength	i. What advantage (strength) does Nigeria have over other countries in agriculture? ii. How strong is Nigeria's institutional framework for achieving sustainable agriculture and competitive agribusiness?
	Weakness	i. What characteristics (weaknesses) are impeding agribusiness entrepreneurship in Nigeria? ii. In which area (of weakness) does Nigeria need to improve in order to boost agricultural productivity?
	Opportunities	i. What agricultural products from Nigeria are in high demand both locally and internationally (opportunities)? ii. Can Nigeria sustain a steady increase in agricultural production while maintaining a competitive advantage (opportunity for expansion)?
	Threats	i. Is there any risk or difficulty in exploring these agribusiness opportunities in Nigeria? ii. What factors/issues are causing people to miss out on agribusiness opportunities?

*Source= compiled by author, 2023.

The other sections of the paper are organized as follows: after discussing agribusiness model innovation, section two discusses the materials and methods, section three discusses the results and discussion, section four discusses the discussion, and section five discusses the conclusion and recommendations

1.3. The New Concept in Agribusiness

The emerging trend in agribusiness involves a re-evaluation of value generation, urging companies in the agri-food industry to move beyond a manufacturing-centric role. Participants often perceive themselves as manufacturers rather than innovators, leading to a tendency to overlook the need for a sustainable business model, which is crucial for future growth. The evolving focus emphasizes a dynamic understanding of value creation, urging active involvement and innovation throughout the entire value chain. Neglecting a resilient business model is identified as a critical oversight that potentially impedes enduring growth. This shift underscores the evolving nature of agribusiness, urging firms to adopt a comprehensive and forward-thinking approach beyond traditional manufacturing roles.

The potential of agritourism as an alternative for boosting the economies of developing countries is often highlighted, with its strong association to various sustainability benefits (Ndhlovu and Dube, 2024). According to Lane (2009), this model can promote the sustainable growth of rural areas by allowing farms to remain productive despite facing socio-economic and environmental difficulties. Its value extends beyond financial gains to include job creation, fostering community pride, and motivating farmers to embrace

innovative business practices within local economies (Lane, 2009). Agritourism is defined by the reconnection between producers and consumers, driven by broad ethical and political objectives such as revitalizing local identities, strengthening rural community ties to agriculture and local foods, fostering sustainable agricultural practices, and promoting both economically viable and socially responsible approaches (Ammirato et al., 2020). Barry and Hellerstein (2004) noted that agritourism offers both economic and social benefits while being ecologically advantageous, as it supports tourism that respects the natural environment, cultural traditions, and the economic needs of specific.

Nigeria, endowed with abundant agricultural and human resources, must establish a framework conducive to agribusiness entrepreneurship, facilitating innovative and cost-effective approaches to deliver and capture value. This approach is pivotal for agribusiness entrepreneurs to play vital roles in addressing global challenges, exploring new opportunities, and adapting to evolving consumer behaviours, constituting the essence of the new deal for the agribusiness concept.

According to the Harvard Business Review, this new deal pertains to efficiently delivering traditional goods to current markets without requiring entirely novel inventions or sectors. This concept has transformative potential for distressed economies, reshaping agri-food systems and contributing significantly to economic prosperity. Embracing the new deal allows food firms to carve a distinctive niche, often involving subtle modifications that offer unique, hard-to-imitate benefits in the industry. To thrive internationally, agri-firms need an organically structured agribusiness concept capable of responsive adaptation to new inventions, a gap existing in Nigeria's current agricultural education curriculum (Ikuemonisan et al., 2022a).

Any agri-firm that wants to be relevant in the international market and grow sustainably must have an organically structured agribusiness concept that can adequately respond to new inventions on a regular basis. However, Nigeria's current agricultural education curriculum does not adequately address this issue.

2. Materials and Methods

2.1. Study Area, Sampling Technique, and Data Collection

The primary aim of this research was predominantly exploratory in nature, making it well suited for qualitative methodology (Corbin and Strauss, 2015; Cohen et al., 2018; Saunders et al., 2019). The approach to the study is qualitative, and data were gathered over a period of three months through a series of 28 in-depth interviews. Four focus group discussions (FGDs) and interviews with key informants (KIIs) were conducted in the agribusiness subsector in Nigeria. Two states were randomly selected from the north (Kano and Benue States), and two states were selected from the south (Cross River and Oyo States). Based on the given criteria of nutritional values, gender inclusiveness and participation, youth empowerment, marketability, and socioeconomic values, one agricultural crop was selected for each state. The selected crops were rice and maize for Kano State, yam and oranges for Benue State, cassava and palm oil for Cross River State, and maize and cassava for Oyo State.

Major actors in the various agribusiness groups in the selected states were invited for interactions, including owners of commercial farms, agricultural exporters, participants in agricultural value chains, agricultural input dealers, agricultural service providers, smallholder farmers, farm equipment fabricators, agricultural traders, agricultural produce processors, and policymakers. The following locations were selected for interactions with stakeholders (FGDs and KIIs): Kure in the Kure Local Government Area (Kano State), Zaki Biam Yam Market in the Ukum Local Government Area (Benue State), Obubra in the Obubra Local Government Area (Cross River State), and Oke Ogun in the Oke-Ogun Local Government Area (Oyo State).

The interviews were conducted at different locations within each state.

An interview protocol consisting of open-ended questions and probing inquiries was developed to guide the interviews. Participants were provided with a clear definition of agribusiness entrepreneurship before the interviews to establish a common understanding.

The focus group discussions and key informant interviews aimed to gain insight into the potential role of the agricultural sector for nutritional diets and increased income generation, particularly among youth involved in agribusiness opportunities along the value chains of the selected crops. The research followed a theoretical sampling approach, initially employing purposive nonprobability sampling and then including additional cases based on their theoretical relevance. A total of 363 individuals participated in the focus group discussions, and 36 key informants were interviewed.

The distribution of respondents by age and gender revealed that 20% were between the ages of 25 and 35, with 42% female and 58% male participants in the focus group discussions. Six participants from each state were chosen as key informants based on their level of participation in the focus group discussions.

Table 2. Distribution of respondents by stakeholdership and sector activities*

S/N	Sector Activity	Respondents	Frequency
1	Input Supply	Private Seed Companies;	2
		From the Crop Protection Products Market	25
		From Fertilizer Market	5
		Government Input Supply Centres	12
2	Production	Farmers without land titles	36
		Subsistence Farmers with land title (<1ha)	21
		Smallholders Farmers with land title (1 – 3ha)	44
		Commercial Farmers with land title (>3ha)	5
3	Trading	Farmers' Group	18
		Wholesalers/Exporter	10
		Middlemen	18
		Retailers	22
4	Marketing/Selling	International Market	1
		National Market	10
		Regional Market	15
		Urban Market	11
		Supermarket	3
		Local Market	14
		Online Market	10
5	Service	Private	11
		Public	15
6	Prospective Trainees	Students	24
		Unemployed Graduates	31

*Source= computed from field survey, 2022.

To address objective 2, a list of global agribusiness practices and strategies for fostering sustainable agribusiness development was generated based on an extensive review of relevant literature, and journal articles, research papers, annual reports, theses, books, survey reports, and stakeholders (respondents) were asked to assess the relevance of these practices to agribusiness development. Second, an evidence-based assessment of the internal (strengths and weaknesses) and external (opportunities and threats) factors that can shape agribusiness entrepreneurship in Nigeria was conducted.

2.2. SWOT Analysis Framework

The SWOT analysis framework provides a structured approach to organize and analyse the data collected from stakeholders. The framework was designed to address specific research questions related to internal and external factors influencing agribusiness entrepreneurship development in Nigeria.

2.3. Strength and Weakness of the Methodology

The research methodology's strengths lie in its alignment with the study's exploratory objectives, leveraging qualitative approaches such as in-depth interviews and focus group discussions to capture nuanced stakeholder perceptions. Its diverse data collection methods, including key informant interviews and theoretical sampling, ensure a rich and triangulated dataset. Geographic and demographic diversity is achieved by involving stakeholders across northern and southern Nigeria, representing a wide range of agribusiness activities and addressing variations in regional challenges. Additionally, the integration of literature to evaluate global agribusiness practices contextualizes the research within both local and international frameworks, enhancing its relevance.

However, the study's reliance on purposive nonprobability sampling raises concerns about potential bias and the exclusion of diverse regional challenges. The absence of quantitative methods limits the generalizability of findings, while reliance on self-reported data risks subjective inaccuracies. The focus on specific crops restricts the comprehensiveness of the analysis, potentially overlooking other critical sectors. Furthermore, the three-month data collection period may not account for seasonal variations, and the lack of clarity in evaluating global strategies could hinder the reproducibility and applicability of the findings.

2.4. Statistical Analysis

This study utilized qualitative research methods, specifically focus group interviews, to collect a substantial amount of data on the strengths, opportunities, weaknesses, and threats to the development of agribusiness in Nigeria and the 24 global agribusiness practices and strategies that were presented to the respondents during the interaction. To manage these data effectively, the analysis process followed a structured approach based on Krueger's (1994) framework analysis, supplemented by key stages from Ritchie and Spencer's (1994) framework. This combined approach offered clear steps for both novice and experienced researchers to navigate the complexity of qualitative data analysis.

The analysis process consisted of several key stages:

Familiarization: This stage involved immersing in the data by listening to tapes, reading transcripts multiple times, and reviewing observational and summary notes. The goal was to gain a comprehensive understanding of the interviews before breaking them down into parts.

Identifying a Thematic Framework: The next step was to identify themes by writing memos in the margin of the text, capturing short phrases, ideas, or concepts emerging from the data. This process helped in developing categories and forming descriptive statements based on the research questions.

Indexing: Data were sifted, highlighted, and sorted to extract relevant submission and make comparisons within and between cases. This stage focused on managing data by cutting and pasting similar quotes together, leading to data reduction.

Charting and Triangulation: The extracted submissions were rearranged under appropriate thematic content. This process involved lifting submissions from the discussants' original context and organizing them based on the thematic framework developed earlier. Additionally, triangulation was adopted to compare the data obtained from the literature, informants, and discussants in the group discussion.

Mapping and Interpretation: The final stage involved interpreting the data by making sense of individual submissions and identifying relationships and links between data points. Krueger (1994) established criteria, such as words used, context, internal consistency, frequency, specificity, intensity of comments, and big ideas, to guide the interpretation process.

Following this systematic and sequential approach to data analysis, the study aimed to ensure the dependability, consistency, and conformability of the data, enhancing the overall quality and rigor of the research findings. Keeping reflective diaries, maintaining observational notes, and utilizing audiotapes or videotapes also contributed valuable dimensions to the data analysis process.

2.5. Thematic Organization and Reporting of Findings

The findings from the SWOT analysis were thematically organized into four sections: strengths, weaknesses, opportunities, and threats, following the pattern suggested by Regmi and Naharki (2020). This thematic organization facilitated a comprehensive presentation of the research findings and allowed for a deeper exploration of each aspect of the SWOT analysis.

2.5.1. Reporting findings

Table 3 presents the summarized findings of the SWOT analysis, highlighting the agreement among respondents on key research questions related to strengths, weaknesses, opportunities, and threats in agribusiness entrepreneurship development in Nigeria. The responses were obtained through a combination of FGDs and key informant interviews, ensuring a diverse range of perspectives and enhancing the credibility and applicability of the research findings.

2.5.2. Interpretation and discussion

The sections following Table 3 delve into the interpretation and discussion of the SWOT analysis findings. Each subsection corresponds to one of the SWOT categories (strengths, weaknesses, opportunities, and threats) and provides detailed insights derived from key informant inputs and relevant literature.

3. Results

The importance of assessing both internal (strengths and weaknesses) and external (opportunities and threats) factors is to enrich the literature on agribusiness on the one hand and to stimulate the business community for long-term scientific actions and business-oriented investment in agribusiness on the other. The pattern for thematically organizing and reporting the findings in this study is provided by Regmi and Naharki (2020). The findings of this study's SWOT analysis on Agribusiness Entrepreneurship in Nigeria are presented in four sections: Strengths, Weaknesses, Opportunities, and Threats.

3.1. SWOT Analysis

Table 3 presents the agreement among respondents regarding the key research questions they were presented with. Notably, the substantial agreement and consistency observed in the responses from both the participants in the Focus Group Discussion (FGD) and the Key Informants strongly support the accuracy and broader applicability of the research findings (Flick, 2014). Previous research identified four leverage points-strength, weakness, opportunities, and threats-as well as associated research questions. The respondents were allowed to make subtle comments to the questions in Table 3 based on their experiences in their respective local agribusiness environments. Table 4 provides a summary of the responses.

Table 3. SWOT analysis*

Strength i. What competitive advantage (strength) does Nigeria have over other countries in Agribusiness Entrepreneurship? ii. What is the current state of the institutional framework (strength) in Nigeria for achieving competitive and sustainable agricultural value chains?	Weakness i. What characteristics (weaknesses) are impeding agribusiness entrepreneurship in Nigeria? ii. In which area (of weakness) does Nigeria need to improve in order to boost agricultural productivity?
● 43.3 million hectares of agricultural land spread across complementary agro ecological zones to support massive agricultural production. ● Enormous, energetic, ingenious, active, and mobile youth population (54%) with a projected population of 262 million in 2050. ● Potential for growth and stability as a member of N-11 countries. ● Indigenous and traditional knowledge ● Plant Biodiversity influenced by enormous anthropogenic forces and floral diversity (A function of Organic residues) ● Plant Biodiversity influenced by enormous anthropogenic forces and floral diversity (A function of Organic residues) Government institutions are available to develop the agribusiness subsector through various government agencies such as the Ministry of Agriculture, the Ministry of Commerce and Industry, the Central Bank of Nigeria, Small, and Medium Development Agency (SMEDAN). ● Institutional Policy Framework for Managing Risk in Agriculture.	● The upstream sector is dominated by traditional agricultural actors. ● Low penetration (adoption) of technology and, as a result, low productivity. ● Farm mechanization is hampered by difficult farm landscape terrain. ● Inadequate technological solutions to agricultural and related issues ● General perception of agricultural drudgery ● Lack of understanding and incentives to start an agribusiness a general perception of agricultural drudgery ● Lack of understanding and incentives to start an agribusiness ● Lack of infrastructure (storage, transport, etc.) ● Postharvest losses and agricultural products with low added value ● Inadequate education and information on export management and international agribusiness; ● Inadequate education and information on export management and international agribusiness; ● Insufficient access to quality data for making informed agribusiness decisions. ● Weak judicial system ● Agribusiness corridor that is unorganized and unregulated agribusiness corridor.
Opportunities i. What agricultural produce/products from Nigeria are increasingly in demand in both the domestic and international markets (opportunities)? ii. Does Nigeria have the capacity to sustain a consistent increase in agricultural production while maintaining a competitive advantage (opportunity for expansion)?	Threats i. Is there any risk or difficulty in exploring these agribusiness opportunities in Nigeria? i. ii. What factors/issues are causing people to miss out on agribusiness opportunities?
● Commercial production of cassava, yam, cocoyam, cereals, cocoa, plantains, oil palm, and agricultural products (Comparative Advantage) ● Agrotourism destinations, both natural and man-made ● Significant domestic and international market (free trade). ● Good bilateral relationships that can aid in the promotion of agribusiness ● Institutions of higher learning where improved and market-driven agribusiness entrepreneurship and other related courses can be taught. ● Agriculture research institutes that promote new agricultural innovations. ● Enormous amounts of foreign exchange from crude oil can be used to fund agricultural research institutes and new agribusiness innovations. ● Increasing international organization support, such as CIDA, FAO, World Bank, AfDB, and others. ● Growing opportunity for virtual businesses	● Insecurity and recurring conflicts ● Low insurance uptake by actors in agricultural value chains as a result of social stigma and insufficient motivation ● Skill gaps, particularly in the development of new products and modern marketing strategies ● Education in modern agriculture and agribusiness is of poor quality. ● Lack of adaptable and inclusive industry-linked pedagogy to train the next generation of competitive agribusiness entrepreneurs. ● Ineffective climate change management strategies ● Lack of irrigation infrastructure Deforestation Pests and diseases ● There is an overemphasis on the upstream sector, with little or no attention given to the midstream and downstream agribusiness sectors. ● Lack of knowledge about export buses; industrial expansion; and a lack of start-up capital.

*Source: Compiled from field survey, 2023.

3.1.1. Strengths

The strengths identified in Nigeria's agribusiness landscape, such as climate suitability, agricultural land suitability, agro-ecological niches, rich plant biodiversity, government prioritization of the sector, and the institutional policy framework, are intricately connected to broader challenges and opportunities within the country's agribusiness sector. Integrating these findings with the theoretical frameworks of Krueger (1994) and Ritchie and Spencer (1994) provide a deeper analysis of these connections.

Nigeria has a strong advantage in terms of climate suitability and agricultural land suitability. The country's diverse climatic regions provide favourable conditions for growing a wide range of crops, giving Nigeria a comparative advantage in farming. This diversity also attracts immigrants to the country. However, despite these advantages, Nigeria's agricultural productivity is currently low, indicating the underutilization of agricultural factors such as land and capital. This low productivity is attributed to factors such as the lack of mechanized agricultural systems and low technological adoption. Overall, Nigeria's climate suitability and diverse agricultural land offer great potential for farming, but there is a need to address the challenges that hinder productivity (Poudel, 2016; USDA-Economic Research Services: International Agricultural Productivity, 2022).

Nigeria also possesses rich plant biodiversity, which presents business potential due to the combination of species and ecologies. Although plant diversity in Nigeria is not fully known, the country is home to a wide range of bird species, reptiles, amphibians, and fish species. Nigeria accounts for only 0.7% of the Earth's surface area, but it has diverse biodiversity and ecology, with over 900 bird species, 135 reptile species, 109 amphibian species, and 648 fish species (Altiparmak, 2022). Nigeria's biodiversity is unique and has high business potential due to the combination of species and ecologies (Luiselli, 2003; Ugochukwu, 2008).

The agro-ecological niches in Nigeria's different climatic zones offer opportunities for specific commodity production and agribusiness. The types of crops and animals raised in each region are determined by Nigeria's agroecological niche. Different ecological zones have their own specific commodities and potential for agribusiness entrepreneurship. Modern innovations in the value chains of these crops can enhance agribusiness opportunities for young entrepreneurs. For example, the savanna zone is suitable for crops such as cowpeas and soybeans, while the drier Sahel and Sudan savanna zones are suitable for drought-tolerant crops such as corn, groundnut, sorghum, and millet. The climatic belt of the savanna zone is also known to be the natural habitat for various tree species. The concept of thematic frameworks is used to categorize crops and livestock based on their suitability for different ecological zones. Integrating modern innovations along these value chains aligns with an entrepreneurial approach, encouraging farmers to adopt innovative practices and enhance productivity.

The concept of thematic frameworks, as outlined by Krueger (1994), was applied here to categorize crops and livestock based on their suitability to different ecological zones. For instance, crops such as maize and rice thrive in monsoon regions, while millet and sorghum are more suitable for drier savanna zones. Integrating modern innovations along these value chains aligns with the entrepreneurial approach advocated by both frameworks, encouraging farmers to adopt innovative practices and enhance productivity.

The government's prioritization of the agricultural sector, as highlighted in the study, reflects a strategic shift towards commercialized agriculture and holistic agricultural development. This aligns with Krueger's framework, emphasizing the role of external factors such as government policies in shaping business environments. The Agricultural Promotion Policy (APP) serves as a roadmap for increasing agricultural input and output, fostering local and global market competitiveness, and promoting entrepreneurship within the sector.

The state of the agri-food system in Nigeria has undergone transformation in recent years due to factors such as population growth, urbanization, environmental consciousness, and changing occupational profiles. The government plays a crucial role in ensuring consistent production and food supply through interventions such as the supply of certified seeds, fertilizers, and funds for agricultural value chains. To manage agricultural risk, the Nigerian government has implemented institutional policies such as the Nigeria Agricultural Insurance Corporation (NAIC), which provides indemnity to farmers and covers a wide range of commodities. Premium subsidies are available for most agricultural insurance funded by federal and state governments.

In terms of future prospects, the demand for agricultural products in Nigeria is expected to be influenced by agro-industrial demands, increased food demand driven by population growth and rising income.

3.1.2. Weaknesses

The weaknesses identified in agribusiness entrepreneurship in Nigeria are intertwined with several dimensions, including land holding, infrastructure, competitiveness, trade, research and development, knowledge transfer, and a weak justice system. The decline in per capita land holdings over time reflects a reduction in agricultural land availability, influenced by various socioeconomic, cultural, and political factors. These complexities hinder the development of agribusiness in Nigeria, as supported by Oluwatayo et al. (2019) and other scholars who cite environmental degradation as a significant threat to agricultural productivity.

Furthermore, high overhead costs due to inadequate power supply and limited access to cheap energy sources were highlighted as concerns by respondents. This issue is exacerbated by the lack of implementation of smart

energy solutions in rural areas, which impacts the sustainability of agro-ecosystems. Integrating these findings with the theoretical framework underscores the challenges faced in agribusiness development in Nigeria, particularly in terms of land availability, infrastructure, and energy sustainability. Addressing these weaknesses is crucial for enhancing the competitiveness and sustainability of agribusiness ventures in the country.

3.1.3. Opportunities

3.1.3.1. Pre-Farm (input), farm and post-farm (processing) and marketing segments

The opportunities identified in Nigeria's agriculture sector align with broader agribusiness challenges and prospects. The sector offers avenues for business expansion, rural employment, income improvement, and poverty reduction if a new orientation emphasizing a favourable business climate is adopted (FAO, 2022). However, existing agricultural structures and programs lack integration with national goals, leading to a decline in employment within the sector and heavy reliance on agricultural imports due to low productivity among subsistence farmers (Cohen et al., 2018; Dzimba and van der Poll, 2022).

The theoretical framework supports these findings by highlighting the importance of productivity-driven innovations and efficient production and supply chains in enhancing agricultural output and job creation (Krueger, 1994). Integrating the results with the theoretical framework underscores the need for agribusiness entrepreneurs to focus on producing crops in which Nigeria has a comparative advantage, such as wheat, sugar, fish, milk, sesame, cashew nuts, cocoa beans, ginger, frozen shrimp, and cotton (Ritchie and Spencer, 1994). Optimizing resource use, including labour and land, is crucial for achieving increased productivity and competitiveness in the global agricultural market (FAO, 2022).

Furthermore, FAOSTAT data indicate that Nigeria has the potential to become a major player in trading various crops globally, including roots and tubers, cassava, yams, cereals, vegetables, fruits, oil palm, maize, and sorghum (FAO, 2022). However, this can only be realized through the adoption of productivity-driven innovations and efficient resource utilization across agricultural value chains (Krueger, 1994). Addressing these opportunities in line with the theoretical framework can contribute significantly to overcoming agribusiness challenges and harnessing Nigeria's agricultural potential.

3.1.3.2. Agrotourism

The potential of agrotourism in Nigeria intersects with broader agribusiness challenges and opportunities, offering a promising avenue for economic growth and development. Scholars and industry experts have highlighted Nigeria's substantial economic potential from agrotourism (Nnadi and Akwiwu, 2005; Khidir, 2020). This perspective suggests that leveraging the tourism sector could address the economic challenges faced by Nigeria and its citizens (Adebayo and Butcher, 2020; Banki and Ismail, 2015).

Khidir (2020) defines agrotourism as a platform for encouraging engagement in agricultural activities and fostering physical development. Data from the Bureau of Statistics indicate that the contribution of the tourism sector to Nigeria's aggregate gross domestic product fluctuated between 2.8% and 4.5% between 2019 and 2020. As of 2021, it stood at 3.6%, and the buck of this this came from travel tourism. According to entrepreneurs in this sector, the conversation is still about how to help the government create an enabling environment so that doing business in the sector is easier. This call is based on the fact that agrotourism can be used to boost the branding of local, organic, and traditional products, as well as the development of rural entrepreneurship and the creation of job opportunities in many rural areas, where rural unemployment is expected to reach 33% by 2022.

3.1.4. Threats

Agribusiness entrepreneurs in Nigeria encounter a myriad of challenges that intersect with broader economic and environmental issues, particularly prevalent in sub-Saharan Africa. Climate change poses a significant challenge to agricultural productivity and sustainability (Ogidi, 2014; Raimi et al., 2021; Pratt et al., 2022). Additionally, the government's failure to establish an enabling business environment hinders entrepreneurship and economic growth (Regmi and Naharki, 2022).

Unorganized trade practices, price fraud, and market volatility further complicate agribusiness operations (Ikuemonisan et al., 2022b). These challenges contribute to a stagnant skilled migration rate, as professionals hesitate to seek opportunities abroad due to uncertainties and limitations within the domestic agribusiness landscape, thereby exacerbating the risk of brain drain.

In light of these challenges, agribusiness entrepreneurs in Nigeria must navigate complex operating environments while also capitalizing on emerging opportunities. Integrating findings with theoretical frameworks can provide insights into strategies for mitigating challenges and leveraging opportunities for sustainable agribusiness development.

3.2. Respondents' Consensus to Strategies for Sustainable Agribusiness Development

Table 4 presents a detailed assessment of agribusiness development strategies in Nigeria, highlighting a strong consensus among stakeholders regarding the urgent need for enhancing the agricultural industry (AI) through a comprehensive approach. This collective recognition underscores the vital importance of significant improvements in Nigeria's agri-food system to achieve efficiency and sustainability.

The theoretical framework supports these findings by emphasizing the role of government intervention, particularly through liberalization measures, in creating an enabling environment for sustainable agribusiness

development (Chao et al., 2022). Liberalization, involving market opening, reduced trade barriers, and increased competition, is seen as essential for fostering efficiency and sustainability in Nigeria's agri-food system.

Table 4 further outlines sustainable strategies that have garnered widespread agreement, with over 80% of stakeholders expressing consensus on these approaches. The consensus reaching processing (CRP) methodology, as developed by Chao et al. (2022), was employed to determine the levels of consensus among respondents. Out of the 24 global agribusiness practices and strategies that were presented to the respondents during the interaction, only seven reported consensus levels of 80% and above.

These strategies, which have received increasing support from stakeholders, serve as key pillars for fostering sustainable agribusiness development in Nigeria. They represent a shared vision among stakeholders and provide a roadmap for policymakers and industry players to prioritize and implement to propel the nation's agribusiness sector towards a more sustainable and efficient future.

Table 4. Respondents' consensus of the assessment of strategies for sustainable agribusiness development in Nigeria*

S/N	Strategies	Approach	To be facilitated by
1	A favourable macropolicy environment must be created.	Appropriate monetary and fiscal policies to stabilize the value of the domestic currency	Central Bank of Nigeria
2	Declare and follow food safety and market supply consistency.	Marketing Freedom to show its support and commitment to the liberalization policy	Government and her agencies
3	Increase human capacity for agricultural development.	A framework for the facilitation of a consistent business and technical training assistance programme to scale up the required skills for actors along agricultural value chains.	Public Sector and Private Sectors
4	Increase access to credit and insurance	Access to finance and insurance for the development of agribusiness through a financial market in which agro-actors have high accessibility and penetration of finance outlets into rural areas.	Finance Institutions
5	Quality assurance for the supplied input	Low-quality input and output must be checked on a regular basis. As a result, quality control and anti-monopoly measures are unavoidable for a well-functioning agriculture industry.	Public Sector. Functioning Regulatory System
6	Transparency should be promoted in the agribusiness industry.	An MIS should be created to collect, analyse, and disseminate information about prices, deliveries, and stock levels at various locations.	Public Sector: Functioning Regulatory System
7	Increase research capacity for the promotion of private agro-companies, and Promote Technology Transfer activities.	Because modern agriculture is science-based, research institutes, universities, vocational centers, and so on must be strengthened, and industry-linked pedagogy developed.	Public & Private Sectors through NUC & NBTE.

*Source= author's compilation, 2023.

The findings from the survey among stakeholders in Nigeria's agribusiness industry reveal key strategies and approaches crucial for fostering sustainable development. These findings align with broader agribusiness challenges and opportunities in Nigeria and are supported by theoretical frameworks from the relevant literature.

A supportive macropolicy environment is identified by 96% of stakeholders as essential. This aligns with the literature emphasizing the need for strengthened agribusiness development capacity in sub-Saharan Africa (Babu et al., 2016). The Central Bank of Nigeria is recognized as a key facilitator for implementing this strategy.

Strict compliance with regulations for food safety and market supply consistency is stressed by approximately 82% of stakeholders. Balancing regulations and market freedom is highlighted in the literature as positively impacting market competition and economic growth (Gould et al., 1996; Nicoletti and Scarpetta, 2003). The government is seen as the facilitator for ensuring adherence to rules.

Third, enhancing human capacity for agricultural development is emphasized by approximately 89% of stakeholders. Investments in human capital are known to significantly contribute to increased agricultural productivity (Davis et al., 2021). Both the public and private sectors are recognized as facilitators, although issues of incompetence within the government system are noted (Dzimba and van der Poll, 2022; Kineber et al., 2023).

All stakeholders (100%) highlighted the need for

increased access to credit and insurance facilities, which are positively associated with higher agricultural productivity and economic growth (Phiri et al., 2022; Demirgüç-Kunt et al., 2017). Collaboration between the government and financial institutions is crucial for facilitating this strategy.

Close to 99% of stakeholders emphasize quality assurance in input supply, recommending quality control measures and antimonopoly regulations (Klerkx and Begemann, 2017). The public sector is identified as the facilitator of this strategy.

Additionally, more than 90% of stakeholders support transparency promotion in the agribusiness industry, aligning with the World Bank's proposal for a Management Information System (MIS) (Awotide et al., 2015). The public sector, particularly the functioning regulatory system, is recognized as the facilitator.

More than 85% of stakeholders support strategies focusing on research capacity and technology transfer activities in private agro-companies. The literature supports this, linking investments in research and development to increased agricultural productivity (Alene et al., 2013; Graziano da Silva et al., 2019). The National Universities Commission (NUC) and the National Board for Technical Education (NBTE) are identified as facilitators of both the public and private sectors.

4. Discussion

The findings of this study both align with and expand upon the literature on agribusiness in Nigeria. The identification of strengths such as extensive agricultural land, a youthful population, indigenous knowledge, and supportive government institutions resonates with previous studies highlighting Nigeria's considerable potential in agribusiness (Babu et al., 2016). These findings reinforce the understanding that Nigeria possesses the natural and human resources necessary for a thriving agribusiness sector.

However, the study also sheds light on critical weaknesses, including low technology adoption, infrastructure challenges, educational gaps, and a weak judicial system, which are noted as areas requiring significant improvement (Watson and Winfree, 2022). This nuanced analysis expands the current understanding by emphasizing the specific barriers and limitations that hinder the full realization of Nigeria's agribusiness potential. This finding challenges the notion that resources alone are sufficient for success and underscores the importance of addressing systemic challenges for sustainable growth.

Moreover, the study's identification of promising opportunities such as commercial crop production, agrotourism, foreign investments, research institutions, and virtual business prospects aligns with observations from the World Bank and other literature (Ndhlovu and Dube, 2024; Ikuemonisan, 2024). This alignment reinforces existing knowledge about the diverse opportunities present in Nigeria's agribusiness landscape and highlights areas where targeted interventions can yield significant returns.

In contrast, the study's discussion of threats such as insecurity, insurance gaps, skill deficiencies, climate change impacts, and limited knowledge about export opportunities offers a sobering contrast to the optimistic outlook on Nigeria's agribusiness potential. These identified threats challenge the narrative of unchecked growth and underscore the need for strategic risk management and capacity-building efforts within the sector.

This study's proposed sustainable strategies, supported by stakeholder consensus and aligned with theoretical frameworks, contribute significantly to the discourse on agribusiness development in Nigeria. By emphasizing the importance of a favourable macropolicy environment, food safety, human capacity development, financial access, quality control, transparency, and research, this study offers a comprehensive roadmap for addressing challenges and leveraging opportunities.

Overall, this study provides a nuanced analysis of Nigeria's agribusiness landscape, expanding the existing knowledge while challenging some prevailing assumptions. The emphasis on collaborative efforts between the public and private sectors to create an enabling environment further enriches the discourse and underscores the potential for Nigeria's agribusiness sector to drive economic growth, food security, and environmental sustainability.

5. Conclusion

The outlined strategies for sustainable agribusiness entrepreneurship in Nigeria are robust and strategically aligned with the country's agribusiness landscape. Creating a favourable macropolicy environment is crucial given Nigeria's diverse agricultural sector, and it resonates well with institutional economics, highlighting the pivotal role of supportive policies in shaping economic behaviour and outcomes. By fostering an environment that encourages investment, innovation, and market access, Nigeria can attract more entrepreneurs to the agribusiness sector, thereby driving growth and sustainability.

Instituting proper food safety and market supply consistency checks addresses critical challenges in Nigeria's agribusiness, particularly quality control and market access. This strategy is rooted in market failure theory, emphasizing the importance of addressing information asymmetry and externalities. Ensuring food safety and a consistent market supply not only enhances consumer trust but also unlocks opportunities for Nigerian agribusinesses to tap into international markets, enhancing competitiveness and revenue generation. These strategies, coupled with increasing human capacity, improving access to finance, implementing strong quality control measures, establishing robust MIS, and enhancing research capacity, form a comprehensive

framework that can catalyze sustainable agribusiness development in Nigeria, fostering increased productivity, competitiveness, and profitability in the sector over the long term.

To address these challenges and leverage opportunities, this study proposes seven sustainable strategies with strong stakeholder consensus. These strategies include creating a favourable macropolicy environment, implementing checks on food safety and market supply consistency, liberalizing policies, enhancing human capacity, increasing access to finance and insurance, implementing quality control measures, establishing a functioning Management Information System (MIS), and boosting research capacity for technology transfer activities.

On the other hand, threats such as insecurity, insurance gaps, skill deficiencies, climate change impacts, and limited knowledge about export opportunities present challenges that need to be addressed to ensure the sector's resilience and growth.

Author Contributions

The percentages of the author' contributions are presented below. The author reviewed and approved the final version of the manuscript.

	E.S.I.
C	100
D	100
S	100
DCP	100
DAI	100
L	100
W	100
CR	100
SR	100
PM	100
FA	100

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The author declared that there is no conflict of interest.

Ethical Consideration

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. The experimental procedures were approved by the Ethics Committee of Adekunle Ajasin University, Akungba Akoko (Approve number: Ref: AAUA/AGRIC/DEAN/2305168).

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AGRICULTURAL INSURANCE MARKETING AND MANAGEMENT: A STRATEGIC APPROACH USING TIME SERIES ANALYSIS

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Abstract: Differentiation in insurance marketing varies across branches, serving as a significant determinant in shaping the strategies and policies to be adopted. While there are certain similarities between the insurance needs of producers in the agriculture, industry, and service sectors and those of households and consumers, notable differences also exist. The primary aim of this study is to develop a strategic approach to the marketing and management of agricultural insurance, which ranks as one of the foremost measures against risks in the agricultural sector. To this end, the series of policy counts, and premium amounts produced in the branches of crop production and livestock life insurance during the 2013–2023 period were forecasted, and the model types yielding the most successful results were identified. The stationarity of each branch of agricultural insurance was achieved by taking the first-order difference of the time series. The most suitable model was determined for each series. According to the obtained models, it is projected that the number of policies and the amount of premiums for agricultural insurance will increase in the coming periods. To ensure that insurance companies, producers, and intermediaries maximize their benefits from these developments, several recommendations regarding marketing and management practices have been proposed. The findings of this research are anticipated to contribute to the strategic marketing management and planning processes of businesses.

Keywords: Insurance marketing, Management, Time series analysis, Insurance

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1. Introduction

Agriculture is a sector of critical importance not only for economic development and meeting the basic needs of societies but also for food security and social stability. However, the sector faces several threats to sustainable production, including natural disasters, climate change, and various risks. Compared to other sectors, agriculture is a sector that carries a higher risk of farmers losing income and experiencing imbalances in their income (Çukur and Saner, 2008). Consequently, these factors create high risks in agricultural production and endanger sustainability in the sector. One of the most effective methods of protection against these risks, which are common in the agricultural sector, is agricultural insurance (Binici et al., 2003). For this reason, governments attempt to protect farmers against income losses caused by economic risks following yield losses due to natural risks that threaten agricultural production (Dinler, 2000). Agricultural insurance is an effective tool to mitigate the impacts of these risks and ensure sustainability in agriculture. Natural disasters, droughts, floods, diseases, and pests are factors that lead to unpredictable losses for agricultural producers. In this context, farmers need effective risk management strategies to protect their income and sustain their agricultural activities. Agricultural insurance plays a crucial role in this regard by mitigating the impacts of these risks and ensuring the economic stability of farmers. By compensating farmers for potential losses, agricultural insurance provides them with the opportunity to produce more securely and supports agricultural sustainability. Risk management in agricultural production aims to reduce uncertainties faced by farmers and ensure a more stable income flow. Agricultural insurance offers various policies for both crop and livestock products, helping farmers protect themselves from natural disasters and other risks. These insurances not only aim to protect farmers' products and income but also contribute significantly to sustainability efforts in the agricultural sector. However, the widespread adoption of agricultural insurance is not always easy. Farmers' lack of sufficient knowledge about insurance products and their inability to properly assess risks result in low demand for insurance. This situation makes it difficult for insurance companies to invest in agricultural insurance and, consequently, leads to a vicious cycle in the sector. Raising awareness and educating farmers about these insurance products will both increase insurance demand and contribute to ensuring sustainability in the agricultural sector. The agricultural sector requires effective risk management strategies and efficient marketing of agricultural insurance to achieve sustainable development goals. This

study aims to emphasize the importance of agricultural insurance and develop strategies to encourage farmers to adopt these products. By doing so, risks in agricultural production can be minimized, and food security and economic stability can be ensured. The aim of this study is to approach the marketing and management of agricultural insurance from a strategic perspective and offer the necessary solutions to encourage farmers to take out insurance. By examining data from the past decade, strategies that can be implemented to prevent risks in agricultural production and ensure the continuity of farmers' incomes will be identified. Special attention will be given to strategies that can be developed by TARSIM (Insurance of Agriculture). Effective marketing of agricultural insurance is of critical importance in increasing farmers' interest in these products and ensuring the effectiveness of the insurance system. Insurance marketing involves not only promoting products but also understanding the needs of farmers and offering solutions that meet those needs. Increasing farmers' knowledge about insurance policies will help them better understand the value of the services offered by insurance companies.

1.1. Literature

1.1.1. Agricultural insurance: sustainability and risk management

Global climate change and the greenhouse effect are increasing the frequency and severity of natural disasters, leading to significant losses in farmer incomes and national economies. Additionally, highly lethal epidemics, such as the recent pandemic, have disrupted agricultural production, leading not only to income losses for farmers but also to a decline in production volume, causing significant economic damage. For example, a recent study by Setyawan et al. (2023) states that the pandemic has further worsened and destabilized production and income stability. As a result, agricultural insurance has become a vital tool for risk management during such unexpected crises. The agricultural sector is developing various methods to prevent these risks or minimize their impact. As the most common risk management tool, crop insurance plays a significant role in this regard. This type of insurance financially protects farmers from potential risks they may encounter during agricultural activities. Moreover, it helps farmers protect their crops and income against unforeseen circumstances such as natural disasters, diseases, pests, and droughts. Agricultural insurance offers farmers the opportunity to produce more securely, thus supporting the sustainability of agricultural activities. Therefore, agricultural insurance is considered a critical safeguard for those working in the agricultural sector. To ensure the continuity of agricultural production, it is necessary to implement agricultural insurance to stabilize crop prices and farmer incomes (Akçaöz et al., 2006). Agricultural insurance is offered in various branches such as crop products, cattle, sheep, poultry, bee hives, aquaculture, and greenhouse farming. Management functions such as planning, organizing, directing, and controlling are also of great importance in the insurance sector. Business managers in both crop and livestock production should make plans by considering meteorological factors and benefit from this information. Especially in crop production, there is a heavy dependence on natural conditions such as climate change and drought. Under these conditions, catastrophic damage may occur, leading to consequences that are difficult to compensate for. Another responsibility of insurance managers is to ensure the standardization of rules and methods and support the specialization of roles. Today, due to global warming, there has been an observed increase in the types and frequency of natural disasters, which makes specialization and diversification in the insurance sector essential. Uncertainty and increasing risks can complicate the decision-making processes of business owners. To manage effectively in line with their goals, business owners must have the necessary information. As noted by Akalın (1970) and Emhan (2009), risk refers to situations where the probability distribution of an event is known, but its uncertainty is not. Risks in agricultural production can be classified into categories such as diseases, price fluctuations, theft, and adverse weather conditions (Pehlivan and Akpınar, 2022). Şahin et al. (2008), Sayılı and Uzunoz (1998), and Karahan (2002) have divided these risks into two main groups: production risk, which originates from nature, and market risk, which arises from the market. Agriculture is an extremely vulnerable sector to unpredictable natural disasters, which can cause substantial damage to human life, property, the environment, and agricultural products (Yalaz, 2023). Moreover, due to the effects of climate change, the potential costs associated with these events are likely to increase in the future. Therefore, instability in the agricultural sector and fluctuations in farmers' incomes arise from these risks and uncertainties. Due to these risks, producers face difficulties in determining which crops to grow. Agricultural businesses, as a result of the risks they encounter throughout the production and marketing process, are unable to expand their operations (Şahin et al., 2008). The widespread adoption of agricultural insurance is not always easy due to factors such as farmers' insufficient knowledge about insurance alternatives and their inability to objectively assess the risks they face. Insufficient demand for insurance creates a vicious cycle, preventing insurance companies from investing in agricultural insurance (Meuwissen et al., 2003). Agricultural enterprises face constant risks in both crop and livestock production. Holloway (1979) categorized farmers' risk behavior into three groups: risk-seeking, risk-averse, and indifferent. The risks faced by businesses in crop production include factors such as hail, fire, floods, and drought, while those in livestock farming include risks like animal death, calf mortality, sunstroke, and fire. To manage risk, business managers in both crop and livestock production may seek to spread or eliminate risks. In this regard, insurance companies play a key role in mitigating these risks. According to the World

Commission on Environment and Development (WCED), sustainable development can be defined as the practice of meeting present needs without compromising the ability of future generations to meet their own needs (Brundtland, 1985). In terms of specialization for sustainable development, significant progress has been made in ensuring sustainability in both crop and livestock production. The more sustainable the production, marketing, and management activities are, the more market balance will be achieved, and competition in the sector will increase. Increased competition will enhance confidence in the sector, improve efficiency, and positively affect the overall health of the agricultural sector. For the effective implementation of agricultural insurance, it is crucial for farmers to be well-informed and able to evaluate insurance alternatives. In this context, the marketing of agricultural insurance plays a critical role in offering products suited to farmers' needs and raising insurance awareness. The next section will discuss strategies for marketing agricultural insurance and evaluate their impact on farmers. Through this, the aim is to increase the widespread adoption of agricultural insurance and support sustainable agricultural production.

1.1.2. Insurance marketing

Insurance marketing refers to the process of offering insurance products to consumers and developing strategies for this process. The complex nature of insurance products reduces consumers' interest in these products, making it difficult to explain the necessity of insurance. Since insurance is a service sector, customer experience and trust-building are critical in-service marketing. The competitive environment in the sector is a significant factor affecting marketing strategies. Insurance marketing should primarily be regarded as a form of service marketing; the differences and characteristics in this field are also valid for insurance marketing (Özgüven, 2008). The only tangible element in the presentation of insurance services is the insurance policy. In fact, the marketed value is the insurance coverage stated in the policy, which is an abstract concept (Akpınar, 2017). The benefit that the customer derives from the insurance policy is the main area of focus for marketing. In the event of damage, the speed of resolution is crucial for customer satisfaction and the evaluation of the service. In insurance sales, an opportunity for a sale arises only when contact is made with individuals who have a need for insurance. This process is quite challenging and complex, as it involves selling an unknown product in an uncertain environment, with no clear idea of when the event may occur. The invisibility of the service offered by insurance companies decreases customers' willingness and interest in purchasing. Although the service becomes tangible when damage or loss occurs, if no damage happens, the service is often considered a wasted resource by the customer. Therefore, it is important to raise awareness among customers about insurance. The foundation of insurance marketing lies in the concept of service marketing, and in this context, educating and informing potential customers about the benefits and necessities of insurance products is crucial (Özgüven, 2008; Akdoğan, 1983). Insurance companies' analysis of customer data and prioritizing customer relationship management will be beneficial in developing marketing strategies focused on target consumers. Digital marketing channels play an essential role in reaching customers and establishing effective communication with them. Insurance companies try to meet customers' expectations in the best possible way by designing policies that are most suitable for their needs. Since these products cannot be produced or stored, customer-focused service delivery is necessary. Therefore, insurance companies must stay in contact with their customers in a competitive environment and act more sensitively to meet their expectations. As a result, in the highly competitive insurance sector, the concepts of customer, service, and quality are becoming increasingly important (Akpınar, 2017). Insurance companies provide many services to their consumers. These services include:

i. Guarantee: These are the guarantees provided by the insurance contract against uncertainties, and they are a distinguishing feature of the insurance contract.
ii. Organization: This refers to the organization of the insurance pool.
iii. Investment: This involves turning the funds accumulated in the organized insurance pool into the most profitable investment.
iv. Advice: Developing recommendations about the precautions that can be taken against possible damage.

In order to achieve profit, insurance companies adopt various strategic marketing approaches. Achieving customer satisfaction, implementing loyalty programs, designing different products, adding compensation products to policies that appeal to the customer's preferences, and creating a competitive edge are considered success criteria (Öztürk and Güven, 2013). In the insurance sector, distribution mix strategies are generally implemented through branches or agents. With the powers granted to the banking system, insurance products can be offered to customers through each branch. As the frequency and damage caused by natural disaster risks increase, premium prices rise. Producers tend to avoid purchasing crop insurance due to the high premium costs. Therefore, a portion of the insurance premiums is subsidized by the government. The amount of this subsidy, aimed at encouraging insurance purchases, is set at fifty percent (50%) of the premium cost and is updated annually by the Council of Ministers (TARSIM, 2013).

1.1.3. Time series analysis

The use of time series analysis in agricultural certification is seen to be important for the dissemination of effective insurance products, ensuring that these products are accepted by the information within the target market and comprehensively monitoring the outcomes and

consumption patterns in payments for management software. For example, Ngungu et al. (2018) emphasize the enhancement of time series techniques in the management of insurance investments and risk assessment strategies. Time series are numerical values that represent the sequential observation of variables over a specific period. It is not mandatory for the observed data to occur consecutively over time; however, regular intervals are necessary to observe the development of the series (Sevuktekin and Nargelecekenler, 2007). This analytical method is frequently used in examining time-dependent data and holds significant importance in various scientific fields, including economics, geophysics, agriculture, and medicine. To achieve the goal in a time series study, sufficient, high-quality, and reliable data are required (Çelik, 2013). In the process of time series analysis, accurately determining the model and testing its suitability for the data are crucial. A poorly determined model will not provide reliable results. After model selection, the appropriateness of the chosen model for the data should be carefully tested. When an appropriate model is established, it will be possible to make accurate predictions based on this model (Çelik, 2013). Time series analysis is an approach aimed at making predictions based on a variable's behavior in past periods (Kennedy, 2006). The observed data set can be predicted as a stochastic process consisting of random variables. This stochastic process is formed by y1, y2, .., yt values at period t. By modeling such a process, probabilities about the future behavior of the relevant series can be obtained (Cryer and Chan, 2008).

Time series analysis is based on examining past data for a specified period to identify certain trends. This analysis includes four main components (Şeker, 2015):

i. Trend Component: The stable state that emerges after a period of increase or decrease in the observed series over the long term. If the series shows an upward or downward direction, it indicates a trend. If the curve fluctuates, it is concluded that there is no trend in the series.
ii. Seasonality Component: The presence of seasonal variations in the observed series. The presence of fluctuations in the series at specific time intervals is examined.
iii. Cyclical Component: The presence of non-seasonal periodic changes observed in the economy. It refers to the rise or fall occurring in the medium term.
iv. Irregular (Random) Component: Changes characterized by uncertainty and expressed through error terms. It should be considered that there may be sudden increases or decreases in data at any time. For example, the sudden increase in mask sales during the pandemic does not mean that such changes will occur at all times.

The complex structure of insurance products, customer needs, and the competitive dynamics in the sector play a critical role in developing insurance marketing strategies. In this context, by using time series analysis, secondary data on the number of policies and insurance premiums for ten years of crop insurance and livestock life insurance will be examined.

2. Materials and Methods

2.1. Main Objective of the Study

The primary aim of this study is to contribute to the development of effective strategies for the marketing and management of agricultural insurance, and to create solution proposals that will increase efficiency and effectiveness in these processes. In line with this goal, the number of policies and insurance premiums for selected agricultural insurance branches (crop insurance and livestock life insurance) covering the past 10 years (2013-2023) have been examined in detail. Using the obtained time series, the study aims to forecast the number of agricultural insurance policies and premiums until 2030. By determining an appropriate time series model, predictions for future policy numbers and insurance premiums will be made, with the goal of supporting companies in developing their marketing and management strategies. Additionally, various recommendations will be made to guide the policies to be followed for the widespread adoption of agricultural insurance and the uptake of insurance by producers. In this context, the findings obtained through secondary data analysis will provide valuable insights into trends and customer behaviors in the agricultural insurance sector. Based on these insights, the study aims to develop concrete recommendations for insurance marketing and management.

2.2. Methodology

The study utilizes secondary data sources. For this purpose, annual data for the period 2013-2023 have been obtained from TARSİM reports and the Turkish Statistical Institute (TUIK) database. Time series analysis is a quantitative method frequently used to predict future data by examining data collected at regular intervals (Özcan and Yıldırım, 2021). The data analysis was conducted using SPSS 27 and Stata software packages. Initially, the time series were individually subjected to unit root tests. The series were tested for stationarity using the Augmented Dickey-Fuller (ADF) test. If no stationarity was found at the level of the series, differences was applied to achieve stationarity (Çamoğlu and Akıncı, 2012). The differencing process eliminates the issue of spurious regression and makes the analysis results more reliable (MacKinnon, 2010). To achieve stationarity, if differencing is performed d times, the variable is considered integrated of order d, denoted as I(d) (Kennedy, 2006). After testing the series with the ADF unit root test, forecasts for each series for the years 2013-2023 were made using the ARIMA, Holt, and Brown models. The Box-Jenkins approach provides a systematic framework for the analysis of time series data and is particularly suitable for non-stationary series. It is widely used in time series analysis because it has the ability to

solve a dataset, regardless of whether the series is stationary or contains seasonal components (Sevuktekin and Nargelecekenler, 2007). This approach may require methods such as differentiating or transformation to make the series stationary. Within the framework of time series analysis, the ARIMA model was used to determine the values of p (autoregressive), d (differencing), and q (moving average) by examining the autocorrelation (ACF) and partial autocorrelation (PACF) functions for each series. The Akaike Information Criterion (AIC) and Schwartz-Bayes Information Criterion (BIC) were used for selecting the appropriate model (Çelik, 2015). The Holt and Brown models, effective in forecasting data with trends and levels, were used to predict future values based on historical data. The model that produced the best result was selected as the forecasting model, and predictions for the future were made. In this process, the validity of the modeling was enhanced by focusing on the presence and nature of the trend.

3. Results

The distribution of the number of policies created in agricultural insurance and the distribution of insurance premiums according to branches are detailed in Figure 1 below.

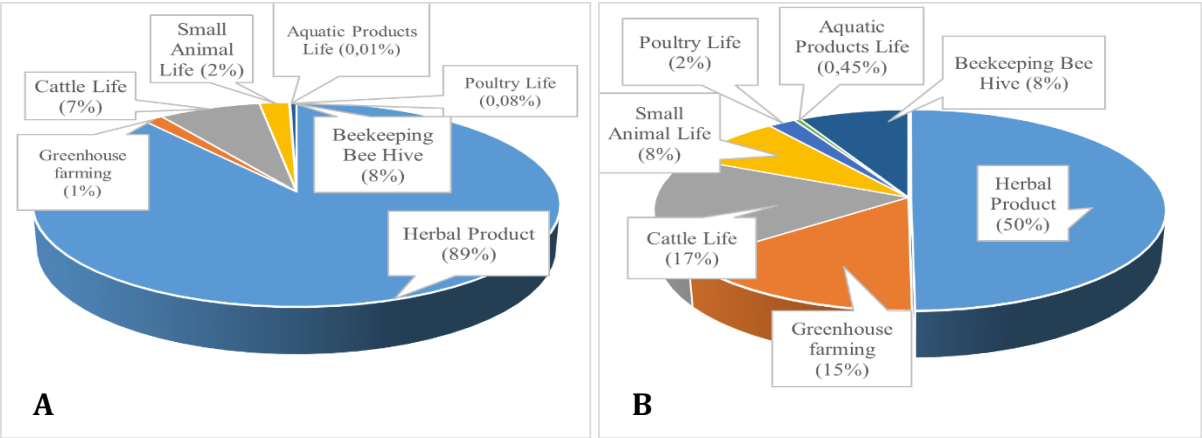

Figure 1. A) Policy distribution rates (%) and B)Insurance cost distribution rates (%) between 2013-2023 years.

Upon examining the distribution graphs, it is evident that the highest number of policies created between 2013-2023 was in the Crop Insurance branch, accounting for 89%. The share of crop insurance in the total insurance premium was calculated at 50%. The Livestock Life Insurance branch ranks second in terms of policy distribution, making up 7% of the total number of policies, while it accounts for 17% of the total insurance premium. Although the number of policies in Greenhouse Farming is 1%, it ranks third in terms of insurance premiums, representing 15% of the total premium. Small Livestock Life Insurance policies constitute about 2% of the total, with an 8% share in the insurance premium. Other branches, such as Beekeeping, Poultry, and Aquaculture, have policy numbers under 1%, with respective shares of 8%, 2%, and 0.45% in the total insurance premium. Agricultural insurance consists of various branches. This study specifically analyzes Crop Insurance, which holds the highest share in both policy numbers and premiums, and Livestock Life Insurance, which holds the second-largest share in terms of insurance premiums. The analysis conducted in this study utilizes the annual data set from 2013-2023. This data set includes the number of policies and insurance premiums categorized by agricultural insurance branches. Table 1 below presents the number of policies created by branch for each year, while Table 2 shows the insurance premiums by branch.

Table 1. Number of policies issued according to agricultural insurance branches by year (Number)

Branch / Year	Herbal Product	Cattle Life
2013	841.694	25.683
2014	1.029.586	23.320
2015	1.311.373	26.636
2016	1.366.550	35.777
2017	1.493.392	54.856
2018	1.607.121	90.904
2019	1.900.609	117.920
2020	1.952.825	181.773
2021	2.147.758	241.012
2022	2.654.588	278.199
2023	2.525.426	414.802

Source: Turkish Statistical Institute (TUIK,2024).

Table 2. Insurance costs by agricultural insurance branches by year (Million TL)

Branch / Year	Herbal Product	Cattle Life
2013	7.567	1.747
2014	9.288	1.950
2015	12.568	2.475
2016	15.861	3.497
2017	18.655	5.441
2018	23.153	9.892
2019	29.741	12.221
2020	39.305	21.785
2021	55.578	32.187
2022	143.568	76.289
2023	222.859	88.249

Source: Turkish Statistical Institute (TUIK,2024).

Between 2013 and 2023, Crop Insurance experienced an average growth rate of approximately 12% in the number of policies, while the growth rate for insurance premiums was significantly higher, at an average of 44%. The increase in policy numbers can be attributed to farmers' growing awareness of the risks they face in crop production and their desire to protect themselves against such risks. The substantial rise in crop insurance premiums is largely due to the sharp increase in insurance premium amounts, driven by inflationary pressures. For Livestock Life Insurance, the average growth rate in policy numbers between the years was 33%, while the average increase in insurance premiums was also 44%. This increase can be explained by the growing professionalism in the livestock sector and the heightened threat posed by animal diseases, which have become a more significant risk for farmers.

3.1. Crop Insurance Policy Numbers and Premiums

The time series of policy numbers created for crop insurance between 2013 and 2023 has been analyzed. It is observed that there is an increasing trend over time for crop insurance policies. To check the stationarity of the series, an analysis of autocorrelation and partial autocorrelation has been conducted. It was found that the series is not stationary and still contains certain correlations at varying levels. Figure 2 shows the autocorrelation plot of the crop insurance policy numbers of series, and the partial correlation.

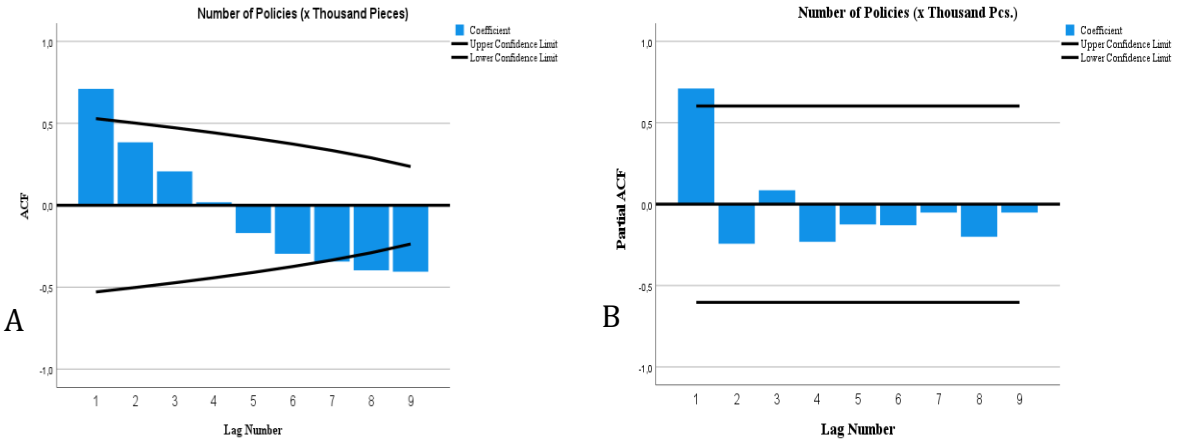

Figure 2. A) Plant products insurance policy number autocorrelation and B) Partial autocorrelation in the number of plant insurance policies.

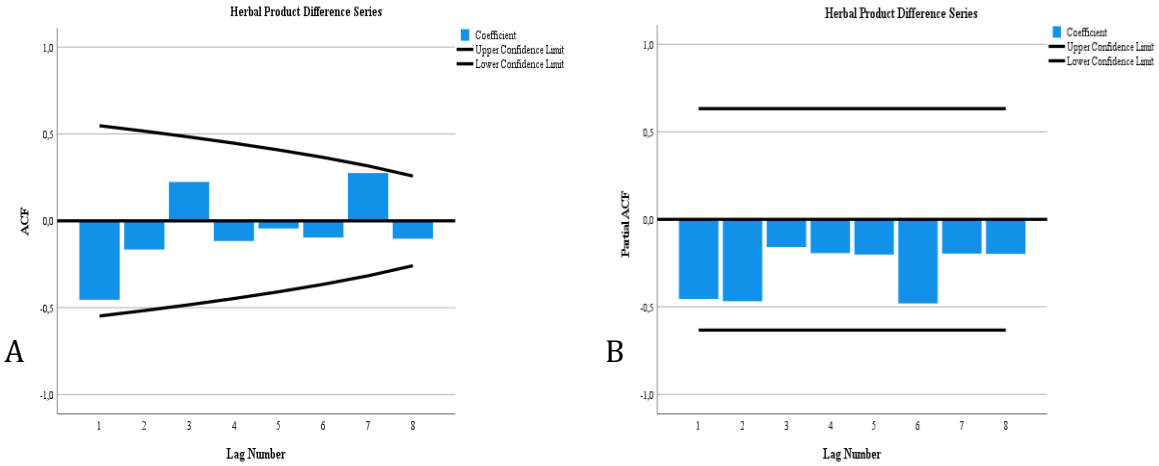

Figure 3. A) Crop insurance policy number difference series partial autocorrelation and B) Crop insurance policy number difference series partial autocorrelation.

Therefore, the first difference operation was applied, and a differenced series was created. Subsequently, stationarity was achieved by checking the ACF and PACF. After the differencing operation, the autocorrelations weakened significantly, and the p-value was high, indicating that the series became stationary. The autocorrelation test results for the different series are shown in Figure 3. below. The Box-Ljung test results confirm that the autocorrelations of the series are zero. Based on these results, the series modeling phase was initiated, and the ARIMA model was chosen. For the p (autoregressive) parameter, the PACF graph was examined, and the first sharp jump was selected as the value for p. In this graph, p could be 1, 2, or 6. For the d (differencing) parameter, the number of differences applied is taken, and since stationarity was achieved after the first difference in this series, the value of 1 was chosen. The "q" parameter, which represents the moving average, was determined by examining the ACF graph. This parameter indicates the predictive power of past error terms for future values. The first jump point in the ACF graph helps in determining the suitable value for q. In this case, 1 and 7 were identified as suitable values. ARIMA models were created for each combination, and the goodness-of-fit statistics were checked. The ARIMA (1,1,1) model provided the lowest MAPE value, and predictions were made using this model. The model's fit statistics are as follows: Constant R^2: 0.443, R^2: 0.998; RMSE: 114.694; MAPE: 3.816; MAE: 79.315; and Normalized BIC: 9.985. Subsequently, the trend in the number of agricultural insurance policies for crop insurance was identified and forecasting was performed until 2030. The results of the trend analysis for crop insurance policy numbers are shown in Figure 4 below.

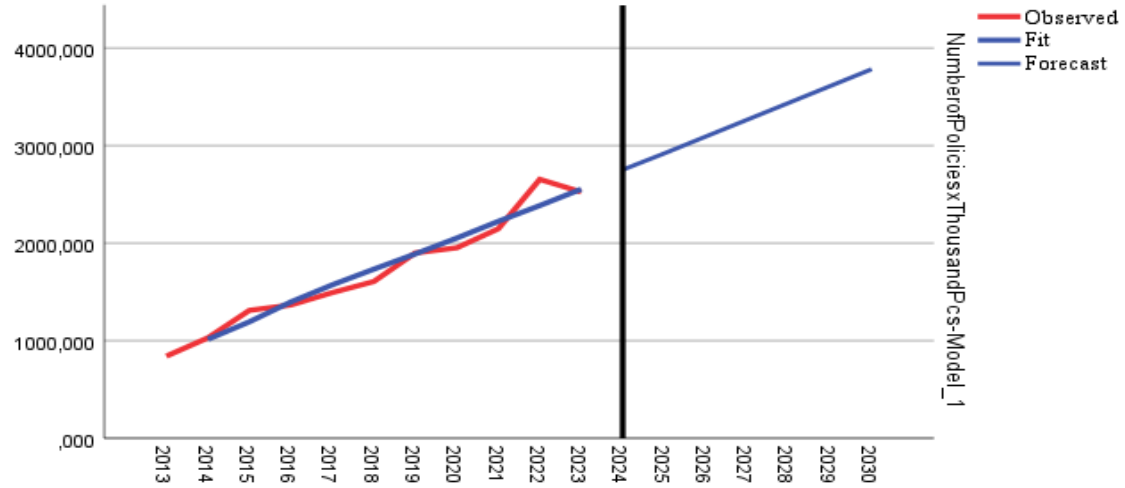

Figure 4. Trend analysis of the number of plant products insurance policies (thousand units).

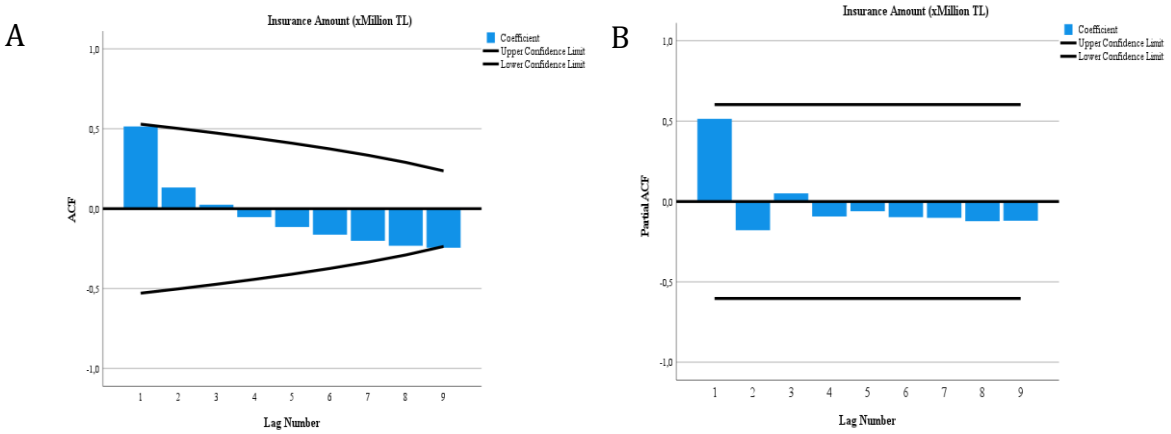

Figure 5. A) Autocorrelation of plant product insurance cost and B) Partial autocorrelation of crop insurance cost.

The number of policies produced in crop insurance is expected to be around 2.752 million in 2024, and this number is projected to reach 3.783 million by 2030. This represents an estimated growth rate of approximately 38%. The same analysis protocol was applied to the series of crop insurance premiums. The obtained autocorrelation and partial autocorrelation are shown in Figure 5. below. As can be seen from the figures, due to the stationary nature of the series, the model selection phase was initiated. In the analysis where the Brown model was preferred, the fit statistics were calculated as follows: R^2: 0.887, RMSE: 22,979.250, MAPE: 9.409, MAE: 8.619, and Normalized BIC: 20.303. While the model generally fits the data well, it indicates areas where improvements are needed. Expanding the data set to cover a broader time period would likely lead to better results. Additionally, since the insurance premiums are calculated in Turkish Lira, the rapid premium increase observed between 2021 and 2023, as shown in Figure 6, may have caused deviations in these values. Despite this, the model was accepted for forecasting, as the indicators for prediction performance are positive, even though this aspect was not covered in the scope of this study. Figure 10 below illustrates that there will be an increase in the premiums for crop insurance in the coming years. It is projected that crop insurance premiums will rise by approximately 157% by 2030. This indicates that a significant accumulation in the insurance pool is expected.

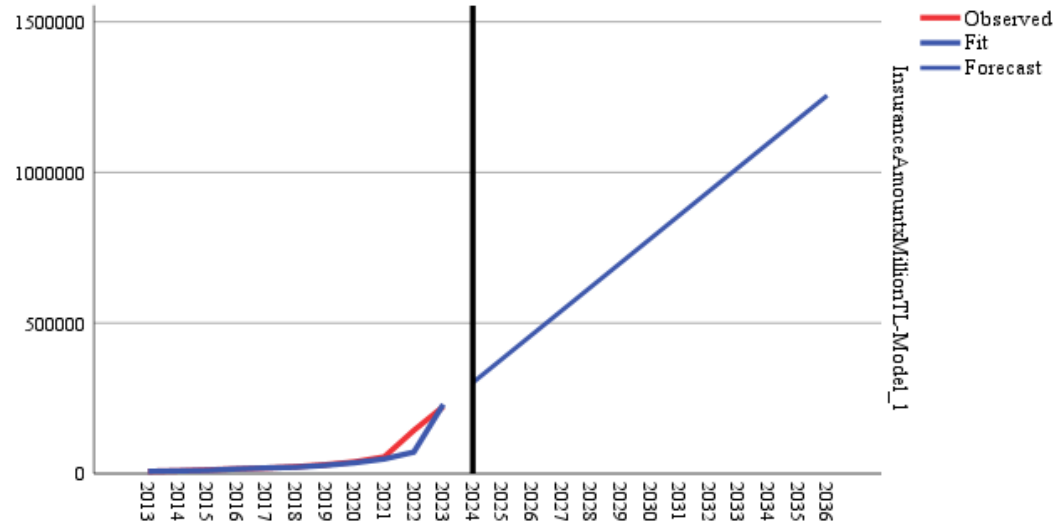

Figure 6. Plant products insurance fees trend analysis (million TL).

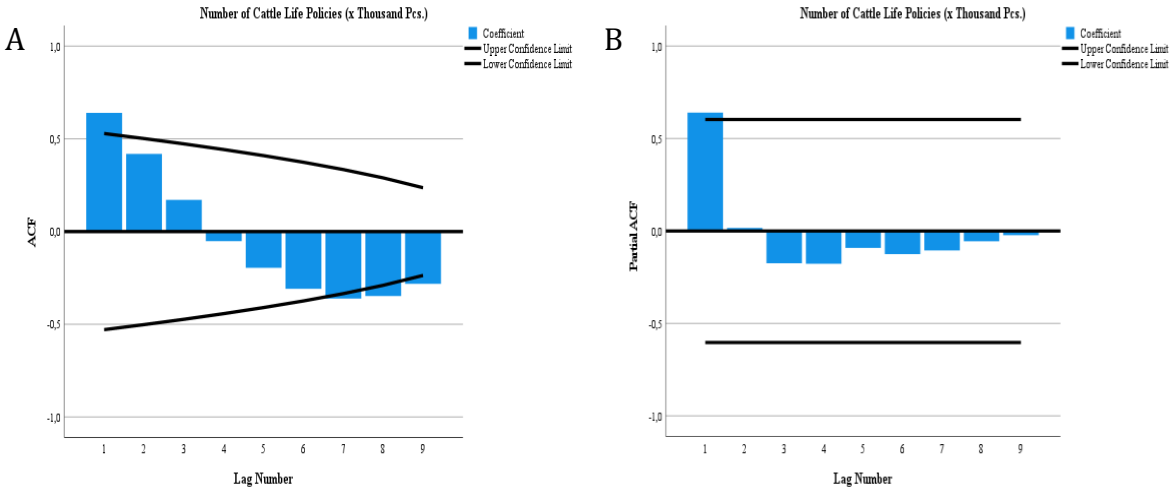

Figure 7. A) Cattle life policies autocorrelation and B) Partial autocorrelation.

3.2. Cattle Life Insurance Policy Numbers and Insurance Premiums

The observed series for the number of policies created in cattle life insurance between 2013 and 2023 was first analyzed. When looking at the number of policies produced for cattle life insurance between 2013 and 2023, it is observed that there has been an increase over time, indicating the presence of a trend effect. To check the stationarity of the series, the autocorrelation and partial autocorrelation analyses shown in Figures 7 below were conducted. It was found that the series was not stationary and still contained certain correlations. Therefore, a first difference operation was performed to create the difference series. After conducting ACF and PACF checks, stationarity was achieved in the series. Following the differencing, the autocorrelations weakened significantly, and since the p-value was high, the series became stationary. Figure 8 resulting from the difference operations are shown below. The results of the Box-Ljung test (after the first lag, with a p-value of 0.443, P>0.05) indicate that the autocorrelations of the series are zero.

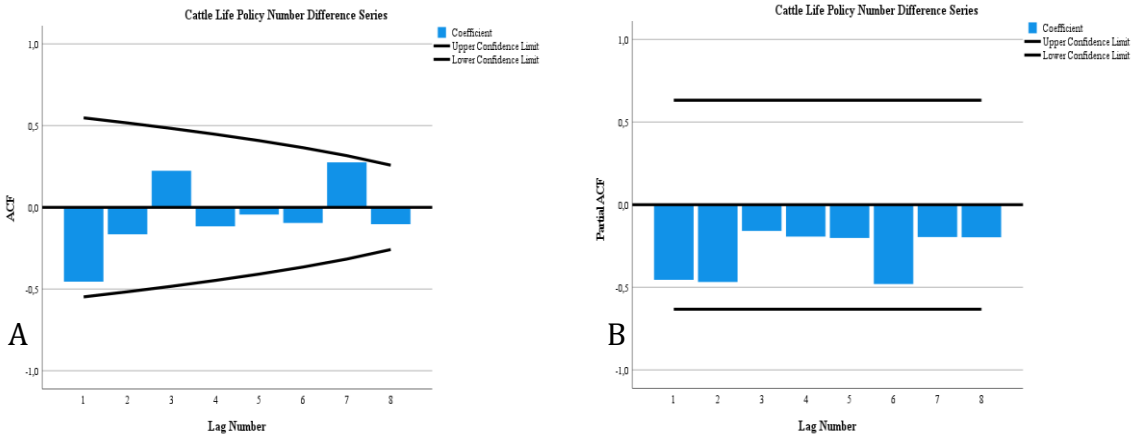

Figure 8. A) Cattle life policy number difference series autocorrelation and B) Cattle life policy number difference series partial autocorrelation.

Based on these results, the modeling process was initiated, and the ARIMA model was selected. For the p (autoregressive) parameter, the PACF graph was examined, and the first sharp jump was chosen as the value. In this graph, p values of 1, 2, and 6 could be possible. The parameter d (differencing) refers to the number of differences applied, and since stationarity was achieved after the first difference in this series, the value 1 was selected. For the "q" parameter, which represents the moving average, the ACF graph was used. This parameter indicates the power of past error terms to predict the future. The first jump point in the ACF helps determine the appropriate q value. In this case, 1 and 7 are suitable values. The ARIMA model was constructed for each combination, and the fit statistics were checked. Among the models, ARIMA (2,0,1) produced the lowest MAPE value, so forecasting was performed using this model.

The model's fit statistics are as follows: *Stationary R^2: 0.964, R^2: 0.972, RMSE: 13.535, MAPE: 3.983, MAE: 5.759, Normalized BIC: 11.876.* Since the fit statistics of the ARIMA (2,0,1) model were considered acceptable, forecasting was carried out. The trend analysis and predicted policy numbers are detailed in Figure 15. The trend in the number of policies for livestock life insurance is expected to continue upwards until 2030, with an estimated increase of around 83%. Lastly, the series for the insurance premiums of livestock life insurance policies was analyzed. To first determine whether the series is stationary, a unit root test was applied. The results obtained are presented in detail in Figures 16 and 17. Upon examining these graphics, the Box-Ljung test results show that the p-value after the first lag is greater than 0.05 (P>0.05; P=0.104), indicating that the series is stationary.

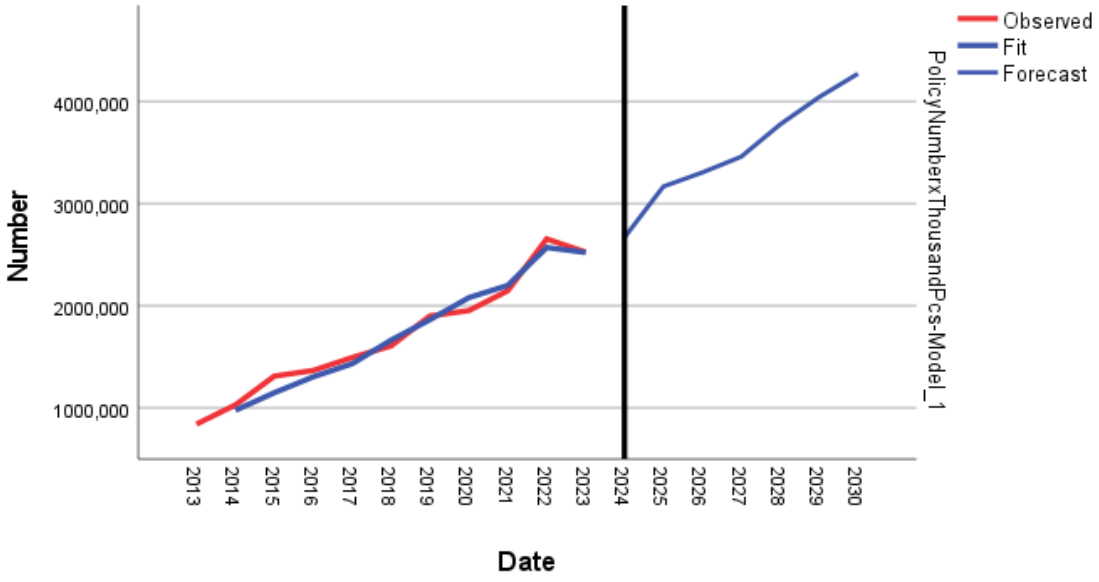

Figure 9. Cattle life insurance policy number trend analysis (thousand units).

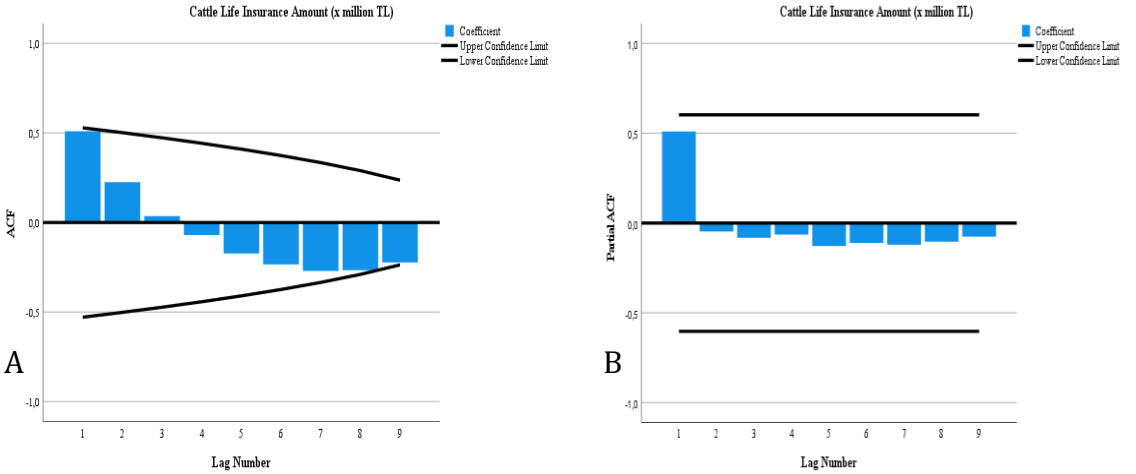

Figure 10. A) Cattle life insurance cost autocorrelation and B) Cattle life insurance cost partial autocorrelation.

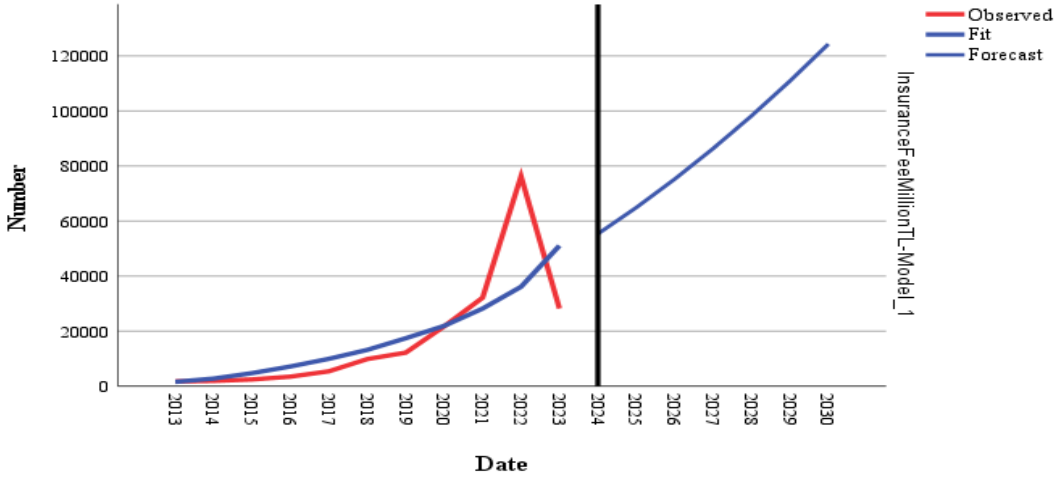

Figure 11. Cattle life insurance cost trend analysis (million TL).

Since the series is stationary, the model selection process proceeded, and analyses were conducted on the Holt, Brown, and ARIMA models to achieve the best MAPE value. The ARIMA (1,0,1) model, which showed the best fit, was selected for forecasting. The ARIMA model's fit statistics are as follows: Stationary R^2: 0.772, R^2: 0.842; RMSE: 16,461.786; MAPE: 21.798; MAE: 5,792.836, and Normalized BIC: 19.041. Although the model requires some improvements, the indicators suggest that it can be considered acceptable in terms of fit. The insurance premiums are calculated in Turkish Lira. As reflected in Figure 8, high inflation in macroeconomic indicators has also affected insurance premiums. Since this aspect is beyond the scope of this study, it has been disregarded, and the focus has been on evaluating the prediction scores. Although a sharp increase is observed from 2021 to 2022, such an extraordinary development has been taken into account in the analysis, and the trend analysis has been normalized. The trend graph indicates that there is an expected increase of approximately 65% in livestock life insurance premiums by 2030.

4. Discussion

Agriculture is a vital element of human civilization, deeply intertwined with global economies, biodiversity, and the historical evolution of human life. It also plays a crucial role in poverty alleviation, economic growth, and environmental sustainability. Agricultural insurance aims to compensate for losses that may occur during agricultural production processes. Drought, floods, frost, hailstorms, and other disasters significantly impact farmers' income and production capacities. Insurance allows for the management of these risks. Therefore, reducing risks and compensating for losses contribute to ensuring stability in the sector and fostering economic development. Agricultural insurance has consistently been a subject of interest among researchers from past to present. In his study, Glauber (2004) emphasized the importance of strategic approaches in the marketing of agricultural insurance. This study develops a strategic approach and provides businesses with strategic recommendations from both marketing and management perspectives, considering future trends in agricultural insurance. This study offers several practical findings and recommendations. One of the key findings is that *time*

series analysis is a valuable method for understanding changes in agricultural insurance demand, conducting risk assessments, developing marketing strategies, and making management decisions. This observation has also been emphasized in previous studies by Skees and Barnett (1999) and Goodwin (2001). The findings of the research indicate that the number of agricultural insurance policies and premium amounts will continue to follow an upward trend in the coming years. A similar study was conducted by Çekici (2009), in which the impact of climate change and global warming on agricultural insurance in Türkiye was examined. The study utilized a broad dataset, employing a time series of annual agricultural insurance premiums from 1986 to 2007. Using Pegels' exponential smoothing technique, projections for 2008 and 2009 were made. The results indicated that agricultural insurance in Türkiye had begun to spread, with the trend expected to continue upward. The use of data from 2013 to 2023 in this study is particularly significant, as it extends the forecasts made up until 2009, bringing them into the present. As noted by Çekici (2009), the continuation of the increasing trend has, in a sense, been confirmed by this study, adding an original contribution to the literature. Kızıloğlu (2017) conducted an applied study to identify the factors influencing farmers' decisions to purchase agricultural insurance. The study found that farmers' education levels, land sizes, annual incomes, and disaster risks significantly affected their insurance adoption. Similarly, Başer et al. (2023) examined the factors influencing farmers' decisions to ensure perennial crops and concluded that education level, experience, crop type, and land size were key determinants. The findings of this study also support existing literature. Over time, the inclusion of more educated individuals in the agricultural sector, land consolidation efforts allowing for larger and more diverse cultivated areas, and farmers' increased awareness of disaster-related damages have likely contributed to the rise in the number of insurance policies. This trend is also observable in time-series analysis graphs. With this, over the years, the strategies implemented by insurance companies to understand consumers and to meet their needs have had a positive impact on the number of policies produced. Insurance premiums have also increased in proportion to the number of policies. This indicates that farmers' trust in the insurance system has increased. In the future, it is important for insurance agents to maintain open communication channels with consumers, informing them about risks and compensation for damage. Employees play a key role in this process, and it should be emphasized that no customer should be lost. Employees should be encouraged to provide clear and transparent information during insurance sales, and motivational practices should be implemented. In their study conducted by Kaygısız et al. (2022), it is stated that in order to ensure and encourage more producers to benefit from insurance in agricultural production, the insurance fee in the insurance policy should be reduced to a reasonable level, the insurance policy fee should be discounted and some other necessary arrangements should be made. At this point, it is obvious that the support given by the state is of great importance in the spread of agricultural insurance. In Turkiye, agricultural insurance is 50% state subsidized. In recent years, producers have become more conscious, which is reflected in the increase in the number of policies and premiums. Financial support incentivizes producers to insure their products. Without this support, the high premium costs could lead to difficulties in payment. Therefore, the state should continue to improve and sustain this incentive system. Producers who benefit from the insurance system are increasingly insuring every aspect of their agricultural activities. This system not only protects the producers but also accumulates significant funds in the insurance pool. The accumulated premiums contribute to societal development and provide financing for sectors in need. Therefore, it is important to keep insurance premiums at reasonable levels for producers and to inform them about the benefits of insurance to encourage more farmers to integrate into the system. For agricultural insurance to be widely adopted and embraced by farmers, it is necessary to convince them of the benefits of the products. Insurance companies should carefully analyze farmers' socio-economic structures, agricultural activities, and risk perceptions. Based on this analysis, the structure and marketing strategies of the insurance products should be shaped accordingly. It is essential to convey the benefits of the insurance product to farmers. Otherwise, they will not show interest in or demand the product. Therefore, farmer education programs should be organized, and the insurance system should be explained in detail. Farmers should be encouraged to view insurance products as a solution rather than a burden. To achieve this, messages should be delivered through communication channels suited to their needs. Tailored products should be developed, taking into account regional differences, and specific policies should be offered. The adoption of technology-based approaches in damage assessment will increase farmers' trust in insurance, ensure objectivity in damage assessments, enhance transparency, and speed up the process. Drones and satellite technologies could be utilized for this purpose. Another recommendation is to increase farmers' motivation by sharing the experiences of other farmers who have benefited from insurance. Insurance companies can direct their efforts to these testimonials, which can significantly contribute to the persuasion process. The marketing of agricultural insurance is not just about selling a financial product; it also involves raising awareness among farmers, ensuring the sustainability of the sector, and contributing to economic development. Effective marketing strategies that increase farmers' interest in insurance can help manage risks more effectively. The success of these efforts will be enhanced by using education, personalized communication, and government support. Ultimately, the widespread adoption of agricultural insurance will provide multifaceted benefits to both farmers and the agricultural sector. Kamilcelebi (2012) highlighted the issue of insurance

sector mergers or sales to foreign companies. This issue needs significant attention. It is important to conduct necessary studies within the framework of organizational theory in the insurance sector and address the potential challenges due to an inability to adapt to environmental changes. The concept of "organizational aging" describes how organizations with old structures struggle to adapt to environmental changes. If organizations fail to keep up, they may be forced to close down according to the "decay theory." As seen in the data, insurance organizations are experiencing growth in their structures and transaction volumes. This growth will bring about environmental changes. Insurance managers in Türkiye are advised to adapt to these changes and tackle potential entropy situations. According to the contingency strategy in organizational theory, the growing agricultural product market is expected to expand the insurance market as well. Another responsibility for insurance managers is to standardize rules and methods and ensure the specialization of roles, with centralized decision-making. According to Akçaöz et al. (2006), accurately identifying the damages caused by risks in agricultural production will allow for rational determination of premiums that are in line with farmers' payment capacities. For agricultural insurance to be accepted by farmers, it is essential to involve experts in the damage assessment process. This will help prevent errors and increase farmers' trust in insurance companies. To boost entrepreneurship and risk-taking in the agricultural sector, it is important to create environments where producers feel more secure. In agriculture, risk management plays a key role in creating such environments. In addition to income insurance, it should also be considered that damage caused by disasters can be compensated through agricultural insurance. Addressing farmers' social security issues is also essential. This study contributes to identifying future trends in agricultural insurance and provides insights into the management and marketing strategies of insurance companies. However, it has certain limitations. The scope of the study is limited to analyses conducted on the number and value of crop production and cattle farming insurance policies. Agricultural insurance also encompasses other product categories such as greenhouse farming, aquaculture, beekeeping, small ruminants, and poultry. Therefore, incorporating these additional product groups in future research could yield different findings. Another limitation of the study is that the dataset covers the period from 2013 to 2023. This period was selected primarily to examine the prolonged adverse global economic conditions and their impact on agricultural insurance in greater depth. However, it also captures the effects of the COVID-19 pandemic, making the study period particularly relevant. Nevertheless, extending the time series to cover a longer period in future studies could lead to different insights. Additionally, the study employs the ARIMA model for modeling policy values. Since the time series analysis of policy values was conducted using current prices, there is a potential concern that this approach might negatively influence the results. To address this issue, researchers tested the validity and reliability of the models and confirmed that no adverse effects were present in the series, effectively controlling for inflationary effects. Specifically, stationarity tests (Dickey-Fuller ADF) were conducted to ensure the stability of the series. Ljung-Box tests were performed to assess autocorrelation, and the model's forecasting performance was evaluated using AIC, BIC, and RMSE criteria, all of which indicated low error rates. As a result, the models demonstrated good fit, low error values, and consistent outcomes, reinforcing the reliability and validity of the analysis conducted with current prices. Furthermore, the sharp increase observed during a specific period was identified as a *temporary shock*, as the series returned to its previous pattern in subsequent years. The shock effect observed between 2021 and 2022 did not result in a permanent structural break in the series. This is evident from the fact that the series resumed its normal course afterward. Additionally, the Ljung-Box test confirmed that no permanent deterioration occurred in the series following the anomaly. The analysis suggests that the sharp rise observed in a single year within the 2013–2023 dataset did not disrupt the overall trend and that such temporary fluctuations are inherent to economic systems. Thus, the inflationary surge had a limited impact on the model's ability to capture the overall trend. Nonetheless, future studies are advised to use real prices instead of current prices to enhance robustness. Future research could focus on identifying the factors influencing farmers' adoption and utilization of agricultural insurance. Such studies could provide significant contributions to the development of strategies for the sustainable production of insurance policies. Finally, research could be designed to explore the underlying reasons for changes detected through time series analysis in agricultural insurance. Understanding the factors driving shifts in farmers' insurance product preferences could yield valuable insights for customer segmentation and target market selection strategies, ultimately informing more effective decision-making processes.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	B.A.	H.O.O.
C	60	40
D	60	40
S	50	50
DCP	50	50
DAI	80	20
L	60	40
W	80	20
CR	80	20
SR	70	30
PM	80	20
FA	50	50

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because there was no study on animals or humans.

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EFFECT OF CASHLESS ECONOMY POLICY ON SMALLSCALE AGRICULTURAL BUSINESSES IN ANAMBRA STATE, NIGERIA

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Abstract: This study examined the effect of the cashless economy policy on small-scale agricultural businesses in Nigeria, focusing on the awareness, perceptions, adoption, and factors affecting the adoption of cashless financial transactions by the business owners. A total of 125 respondents were surveyed across various small-scale businesses in the region, using structured questionnaires. The study analyzed the data using descriptive statistics like frequency, percentage and inferential statistics. The findings revealed that 62.4% of respondents were aware of the policy, with mobile transfers being the most commonly employed transaction method (36.8%). A smaller group perceived the policy as politically motivated or intended to cause hardship for the poor. Mobile transfers (36.8%) were the most commonly used transaction instrument, followed by POS (19.2%) and ATM (16.0%). This highlights the reliance on mobile platforms for cashless transactions. However, some factors like age, high cost of the instrument, location of business, technical knowhow and internet network availability affected the utilization of cashless financial transactions instruments among the respondents. The test of hypothesis to ascertain the effect of the cashless financial transactions on smallscale agricultural businesses indicated that there was a positive effect. The study highlights the need for better infrastructure, increased awareness, and tailored solutions to support the smooth transition to a cashless economy, particularly for small-scale businesses.

Keywords: Smallscale businesses, Cashless policy, Perception, Adoption, Factors

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1. Introduction

Until recently, both formal and informal sectors conducted business transactions primarily with physical cash, such as naira notes, coins, cheques, bank drafts, money orders, and tellers. This cash-dependent system provided a straightforward, durable, and trustworthy method for managing financial transactions, allowing businesses to operate effectively within a paper-based economy (Gbalam and Dumani, 2020). However, as international business grew, technological advancements emerged, and global education levels rose, cash-based transactions began encountering significant limitations. Issues included increased corruption, vulnerability to armed robbery, terrorism financing, potential financial losses from natural disasters, the physical burden of handling large amounts of cash, high transaction costs, and even bacterial contamination from handling banknotes (Ezinwane, 2023). Traditional cash transactions face various issues due to increasing globalization, advancements in technology, and rising literacy levels. Cash-based systems have shown limitations related to inefficiency, unreliability, and convenience, with challenges such as corruption, theft risks, and health concerns associated with handling physical currency (Ezinwane, 2023). For example, the risk of bacterial transmission through physical cash poses public health risks. These challenges highlighted inefficiencies and inconveniences in cash-based systems, encouraging a shift towards digital and cashless transaction methods. Over recent years, global trends toward digitalization have significantly influenced economies worldwide, particularly in payment systems. The financial sector has adapted through various organizational changes aimed at supporting the efficient production and trade of goods and services. Driven by deregulation, globalization, and advancements in information systems, new financial practices have emerged, enabling both banks and customers to handle transactions in innovative ways. E-cards and internet banking now allow users to manage their finances with ease, providing 24-hour access to accounts, transaction histories, money transfers, savings, and other banking

services from any location (Adedeji and Oluyemi, 2021). In Nigeria, the Central Bank of Nigeria (CBN) introduced the cashless policy in 2012 to enhance the efficiency of transactions, reduce cash handling costs, foster financial inclusion, and combat illicit financial activities. The policy was envisioned to shift Nigeria's economy from cash-heavy transactions to electronic payments, aligning with international standards and improving the quality of banking services. Among its goals, the policy seeks to minimize the issues related to high cash usage, including elevated transaction costs, security risks, and inefficiencies (CBN, 2011). A cashless economy refers to a system where transactions are conducted primarily through electronic media, rather than physical cash, using mobile apps, credit cards, checks, and other digital tools. The concept does not imply a complete absence of cash but rather emphasizes an economic structure where the exchange of goods and services relies on electronic payment. The informal economy, consisting of diverse, often unregulated activities, plays a critical role in economic development by fostering employment and generating revenue (Aremu and Adeyemi, 2011). This sector includes micro and small enterprises, which contribute substantially to local capital growth and support higher productivity levels (Xia, Qamruzzaman and Adow, 2022). The implementation of the cashless policy in Nigeria has encountered significant challenges. Issues include delays in confirming transactions at points of sale, non-remittance of funds, losses to payment channel operators, advanced fraud, and poor customer service. According to Institute of Chartered Accountants of Nigeria (ICAN), the policy has faced multiple reversals and amendments by the Central Bank of Nigeria (CBN), particularly since December 6, 2022 (Punch, 2022). These issues were further exacerbated by the near collapse of banking payment systems and the challenging rollout of the currency redesign, which aimed to fully transition Nigeria to a cashless economy by January 9, 2023. This situation led to considerable hardship for both individuals and businesses across the country. Heman and Anna (2023) noted that the cashless policy has introduced certain difficulties for small business owners in Nigeria, including costs for electronic payment systems, inadequate infrastructure, and varying levels of financial literacy. A number of studies have investigated the impact and effect of cashless economy policy in Nigeria. However, none of these studies has focused exclusively on the effect of cashless economy policy on smallscale businesses in Awka North. Therefore, this current study will fill such gap in knowledge by ascertaining effect of cashless economy policy on Small scale businesses in

Awka North. The specific objectives of the study were;

i. identify the types of small-scale agricultural businesses in the study area;
ii. assess the level of awareness of the cashless economy policy among small-scale agricultural business owners;
iii. investigate the perception of the cashless economy policy among small-scale agricultural business owners;
iv. examine the types of cashless financial transaction instruments used by small-scale agricultural businesses; and
v. determine the factors affecting the utilization of cashless financial transaction instruments by small-scale agricultural businesses.

Hypothesis: Ho: There is no effect of cashless financial transactions on smallscale agricultural businesses.

1.1. Literature Review

Micro enterprises in Nigeria are typically defined as businesses with project costs (excluding land) up to ₦500,000, while small enterprises have project costs up to ₦50 million and employ up to 100 workers (Nkwede and Nkwede, 2022). Small businesses, such as food vendors, farmers, artisans, and mechanics, are pivotal for self-reliance and economic growth (Koloti, 2022). Adedeji and Oluyemi (2021) found that while the cashless policy has promoted electronic payments, small businesses encounter challenges, including high transaction fees and limited access to financial services. Similarly, research by Ogbeide and Atanbori (2019), as well as Adegbaju and Oladeji (2020), revealed that despite viewing cashless systems as a means to reduce cash-handling risks, small enterprises struggle with costs associated with maintaining these systems and low financial literacy levels. Further studies by Obi (2023) suggested that the cashless policy provides SMEs with digital payment options, reducing cash dependency and minimizing theft and fraud risks. However, SMEs, particularly those in rural areas with limited infrastructure, experience unique challenges. Phinaonyekwelu and Nnabugwu (2018) conducted a study to evaluate the impact of the government's cashless policy on the performance of Micro, Small, and Medium Enterprises (MSMEs) in Anambra State. The independent variables examined were cashless transaction channels, specifically internet/online banking services, automated teller machine (ATM) services, and mobile banking services. Employing a descriptive survey design, the researchers used summary statistics, Pearson correlation, and multiple regression analysis, with all statistical tests performed at a 0.05 significance level. Initial findings indicated an F-statistic of 23.516, which was significant ($P<0.05$). The regression coefficient of 0.627 suggested a 62.7% relationship between the dependent and independent variables, while the R^2 value of 0.678 implied that 67.8% of the variance in MSME performance could be explained by the selected independent variables. Key results demonstrated that internet/online banking, ATM, and mobile banking services had a positive and significant effect on MSME performance in Anambra State. Odumusor (2023) investigated the impact of the cashless policy on the performance of small-scale enterprises in Nigeria, focusing on selected businesses in the Cross River

Northern Senatorial District. The study aimed to assess three specific objectives: the effect of Automated Teller Machine (ATM) transactions, internet banking, and Point of Sale (POS) transactions on the performance of small-scale businesses in the district. Adopting a descriptive research design, the study targeted 122 employees from Gomara Farms Ltd, Ushie Table Water Ltd, and Blessed Brother Bread Ltd., with a final sample size of 111 determined using Krejcie and Morgan's (1970) table. Simple random sampling was applied to select participants, and data analysis was conducted using Ordinary Least Squares (OLS) regression with SPSS (version 27), employing both descriptive and inferential statistics. Findings revealed that ATM transactions, internet banking, and POS transactions all have a significant positive effect on the performance of small-scale businesses in the Cross River Northern Senatorial District. Humphrey (2017) studied the impact of the cashless policy on small-scale businesses in Ogoni Land, Rivers State, Nigeria. The research aimed to assess how cashless transactions affect these businesses. Conducted in Ogoni, the study utilized purposive sampling to select 250 small business owners and operators who completed questionnaires. Data analysis involved frequency tables, percentages, and regression analysis, conducted using SPSS (Statistical Package for Social Sciences). Findings revealed that most small-scale businesses in Ogoni operate as sole proprietorships with low income, limited banking practices, and minimal reliance on substantial capital. Additionally, many businesses focused on providing services that largely depend on a "cash and carry" approach, rendering bank transactions, ATM usage, and online banking less relevant. The study found that business operators showed minimal use of ICT in business operations and transactions, presenting a significant challenge to the adoption of cashless policy in the area. Overall, the cashless policy introduction had a negative impact on small-scale business processes and growth in Ogoni Land.

2. Materials and Methods

2.1. Area of the Study

The location of this study was Awka North Local Government Area. Awka North LGA was created in 1989 from Awka LGA of old Anambra state, It is bounded on the south by Awka South LGA and on the north by Oji-River LGA of Enugu State. Awka north is one of the 21 Local Government Areas that make up Anambra State; it is made up of ten (10) communities namely: Awba Ofemili, Ugbene, Ebenebe, Achalla (the capital), Urum, Amanasa Amauke, Amansea, Mgbakwu and Ugbenu. In the past, people of Awka North were known for farming and fishing. Today, Awka North LGA prides itself as the food basket of Anambra state. The area in the past was the site of the Nri civilization (Atupulazi, 2016). According to Okafor (2015), the traditional occupation of the people of Awka North has been farming, trading, hunting, fishing and a host of others. Most people in Awka North LGA are farmers (self-employed). However, there are increasing numbers of residents who are now engaged in wage labour. Awka North Local Government Area was chosen for this study because of its strategic location and importance to the problem under investigation. The National Bureau of Statistics (2021) projected the population of Cities and Local Government Areas in Nigeria. Hence, Awka North LGA was projected for 2021 using 3.0 percent annual population growth rate to arrive at 198,065 persons. A breakdown of the population shows 108, 575 males and 89,490 females. The target population for this study were the smallscale agricultural business owners aged I8 years and above. The sample size for this study was one hundred and twenty five (125) respondents. T test analysis was used to test the hypothesis. For all purposes, p-value of 0.05 was considered as the level of significance.

2.2. Sampling Technique

Multistage sampling technique was employed in the study. First, the communities in Awka North LGA were identified as follows: Awba Ofemili, Ebenebe, Achalla (headquarters), Urum, Amanuke, Amansea, Ugbene, Mgbakwu, Ugbenu, and Isu Aniocha. Then, with purposive sampling technique, five communities were selected namely: Mgbakwu, Ugbenum, Amanuke, Ebenebe and Amansea. The third stage was the random selection of twenty five respondents from each of the communities making it one hundred and twenty five (125) smallscale agricultural businesses in the study area. Hence, a total of one hundred and twenty five (125) respondents were administered the questionnaires. The data collected was analyzed using descriptive and inferential statistics.

2.3. Data Analysis Techniques

The data collected were analyzed using descriptive statistical and econometric tools. Objectives (i) (ii) (iii) and (iv) were analyzed using frequency, percentage and mean. Objectives (v) was analyzed using regression analysis; the implicit functional form is specified as follows:

$$Cashless=\beta0+\beta1Age+\beta2Sex+\beta3High\ Cost\ of\ Cashless\ Transaction\ Instruments+\beta4Location\ of\ Business+\beta5Technical\ Knowhow+\beta6\ Internet\ Availability+\epsilon$$

Where:

Cashless = Dependent variable (utilization of cashless financial transaction instruments).

Age = Age of the small-scale agricultural business owner (Independent variable).

Sex = Gender of the business owner (Independent variable, where male = 1, female = 0).

High Cost of Cashless Transaction Instruments = A variable indicating the cost of adopting cashless systems (Independent variable).

Location of Business = A variable related to the location where the business operates (Independent variable).

Technical Knowhow = The technical knowledge of the business owner about cashless financial systems

(Independent variable).

Internet Availability = A variable indicating the availability of internet access for the business (Independent variable).

ϵ = Error term (captures unobserved factors affecting the dependent variable)

2.4. Test of Hypothesis

In order to express the test of hypothesis mathematically, we are working with a t-test for independent samples, where you are comparing the means of two groups (those using cashless transactions and those not using cashless transactions) to determine if there is a significant difference between them.

1. Null Hypothesis (H_0): There is no effect of cashless financial transaction instruments on small-scale agricultural businesses.

Mathematically: H0: $\mu1 - \mu2 = 0$

where $\mu1$ is the population mean for small-scale agricultural businesses using cashless transactions, and $\mu2$ is the population mean for those not using cashless transactions.

2. Alternative Hypothesis (H_1): here is an effect of cashless financial transaction instruments on small-scale agricultural businesses.

Mathematically: H1: $\mu1 - \mu2 \neq 0$

3. Results and Discussion

3.1.Types of Smallscale Agricultural Businesses in The Study Area

The result in Table 1 shows that majority (28%) of the respondents in the study area were into the selling of foodstuff, which was followed by respondents selling provisions (20.8%), 12% of the respondents were selling vegetables, 11.2% of the respondents were selling fast food, 7.2% were into food vendors, minimarket and food stall, and 8% were operating a mini supermarket. The result shows that in the study area, most of the respondents were into the selling of one agricultural goods or the other. The findings from Table 1, showing a predominance of food-related small businesses such as foodstuff, provision shops, and fast-food vendors, align with research that highlights the significance of food and essential goods retailing within local economies. Studies indicate that small businesses in emerging and developing regions often center around daily necessities, with food-related businesses being particularly prominent due to consistent demand for staple and convenience goods. The result supports Onyema et al. (2018) who pointed out that food and provision shops are crucial in fulfilling the daily needs of low-income communities, especially where larger retail infrastructures may be lacking. Similarly, Goyal and Pradhan (2020) observed that small-scale food enterprises provide affordable, accessible options for local consumers, sustaining both livelihoods and food security within the community. Furthermore, research by Tebaldi and Elmslie (2017) noted that micro and small enterprises focusing on essential goods like groceries and prepared foods tend to thrive in densely populated, low-income areas where informal economies play a critical role. This phenomenon is especially observed in sub-Saharan Africa, where small food businesses meet local demands efficiently, with relatively low startup costs and operational flexibility (Agyapong, 2010). Additionally, the prevalence of these businesses, as indicated by Table 1, may reflect a strategic adaptation by entrepreneurs to capitalize on high-demand items, such as basic foodstuffs and fresh produce, which provide a stable customer base and frequent sales turnover, as suggested by Karnani (2011).

Table 1. Types of smallscale agricultural businesses*

Variables	Frequency (125)	Percentage (%)
Mini supermarket	8	6.4
Provision shop	26	20.8
Minimarket	9	7.2
Foodstuff	35	28.0
Food stall/kiosk	9	7.2
Food vendors	9	7.2
Vegetable seller	15	12.0
Fast food	14	11.2

*Field study 2024

3.2. Level of Awareness of The Cashless Policy

The results in Table 2 reveal that a majority of respondents (62.4%) were aware of the cashless economy policy, while 8% were not aware, and 29.6% neither confirmed nor denied their awareness. This finding reflects a relatively high level of awareness among respondents, indicating that the cashless economy policy has been fairly well-publicized. Awareness levels can significantly influence adoption and acceptance, as shown by studies in similar contexts. Adesina and Ayo (2010) demonstrated that high awareness of digital financial services in Nigeria was positively correlated with the adoption of these services, suggesting that knowledge is a critical first step in successful implementation of cashless policies. A similar study by Chukwu and Ezeagba (2017) found that public awareness campaigns were essential in increasing awareness of cashless transactions in Nigeria, but they also highlighted that awareness does not always translate to usage. People may be aware of the policy but still hesitant to adopt cashless practices due to concerns about reliability, accessibility, and digital literacy. The significant percentage (29.6%) of respondents who neither confirmed nor denied awareness could imply partial or superficial knowledge of the cashless policy people may have heard of it but lack sufficient understanding to participate confidently. The finding that 8% of respondents were not aware of the policy aligns with research suggesting that awareness of financial inclusion policies is often lower in rural areas and among

people with limited access to education or technology. Sanusi (2012), in a study on financial inclusion, noted that information dissemination in urban areas generally leads to higher awareness, whereas rural populations may remain uninformed due to limited infrastructure and outreach efforts. This points to the importance of targeted education and training to bridge awareness gaps.

Table 2. Level of awareness of the cashless economy policy*

Variables	Frequency (125)	Percentage (%)
Aware	78	62.4
Not aware	10	8.0
Neither	37	29.6

*Field study 2024.

3.3. Perception of The Cashless Economy Policy

The data in Table 3 illustrates a range of perceptions about Nigeria's cashless economy policy among respondents, reflecting varied attitudes and understanding of its purpose. The majority, 47.2%, view the policy as a measure to reduce looting in the country, suggesting a perception that the cashless system could curb corruption and improve financial accountability. This aligns with studies highlighting the positive impact of cashless policies in enhancing transparency and reducing financial malpractice, as found in research by Akinola (2021) and Okeke and Eze (2020). These studies argue that limiting cash transactions constrains illicit financial flows, making it more challenging for individuals to misappropriate funds. Another significant group, 17.6%, believes the policy is aimed at controlling inflation, which echoes discussions by financial analysts on cashless policies as mechanisms to stabilize currency circulation and reduce cash hoarding (Sanusi, 2012). This viewpoint reflects an understanding that reduced physical cash transactions could alleviate inflationary pressures in the economy by encouraging funds to remain in the banking system, thus providing more stability. Additionally, 16.8% of respondents think the policy has political motives, particularly to curb vote-buying during elections. This perception is supported by studies such as Adekunle (2020), which discuss how cash restrictions may reduce cash-driven election malpractices, fostering fairer electoral processes. A smaller portion of respondents view the policy with skepticism. About 3.2% believe it primarily serves the wealthy, while 2.4% feel it is intended to create hardship for the poor. These perspectives align with critiques noted by Adesina and Ayo (2010), who found that cashless policies may disproportionately affect lower-income populations lacking adequate access to digital banking infrastructure, causing increased financial exclusion. Lastly, the minority who perceive the policy as "a scam" (0.8%) reflects a broader skepticism and mistrust of government initiatives, particularly in contexts where there has been historical mismanagement or policy reversals. Such skepticism can also be tied to the technical and infrastructural challenges observed during the initial phases of implementation, as noted by Chukwu and Ezeagba (2017).

Table 3. Perception of cashless economy policy*

Variables	Frequency (125)	Percentage (%)
It is a scam	1	0.8
It is for the rich	4	3.2
It is to suffer the poor	3	2.4
To check inflation	22	17.6
For political reasons/against vote buying during elections	21	16.8
It is to reduce looting in the country	59	47.2
All of the above	15	12.0

*Field study 2024.

3.4. Cashless Economy Policy Transaction Instruments Employed by The Respondents

The data in Table 4 shows the different transaction instruments employed by respondents within the cashless economy policy framework. The results highlight that mobile transfers are the most commonly used method, with 36.8% of respondents favoring this form of transaction. This aligns with recent studies showing the growing popularity of mobile banking due to its convenience, accessibility, and ease of use, especially as smartphones become more widespread (Sanusi, 2021). Additionally, mobile transfers offer flexibility and allow transactions to be completed anytime, which is particularly advantageous in areas with limited physical banking infrastructure (Oluyemi et al., 2020). The use of POS (Point of Sale) systems follows closely at 19.2%, demonstrating a significant uptake in digital payment infrastructure within small and medium-sized businesses. According to Adesina and Ayo (2020), POS systems have become popular in retail transactions, as they offer a secure and reliable payment method, especially in rural and semi-urban areas where ATM access may be limited. ATM use ranks third at 16.0%, which reflects its continued relevance in cash withdrawal and basic financial transactions. However, studies indicate that reliance on ATMs is gradually decreasing as

more people transition to digital methods like mobile and POS payments (Adeleye, 2019). The presence of checks and trade by barter, both at 2.4%, suggests that while digital options are growing, traditional forms of transaction still have limited usage, particularly in cases where technology or banking services are scarce. Interestingly, 23.2% of respondents reported using "all of the above" transaction methods, suggesting that they are adapting to various tools depending on situational needs. This adaptability is supported by research from Chukwu and Ezeagba (2017), who argue that consumers in developing economies often rely on multiple transaction methods due to inconsistent access to infrastructure and varying transaction requirements.

Table 4. Cashless economy policy transaction instruments employed by the respondents*

Variables	Frequency	Percentage
POS	24	19.2
ATM	20	16.0
Cheque	3	2.4
Mobile transfer	46	36.8
Trade by barter	3	2.4
All of the above	29	23.2

*Field study 2024.

3.5. Factors Affecting The Utilization of Cashless Financial Transactions Instruments

Table 5 outlines the key factors affecting the utilization of cashless financial transaction instruments by small-scale agricultural businesses. Statistically significant variables (P<0.05): Age, high cost of the instrument, location of business, technical knowhow and internet network availability. Not significant: Sex (P=0.520).

Constant (Intercept): B = 3.496: This is the predicted value of the dependent variable (cashless) when all independent variables (Age, sex, high cost of the instrument, location of business, technical knowhow and internet network availability) are equal to zero. t = 9.954, P=0.000: This constant is statistically significant, as indicated by the very low p-value.

Age (age):

B = -2.559: For each one-unit increase in age, the dependent variable (cashless) decreases by 2.559 units, holding all other variables constant. The negative sign indicates a negative relationship between age and cashless. Beta = -0.708: This is the standardized coefficient, showing that age has a strong negative effect on cashless. Age is the most influential variable among all predictors in this model. t = -10.364, P=0.000: The effect of age on cashless is highly statistically significant. Tarek and Ahmed (2019) in their study explores how demographic factors like age influence the adoption of cashless payments. Their findings indicated that older individuals are less likely to use cashless systems in agricultural marketing in Sub-Saharan Africa.

Sex (sex):

B = 0.100: There is a small positive relationship between sex and cashless, where a one-unit increase in the sex variable (e.g., male = 1, female = 0) leads to an increase of 0.100 units in cashless. Beta = 0.035: The standardized coefficient suggests that sex has a very small effect on cashless. t = 0.645, P=0.520: This is not statistically significant (p > 0.05), meaning that sex does not have a meaningful impact on cashless in this model.

3.6. High Cost of The Cashless Transaction Instruments

B = -1.066: For each one-unit increase in cashless transaction instruments, cashless decreases by 1.066 units, indicating a negative relationship. Beta = -0.283: The standardized coefficient shows that education has a moderate negative effect on cashless. t = -3.990, P=0.000: This effect is statistically significant.

3.7. Location of Business

B = -1.318: For each one-unit increase in the location of business variable (possibly an indicator of relationship status), cashless decreases by 1.318 units, showing a negative relationship. Beta = -0.356: The standardized coefficient shows that location of the business has a moderately strong negative effect on cashless. t = -5.830, P=0.000: The effect of the business location is highly statistically significant.

3.8. Technical Knowhow

B = 1.694: For each one-unit increase in technical knowhow, cashless increases by 1.694 units, showing a positive relationship. Beta = 0.450: The standardized coefficient indicates that technical knowhow has a moderate positive effect on cashless. t = 8.053, P=0.000: The effect of awareness on cashless is highly statistically significant. The findings corresponds Moran (2021) who found that educated farmers in rural India were more likely to embrace digital payment systems for agricultural goods, which positively impacts market accessibility

3.9. Internet Availability

B = 2.485: For each one-unit increase in internet availability, cashless increases by 2.485 units, indicating a positive relationship. Beta = 0.568: The standardized coefficient suggests that internet availability has a moderate to strong positive effect on cashless. t = 7.225, P=0.000: The effect of experience is statistically significant.

Table 5: Factors affecting the utilization of cashless financial transaction instruments*

Model	B	Std Error	Beta	T	Sig
Constant	3.496	.351		9.954	.000
Age	-2.599	.247	-.708	-10.364	.000
Sex	.100	.155	.035	.645	.520
High cost of cashless transaction instruments	-1.066	.267	-.283	-3.990	.000
Location of business	-1.318	.226	-.356	-5.830	.000
Technical knowhow	1.694	.210	.450	8.053	.000
Internet availability	2.485	.344	.568	7.225	.000

*Field study, 2024.

3.10. Test of Hypothesis

H01: There is no effect of cashless financial transactions instruments on the smallscale agricultural businesses.

The t-statistic is very high, indicating that the difference between the two sample means is highly significant. Degrees of Freedom (df = 143): This reflects the number of independent pieces of information available to estimate variability. P-Value (1.62745E-38 for two-tailed test): The extremely low p-value indicates that the result is statistically significant at any common significance level (such as 0.05 or 0.01). The null hypothesis (that there is no difference between the two groups) can be rejected. Critical Values: Since the t-stat (17.98) is far greater than the critical value (1.976), the null hypothesis is rejected, confirming a significant difference between the two groups. The data shows a significant difference between the two groups (those using cashless transactions and those who do not). This means that cashless financial transactions have a measurable impact on smallscale agricultural businesses. The high t-statistic (17.98) indicates that this effect is robust, with the mean difference between the two groups being far from zero. The findings is consistent with Olawunmi and Olamide (2019) who investigated the effect of cashless policies on the marketing of agricultural goods in rural Nigeria. The authors found that farmers who used cashless systems were able to reach more buyers, received payments faster, and experienced less difficulty in managing their sales, particularly in urban areas. Nwachukwu and Orji (2020) also supported the findings by stating that the adoption of ICT tools, including mobile payment systems, significantly impacted the efficiency of agricultural marketing in Nigeria. Farmers using digital financial services experienced fewer challenges in getting payments, securing better prices, and reaching larger markets. The study rejects the null hypothesis that cashless financial transactions does not affect the smallscale agricultural businesses and therefore accepts the alternate hypothesis which states that cashless financial transactions does affect the smallscale agricultural businesses.

Table 6: Effect of cashless financial transactions on smallscale agricultural businesses*

	Variable 1	Variable 2
Mean	3.568	0.592
Variance	3.18283871	0.243483871
Observations	125	125
Hypothesized Mean Difference	0	
Df	143	
t Stat	17.97520323	
P(T<=t) one-tail	8.13727E-39	
t Critical one-tail	1.655579143	
P(T<=t) two-tail	1.62745E-38	
t Critical two-tail	1.976692198	

*Field study 2024.

4. Conclusion

This study assessed the effect of cashless economy policy on smallscale agricultural businesses in Anambra State, Nigeria. The location of this study was Awka North Local Government Area where one hundred and twenty five (125) respondents were selected for the study. The general questions was analyzed using descriptive (frequency, percentage and mean) and inferential statistics (regression). To test the hypothesis, t test was

employed. The results from the study revealed various challenges and opportunities associated with the adoption of the cashless economy policy by small-scale businesses. The result findings indicated that a large majority (62.4%) of respondents were aware of the cashless policy, while 8% were not aware. However, a significant portion (29.6%) remained indifferent, suggesting a gap in effective dissemination of information. The majority of respondents believed that the policy was aimed at reducing looting (47.2%) and controlling inflation (17.6%). A smaller group perceived the policy as politically motivated or intended to cause hardship for the poor. Mobile transfers (36.8%) were the most commonly used transaction instrument, followed by POS (19.2%) and ATM (16.0%). This highlights the reliance on mobile platforms for cashless transactions. The factors affecting the utilization of cashless financial transactions instruments were Age, high cost of the instrument, location of business, technical knowhow and internet network availability. The factors reflect infrastructural and awareness challenges in the implementation of cashless systems. The test of hypothesis to ascertain the effect of the cashless financial transactions on smallscale agricultural businesses indicated that there was a positive effect. The findings indicated that while there is a significant awareness of the cashless economy policy, challenges such as poor infrastructure, network issues, and insufficient patronage persist, hindering the effective adoption of digital payment systems in small-scale businesses. The policy's perceived impact on income, sales, and cash flow suggests that the transition to a cashless system has been met with some resistance, especially among businesses that depend heavily on cash transactions. However, the policy has had some positive effects, such as reduced theft and increased security in transactions.

1. Infrastructure Development: There is a need for enhanced infrastructure, particularly in rural areas, to support mobile and online transactions. This includes expanding internet connectivity, ensuring the availability of POS terminals, and improving banking facilities to reach more businesses and consumers.
2. Awareness and Training: Efforts should be made to increase awareness and provide training on the benefits and use of cashless transactions. Government agencies and financial institutions should collaborate to educate small business owners and the general public about the advantages of digital payments.
3. Customer Incentives: To encourage adoption, businesses and financial institutions can offer incentives for customers who use cashless methods. This could include discounts, rewards, or even financial literacy programs aimed at educating the public on the advantages of mobile payments.
4. Addressing Network Issues: Strengthening mobile networks and ensuring reliable internet access is crucial for the smooth operation of cashless transactions. Partnerships between the government and private telecom companies may be beneficial in addressing these issues.
5. Tailored Solutions for Small-Scale Businesses: Given the challenges faced by small-scale businesses, especially in rural areas, the government and financial institutions should create policies and support programs that are specifically tailored to meet the needs of these businesses. These programs should consider the unique challenges faced by these businesses, such as limited access to financial services and digital platforms.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	O.A	O.F	O.E	A.O	O.F
C	90		10		
D	100				
S		20	30	30	20
DCP		100			
DAI	100				
L	20	20	20	20	20
W	100				
CR	20	20	20	20	20
SR	20	20	20	20	20
PM	20	20	20	20	20
FA					

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee permissions for this study were obtained from Nnamdi Azikiwe University Social Sciences Ethics Committee.

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MOLECULAR DETERMINATION OF THE ASSOCIATIONS BETWEEN MYOSTATIN GENE AND SOME GROWTH TRAITS OF RABBITS

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Abstract: This study investigates the myostatin (MSTN) gene polymorphisms and their associations with some growth traits in New Zealand white rabbits. A substitution from cytosine (C) to thymine (T) was observed at base position 847 in the second intron region of the gene, and the genotypes TT and CT were attained only. Genotype frequencies were calculated as 47% for TT and 53% for CT, while allele frequencies were determined as 74% for T and 26% for C. The genotype was found to be not in Hardy-Weinberg Equilibrium (χ^2=7.27, P<0.01). The research revealed that the significant associations of MSTN gene polymorphism on some growth traits such as live weight, shoulder-to-tail length, front leg length, and chest circumference. Rabbits belongs to the TT genotype were found to have significantly higher live weight and front leg length compared to rabbits with the CT genotype, while rabbits with the CT genotype were found to be longer in shoulder-to-tail length and chest circumference compared to rabbits with the TT genotype. No statistically significant differences were found among the genotypes for other growth traits such as neck-to-shoulder length, hind leg length, and ear length. These findings suggest that MSTN gene polymorphism may influence certain growth traits in rabbits, and the observed genotypic differences, especially in traits such as live weight, shoulder-to-tail length, front leg length, and chest circumference, should be considered in genetic breeding programs. The findings of this study contributes to better understanding of the potential of MSTN gene polymorphisms in rabbit breeding programs.

Keywords: Myostatin, Candidate gene, Polymorphism, Rabbit, Growth traits

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1. Introduction

The domestic rabbit (*Oryctolagus cuniculus*) is an animal species with several breeds differing with in their distinct characteristics and appearances. In fact, although it is not immediately visible, rabbit production plays several important roles in agriculture and food industry. Firstly, rabbit meat is rich in essential nutrients including protein, vitamins and minerals such as B12, iron and zinc. As a high-protein and low-fat meat source that rabbit production is more environment friendly than traditional livestock husbandry because they require less feed and water compared to cattle or pigs. Being satisfied with this little input makes it an efficient alternative source for meat production (Sarıçiçek, 1993). Compared to other livestock production, rabbit farming is a remarkable agricultural activity as a sustainable and low-cost source of meat production. In fact, the feeding and care costs, which are very high in other species, are quite low for rabbits. Feeding of fattening kits and labor as the major costs were calculated 26% and 18% of the total cost by Cartuche et al. (2014). Secondly, rabbits are raised for their fur and skin and contributes to the fashion and textile markets by providing materials for clothing and accessories. Additionally, rabbits produce manure that can be used as high-quality and valuable fertilizer, increasing soil fertility and promoting sustainable agricultural practices. Moreover, rabbit farming provides a source of income for many small farmers and rural communities. This requires relatively little investment and can be integrated into existing agricultural systems, thereby improving food security and livelihoods (Olawumi, 2014). The global rabbit meat production was estimated to be around 1.4 million tons in 2018 (Lukefahr et al., 2022). China is the main rabbit meat producing country with a share over 68% in the world followed by DPR Korea and some countries in Europe. Although Türkiye is a country where some rabbit breeds, including the long-haired Angora rabbit, originate, it is not among the top 30 in world rabbit production. The country has a very low rabbit meat production of 35.06 tons in 2022 for both local consumption and some export. Rabbit farming in Türkiye contributes little to the agricultural economy but provides a source of income for small-scale or low-input farmers. The low investment cost and high feed conversion efficiency make it an attractive option for rural

households. Although per capita rabbit meat consumption is still very low compared to other sources of meat, it is gaining popularity due to its health benefits in recent years (Nistor et al., 2013). As in other areas of animal husbandry, breeding practices based on the selection of individuals with high genetic merit are important for increasing efficiency and production in rabbit breeding. For this purpose, the advances in recent new generation sequencing technologies in genomic selection have come to the fore instead of traditional breeding. In this context, genomic selection based on Quantitative Trait Locus (QTL) has been one of the most studied topics for the last three decades (Dentine, 1992). QTL association studies aim to identify the specific regions of the genome that are associated with variation in traits. Candidate gene studies help to determine specific regions of genes that are hypothesized to influence a trait based on prior knowledge of their function, expression patterns, or involvement in related biological pathways. In this regard, myostatin (MSTN) or GDF-8 is a pivotal regulatory factor in muscle growth, belonging to the transforming growth factor-beta (TGF-β) superfamily (Langley et al., 2002; Thomas et al., 2000). It plays a critical role in regulating skeletal muscle growth and development across various animal species including rabbits (Bindu et al., 2011; Rasmussen, 2016). In livestock, understanding functions of MSTN has the importance due to its potential impact on meat production efficiency and overall growth traits. In their comprehensive review, Aiello et al. (2018) reports that MSTN acts at key points during pre- and post-natal life of amniotes that ultimately determine the overall muscle mass of an animal. Mutations have already demonstrated the impact of attenuating myostatin activity on muscle development. As a matter of fact, association analyses between the MSTN and IGF2 genes and phenotypes of farm animals, i.e., sheep (Osman et al., 2021); cattle (Esmailizadeh et al., 2008; Grisolia et al., 2009; Bagnicka et al., 2010; Berkowicz et al., 2011; Lin et al., 2022) have become one of the frequently studied research topics. Although it has not been studied as much as in other species, some studies focusing on the MSTN and IGF2 genes have been conducted in rabbits (Wallis and Wallis, 1995; Fontanesi et al., 2008; Sternstein et al., 2014; Abdel-Kafy et al., 2016; Hristova et al., 2017; Yang et al., 2019; Zhang et al., 2019; Ramadan et al., 2020; Helal et al., 2022; Safaa et al., 2023). Given the increasing demand for meat production and improved growth characteristics, identifying the polymorphic regions of MSTN on growth traits in rabbits could lead to enhanced breeding strategies and better management practices. For this purpose, this study aimed to determine the polymorphisms and their associations of MSTN with some important growth traits such as live weight, shoulder-to-tail length, front leg length, and chest circumference.

2. Materials and Methods

As the study material, phenotypic data and blood samples for genotyping were collected from a total of 60 randomly selected 2 and 3 years old animals (45 male and 15 female) belonging to the rabbit breed New Zealand White raised at the Çukurova University Faculty of Agriculture Research and Application Farm in Sarıçam district of Adana province. In this study, the following protocol was applied for DNA extraction from the blood samples collected. Using a pipette, 200 µl of uncoagulated blood and 600 µl of DP buffer were taken into a 1.5 ml Eppendorf tube and homogenized by pipetting. The prepared samples were centrifuged for 5 minutes at 7600 xg, and then the supernatant was discarded. 200 µl of DA buffer was added to the remaining in the Eppendorf tube and it was mixed by pipetting. 20 µl of Proteinase K and 220 µl of DB buffer were added and incubated at 65 oC for 20 minutes. 220 µl of pre-cooled ethanol was added to the incubated samples and vortexed. The mixture was transferred to filtered tubes and centrifuged at 10900 xg for 1 minute, and the liquid remaining under the filter was poured. 500 µl of DY buffer was added to the filtered tube and centrifuged for 1 minute at 10900 xg, and the liquid remaining under the filter was poured. This step was repeated once more, and then the filtered tube was centrifuged for 1 minute at 10900 xg as empty without adding any reagents to it. The collection tube remaining under the filtered tube was discarded. The filtered tube was placed in a new 1.5 ml Eppendorf tube, 70 µl DE buffer was added, and it was kept at room temperature for 1 minute, then centrifuged for 2 minutes at 10900 xg. The filtered tube part in the Eppendorf was discarded, and the obtained DNA product was placed in an Eppendorf tube.

Table 1. Protocol applied to the PCR instrument for MSTN gene amplification

Process	Temperature (°C)	Time (minute)	Number of cycles
First denaturation	95	5	1
Denaturation	95	0:30	
Annealing	44.7	0:40	30
Extension	72	0:50	
Last denaturation	72	10	1
Waiting	4	∞	1

The protocol given in Table 1 was applied to the PCR device for the synthesis of MSTN gene regions from genomic DNAs. Electrophoresis gel was prepared to visualize and isolate DNA (Gibbs, 1990). Due to the phosphate group contained in DNA, the prepared 5X stock Tris-Borate-EDTA (TBE) solution was diluted to 1X in order to have a negative charge and pH between 5-7 in the prepared gel. While preparing the stock solution, 108g Tris, 55g Boric Acid and 40 ml 0.5M EDTA (pH 8.0) were dissolved in 2 liters of pure water and transferred to glass bottles. The stock solution was diluted by adding 400 ml of pure water to 100 ml TBE 5X. While preparing the gel,

0.2440g agarose was weighed on a precision scale and placed in a 0.50 beaker. 30ml of ready-to-use 1X TBE was added to the agarose. The beaker was covered with stretch film and small holes were made on it. The solution prepared in the beaker was subjected to heat treatment in a microwave oven for 2 minutes at 600W. In order to make the DNA fluorescent under UV light, 1.0 µl ethidium bromide was added to the solution and it was mixed. The prepared solution was poured into the tank without creating air bubbles, and an 8-comb was placed and the gel was waited for to solidify. The comb on the solidified gel was gently removed without tearing it. The gel was placed back into the tank so that it would float in the tank and enough electrolyte solution was added to completely cover the gel. DNA fragments amplified by the PCR method were placed into the gel. After this process was completed, the gel was illuminated with UV light and examined. The PCR products obtained were stored at -20 ^{o}C to be sent for sequencing. The chain termination method developed by Sanger et al. (1977) is widely used in DNA sequencing analyses. In this method, which is based on enzymatic DNA synthesis, the DNA strand to be sequenced is used as a template for the newly synthesized strand. One of the enzymes Klenov, Taq DNA polymerase, reverse transcriptase or sequencing can be used to provide DNA synthesis. The basis of the method is that DNA polymerase uses dNTPs (deoxyribonucleoside triphosphate) as well as ddNTPs (dideoxyribonucleoside triphosphate) that do not carry an OH group at the 3' position of deoxyribose. The addition of a ddNTP to the synthesized DNA stops the synthesis because there is no OH group at the 3' position. Four separate reaction mixtures are prepared during sequencing. Each mixture contains the template DNA strand, a primer, four of the dNTPs and a small amount of one of the ddNTPs. A different ddNTP is present in each reaction for specific chain iv termination. Since very small amounts of modified nucleotides are used in each reaction, new chain synthesis is randomly terminated and a series of DNA fragments are formed (Klug and Cummings, 2000).

Table 2. Descriptive statistics of the studied growth traits according to MSTN genotypes

Traits		Mean.	SD	Min	Max	CV(%)	P-value
LW	TT	2.39	0.15	2.13	2.61	6.28	0.001*
	CT	2.38	0.10	2.08	2.52	4.20	
	Overall	2.39	0.12	2.08	2.61	5.22	
LNS	TT	11.21	1.20	9.00	13.00	10.70	0.850
	CT	11.36	0.89	10.00	12.50	7.83	
	Overall	11.29	1.04	9.00	13.00	9.24	
LST	TT	29.93	1.90	26.50	33.00	6.35	0.019*
	CT	31.02	2.23	28.00	35.50	7.19	
	Overall	30.48	2.13	26.50	35.50	6.99	
LFL	TT	12.64	0.56	12.00	13.50	4.43	0.015*
	CT	12.40	0.62	11.50	13.50	5.00	
	Overall l	12.52	0.60	11.50	13.50	4.77	
LRL	TT	11.32	0.85	10.00	12.50	7.51	0.115
	CT	11.14	0.75	10.00	12.50	6.73	
	Overall	11.23	0.80	10.00	12.50	7.14	
LE	TT	12.25	1.87	9.00	14.50	15.27	0.373
	CT	12.50	1.81	9.50	15.00	14.48	
	Overall	12.38	1.83	9.00	15.00	14.79	
CC	TT	29.00	1.78	25.50	32.00	6.14	0.039*
	CT	28.60	1.26	27.00	31.50	4.41	
	Overall	28.80	1.54	25.50	32.00	5.35	

* P<0.05, LW= live weight (kg), LNS= length from neck to shoulder (cm), LST= length from shoulder to tail (cm), LFL= front leg length (cm), LRL= rear leg length (cm), LE= ear length (cm), CC= chest circumference (cm).

The DNA fragments obtained as a result of the reactions are electrophoresed and run side by side on the gel. With the effect of the applied electric field, a staircase image is created on the gel, with the shortest DNA fragments at the front. According to the labeling method, the detected fragments on the gel are read according to the type of ddNTP added to the reaction mixture (Klug and Cummings, 2000). Before the association analysis, the descriptive statistics in Table 2 of the studied growth traits were calculated, and then a quality control was performed on the phenotypic data. In order to visualize the distribution of the traits and also to check probable outliers, the data distributions were examined by drawing the relevant plots seen in Figure 1. When the probability density function plots of the growth traits along the diagonal in Figure 1 are examined, it is seen that all traits except CC are not normally distributed. In fact, according to the results of the Shapiro-Wilk normality test performed with the shapiro.test function of R, it was determined that the traits LW, LNS, LST, LFL, LRL and LE

(LW: Live Weight (kg), LNS: Length from neck to shoulder (cm), LST: Length from shoulder to tail (cm), LFL: Front Leg Length (cm), LRL: Rear Leg Length (cm), LE: Ear Length (cm), CC: Chest Circumference (cm)) were not normally distributed (P<0.05). There are many methods such as arcsine, logarithmic, square root, Box-Cox etc. for normalizing the data, and which method to use depends on the distribution of characteristics of the data. For example, square root or logarithmic transformation can be recommended for normalizing right-skewed distributions; the methods of cube or square transformation can be better options for left-skewed distributions. However, in practice, it is seen that the traditional methods may not be efficient to normalize many types of data, recently, Inverse Normal Transformation (Shore, 2000; 2002), abbreviated as INT, has gained popularity and is offered as a tool in various software (Cebeci and Gökçe, 2023).

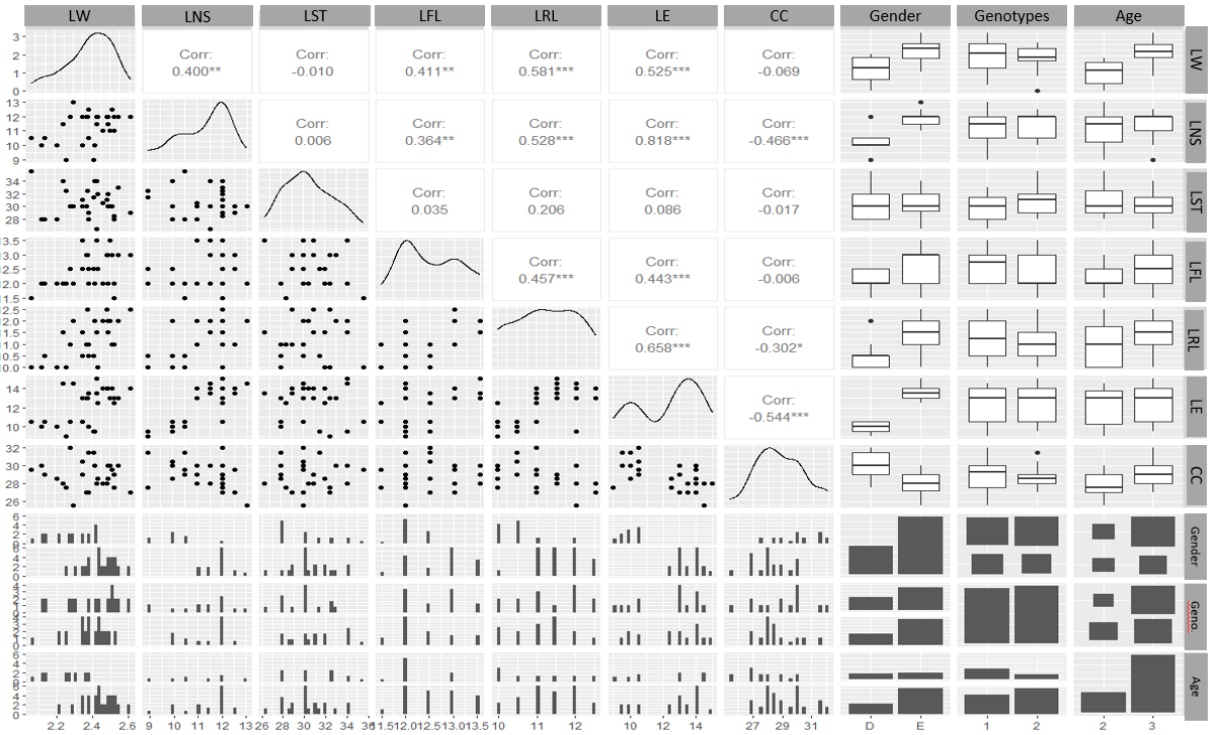

Figure 1. Distribution and scatter plots of the growth traits by the genotypes, genders and age groups.

In this study, thus, INT was applied to normalize the phenotypic data and the results are visualized in Figure 2. According to the post-normalization test results, INT was successful to normalize the phenotypic data. Following the normalization the data was also checked for possible outliers. For this purpose, a procedure based on Tukey's IQR method was run as described in Cebeci (2020), and no outliers found in the data for the all examined traits.

In the present study, a generalized linear model (GLM) shown in equation 1 was used to test the associations between the genotypes and phenotypes.

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ijkl} \quad (1)$$

In equation 1:

Y_{ijkl}: Phenotypic value of the studied trait observed on the *l.* animal with *i.* genotype and *j.* gender and *k.* age group,

μ: Overall mean,

α_i: Effect of i. fixed genotype the MSTN mutation site (TT, CT),

β_j: Effect of *j.* gender (male, female),

γ_k: Effect of *k.* age group (2, 3),

ε_{ijkl}: Random error (or residual), $\varepsilon \sim N(0, \sigma^2)$.

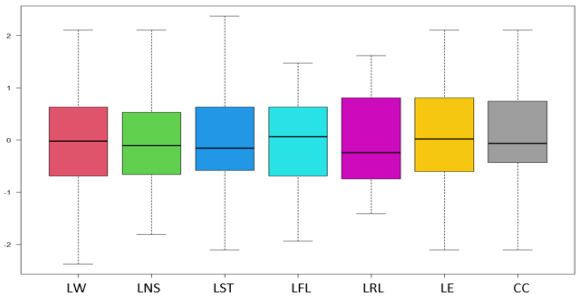

Figure2. Boxplots of the traits after normalization and outlier check. (LW= live weight (kg), LNS: length from neck to shoulder (cm), LST= length from shoulder to tail (cm), LFL= front leg length (cm), LRL= rear leg length (cm), LE= ear length (cm), CC: chest circumference (cm).

All the genomic and statistical analyzes including visualizations were done in the R version 4.3.2 (R Core Team, 2024). While the glm function in the basic stats package of R was used for GLM analysis, the functions plot and boxplot in the basic graphics package; the ggpairs function in the GGally package (Schloerke et al., 2024), and the functions in the corrplot package (Wei and Simko,

2021) were used to draw correlograms, boxplots, histograms and other plots. The R package genetics (Warnes et al., 2021) was used to calculate allele and genotype frequencies, and to test Hardy-Weinberg Equilibrum.

3. Results

In this study, a T>C substitution at 847 bp location of rabbit MSTN was identified by sequencing method as shown in Figure 3. This variant is a newly discovered variant in the intron region 2 of MSTN. According this finding, the associations between two genotypes (TT and CT) formed due to this mutation and the examined body measurements were analyzed. When the allele frequencies at this locus were examined, it was calculated that the T allele had a frequency of 74% and the C allele had a frequency of 26%; accordingly, the genotype frequencies were 47% for the TT genotype and 53% for the CT genotype. According to the χ^2 test, it was determined that the population deviated from the Hardy-Weinberg Equilibrium (HWE) in terms of genotype frequencies (χ^2=7.27, P=0.007). As seen in Table 2, the effect of genotypes was found to be significant for the traits of LW, LST, LFL and CC. The mean LW of the rabbits belonging to TT genotype was determined as 2.39±0.15 kg while the mean LW of rabbits from the CT genotype was found as 2.38±0.10 kg (P=0.001). This finding indicated that rabbits with the TT genotype at this loci might have higher LW compared to those with the CT allele, which could be valuable for breeding programs aimed at enhancing meat production. For the trait LST, the rabbits of the CT genotype were found to be longer with a mean of 31.02±2.23 cm than the rabbits with the TT genotype with a mean of 29.93±1.90 cm (P=0.019). For trait LFL, the rabbits with the TT genotype with an average of 12.64±0.56 cm were longer than rabbits the of CT genotype with a mean of 12.40±0.62 cm (P=0.015). The mean CC for the TT genotype was 29.00±1.78 cm while it was 28.60±1.26 cm with the animals have the CT genotype, and a significant difference was also observed between the genotypes for this trait (P=0.039). On the other hand, no statistically significant difference was determined between the TT and CT genotypes for the traits LNS, LRL and LE (P>0.05). These findings pointed out that MSTN gene polymorphism may affect some growth traits in rabbits. In particular, it indicates that genotypic differences observed for the growth traits such as LW, LFL, LST and CC should be taken into consideration in genetic breeding studies.

4. Discussion

Fontanesi et al. (2008) identified a single nucleotide polymorphism (C>T) in intron 2 of rabbit MSTN gene using a PCR-RFLP protocol designed to investigate this mutation in a larger number of rabbits (15 Checkered Giant, 9 Giant Grey, 6 Dwarf, 4 Burgundy Fawn, 3 Giant White, 3 Lop, 2 Belgian Hare, 1 New Zealand White). Allele frequencies across breeds were 0.51 for allele C and 0.49 for allele T. C. Bindu et al. (2011) reported that CT genotypes were associated with higher body weight but the difference in body weight among different genetic groups was not statistically significant in a sample of 60 animals from New Zealand, Soviet Chinchilla and crossbred rabbits subjected to PCR-based RFLP. Sternstein (2014) identified three SNPs (c.–125T>C, c.373+234G>A, c.747+34C>T) related with carcass composition traits in F_2 animals of the cross Giant Grey × New Zealand White. Hristova et al. (2017) also confirmed the presence of the polymorphisms in MSTN gene in the a80 bp fragment of the intron 2 of MSTN gene of New Zealand rabbits. Navratilova et al. (2018), revealed significant positive effect of allele T (c.747+34C>T) and allele G (c.194A>G) on meat performances in tested rabbit population. In one of more recent studies conducted by El-Sabrout and Aggag (2018), the effects of four novel SNPs identified in the MSTN and MC4R genes on carcass quality traits were examined. It was found that rabbits with the BB genotype had higher live weight, carcass weight, fleece and carcass fat than the rabbits with the AA genotype in their study. They revealed that genetic polymorphisms play an important role in both growth traits and carcass quality. Yang et al. (2019) also found the significant associations between MSTN genotypes and carcass traits, and reported that in the Exon 1 region of MSTN gene, CC genotype rabbits had significantly higher performances than the rabbit of TT genotype. For the genotypes in the Exon 2 region of the gene, AA genotype rabbits were heavier than TT genotype rabbits. Peng et al. (2013) indicated that the association between the genotypes and live weight gain on the 35th and 70th days were insignificant while the CT genotype had higher live weight than individuals with the CC genotype on the 84th day. In the present study, a T>C substitution was found at the 847th base of the second intron of rabbit MSTN gene. The frequencies of the genotypes of TT and CT were determined as 47% and 53% respectively. The frequency of the T allele was 74% and the C allele was 26%. This finding is similar to the findings by Fontanesi et al. (2008) and Hristova et al. (2017) because they reported polymorphisms in the second intron of MSTN gene of New Zealand rabbit breed. In this study, it was also detected that the studied population deviated significantly from the Hardy-Weinberg Equilibrium (HWE) in terms of genotype frequencies as stated in some research studies, i.e. Hristova (2017). Several factors may explain the deviations from HWE. Firstly inbreeding can increase homozygosity and alter allele frequencies. For example, if a small flock is kept in closed stock for generations, deviations from HWE can occur due to an increase in certain alleles. Secondly, artificial selection for certain traits, such as daily weight gain, can favor certain alleles and disrupt HWE. Moreover, random fluctuations in allele frequencies can occur in small populations. This can lead to HWE violations for rare alleles, which can be difficult to detect, especially in small flocks. The introduction of animals or genes into a flock can affect allele frequencies.

For example, the introduction of several high genetic merit males into a breeding stock can significantly alter the genetic make-up of the flock. If animals are mated preferentially for certain traits, this can lead to skewed allele frequencies. For example, if larger genetic merit animals are mated, this can lead to an increase in individuals homozygous for certain alleles. As another factor, new mutations can introduce variation and disrupt HWE. For example, if a new mutation that affects feed conversion efficiency emerges in the herd and provides a survival advantage, it can rapidly change allele frequencies. In this study, potential reasons for deviation from HWE could not be examined because sufficient data were not provided to understand these factors and better examine genetic diversity. However, it is thought that allele frequencies can be calculated more stably by increasing the sample size to increase the power of the analysis and possibly test HWE better. The findings from this and previous studies suggest that the polymorphisms in MSTN gene may affect some growth traits in rabbits. In particular, the genotypic differences observed in the studied growth traits of LW, LST, LFL and CC should be taken into account in genetic breeding studies. If the detected T>C variant affects regulatory elements within the intron, it may lead to changes in the expression levels of the myostatin protein, potentially leading to increased muscle growth. This finding is consistent with previous studies showing that certain mutations in the MSTN gene are associated with the 'double muscling' phenotype in cattle. As a matter of fact, Lv et al. (2016) generated the MSTN knockout (KO) rabbits by co-injection of Cas9 mRNA and sgRNA into zygotes, and they observed the typical phenotype of double muscle with hyperplasia or hypertrophy of muscle fiber in MSTN KO rabbits. Recently Zhang et al. (2019) also studied on double-muscling and pelvic tilt phenomena in rabbits with the cystine-knot motif deficiency of myostatin on exon 3. In rabbits, if the T>C variant is associated with improved muscle or growth rates, it also may be beneficial to increase meat yield. In fact, if there is a mutation in MSTN gene, its negative regulation function is disrupted leading to double muscle phenotype. However, more advanced association analyses such as GWAS or functional analyses can be performed to investigate the specific effects of this intronic variant on muscle traits.

5.Conclusion

A novel mutation T to C was detected at position 847 in the second intron region of the MSTN gene of rabbit. The study revealed the significant differences between the TT and TC genotypes for the growth traits of LW, LST, LFL and CC. For these traits, the animals with TT genotype significantly outperformed than the animals of TC genotype. These findings pointed out that MSTN gene polymorphisms should be considered in rabbit breeding programs targeting the meat and growth related traits. Applying genomic selection programs rabbit breeding based on selection could provide a rapid increase in meat yield. The genomic selection focusing on MSTN gene can contribute to the effectiveness of breeding programs and improve the development of more sustainable rabbit populations. Rabbit breeders could benefit the genetic polymorphisms in genetic breeding programs.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	H.B.D.	Z.C.
C	50	50
D	90	10
S	10	90
DCP	100	0
DAI	50	50
L	90	10
W	100	0
CR	10	90
SR	100	0
PM	10	90
FA	100	0

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. The experimental procedures were approved by the Local Animal Care and Ethics Committee of Çukurova University in Adana, Türkiye (Approval date: March 28, 2024 and protocol code:3).

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Research Article
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THE EFFECT ON MYCOTOXIN DEVELOPMENT OF COMBINED ORGANIC ACID ADDITIVE AT DIFFERENT LEVELS IN DAIRY FEED

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Abstract: Organic acid treatment to prevent deterioration of feeds by exposure to mycotoxins and to extend their storage life is the most important requisite of natural, safe and wholesome feed production. (1) Background: The aim of this study was to determine the effects of an organic acid (OA) combination added in incremental levels to commercial dairy feed, on mycotoxin development in feeds stored for varying lengths of time; (2) Methods: For the trial, a total of 5 treatment groups were formed as control, 10 lt water only (without OA), and 10 lt water with respectively 0.2, 0.3 and 0.4 kg/ton OA addition. The trial conducted in a commercial feed mill for a span of 3 months from March through May, was set-up in a 3x5 factorial plan, taking into account the effect of groups and storage duration in months. Deoxynivalenol, zearalenone, aflatoxin, fumonisin B1, T-2 toxin, ochratoxin, fumonisin B2 and HT-2 levels were measured in the feed samples taken at certain control points of the feed manufacturing process, that is to say after the mixer, conditioner and cooler, and stored for three months; (3) Results: It was determined on the basis of the results that, deoxynivalenol and aflatoxin levels significantly decreased ($P<0.05$) with the addition of both 0.3 and 0.4 kg/ton OA+water into the compound feed, whereas fumonisin B1 levels decreased ($P<0.001$) with the addition of 0.3 kg/ton OA+water. Mycotoxin content was further affected by the length of storage, with the highest contamination detected in May ($P<0.001$). Ochratoxin, fumonisin B2, T-2 and HT-2 remained below thresholds of detection throughout the trial; (4) Conclusions: Adding 0.3 or 0.4 kg/ton OA+water may be beneficial in reducing the development of mycotoxins in feed, however, more research is needed on the subject.

Keywords: Mycotoxin, Organic acid, Feed toxicity, Feed storage, Dairy

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1. Introduction

Efficient and sustainable animal husbandry has become an area of increasing scientific interest. In addition, there is an increasing demand for safe, healthy and residue-free animal production for human and animal consumption. Besides efficient and sustainable livestock production, the growing demand for wholesome and residue-free animal production safe for human and animal consumption has become an area of intensifying scientific focus. Among feed additives used in recent years as alternatives to antibiotics for preventing bacterial infections, especially contagious epidemics, and for growth promoting purposes, organic acids, their salts and/or blends have gained popularity. Organic acids are organic carboxylic compounds containing amino acids and fatty acids with the general structural formula R-COOH and their acidity is connected to the carboxyl group (-COOH). They are weak acids which dissolve in water to release a hydrogen ion (H+) and a carboxylate ion (-COO-) (Lim et al., 2015). The most widely used organic acids are short-chain fatty acids such as formic, lactic, malic, citric, acetic, butyric and propionic acids. As they are substances synthesized by the animal itself, occurring as a result of natural biochemical metabolism, they are recognized as environmentally friendly, residue-free, safe and natural feed additives which do not present any risks when used in feeds (Humer et al., 2016; Gül and Tekce, 2017; Dijksterhuis et al., 2019). Improvements in terms of performance and feed efficiency have been reported in studies on organic acid supplementation in ruminants (Wang et al., 2020; Gallo et al., 2021), swine (Lei et al., 2017; Hossain et al., 2018; Nguyen et al., 2020) and poultry (Adil et al., 2010; Khan and Iqbal, 2016) diets. Furthermore, it has also been suggested that organic acids may regulate intestinal bacterial populations, reduce gas emission, improve nutrient digestibility and gut health in animals. However,

it was emphasized that the type of organic acid, its dosage and overall feed formulation may affect the response to the use of dietary organic acids (Nguyen et al., 2020). On the other hand, feed ingredients such as cereals, by-products thereof and oilseeds carry a potential contamination risk due to the emergence of various mycotoxins and toxic metabolites, usually produced by a range of fungi or mold species (Pearlin et al., 2020). The use of acids is among several tools employed in good management practices to reduce the microbial contamination risk during storage of the feeds. It has been reported that the effect of salts or combinations of organic acids in feeds is mainly related to their protective action, especially against mycotoxin development (Kaya et al., 2014; Humer et al., 2016; Moon et al., 2018). The synergistic effects of pKa values, which is an indication of their undissociated forms, and their strong antimicrobial properties depending on the cell membrane structure and pH range are particularly important for protein-rich feed ingredients such as soybeans (Khan and Iqbal, 2016; Lei et al., 2017). Organic acids, of which antimicrobial effects increase in parallel with their pKa values, suppress the proliferation of pathogens by increasing the acidity of cellular pH through the released hydrogen ions and by disrupting bacterial cellular metabolism (Nguyen et al., 2020). On the other hand, Humer et al. (2016) reported that organic acids, which are frequently used in feed preservation and processing, improve the nutritional properties of feeds by promoting the breakdown of antinutritional factors, enriching the feed with healthier ingredients, thus increasing their health benefits in animal and human nutrition. It has been emphasized that commonly used mold inhibitors contain organic acids in various levels and combinations (Eissen et al., 2010). Surfactants can be added to organic acid blends in liquid form in order to increase their effectiveness, by ensuring that mold inhibitors and water are evenly distributed among the feed particles. Surfactants, which basically consist of mixtures of wetting agents, are compounds that reduce the surface tension at the interfaces between phases insoluble in one another (oil-water, solid-water) by virtue of their hydrophilic and hydrophobic groups (Rosen and Kunjappu, 2012). Surfactants also improve the water binding capacity of feed ingredients and reduce water activity in feeds (Eissen et al., 2010).Mycotoxins that cause various clinical infections in animals, depending on the storage conditions and duration of feeds, are toxic substances produced by certain fungal species such as Fusarium, Aspergillus, Penicillium and Alternaria (Gül and Tekce, 2017). The most common mycotoxins with mutagenic, teratogenic and carcinogenic effects are aflatoxin, zearalenone, deoxynivalenol, T2, HT-2 and fumonisins (Yang, 2019; Jubeen et al., 2020; Yang et al., 2020). Although there are various physical, chemical and biological methods to reduce mycotoxin contamination, not all approaches are suitable for feed manufacturers (Čolović et al., 2019). In the feed milling industry, mycotoxins need to be eliminated or neutralized by easy to store, practical to apply and economical solutions without compromising the technological properties and nutritional value of the products (Zhu et al., 2017; Huss et al., 2018). The aim of this study was to determine the effects of an organic acid (OA) blend on mycotoxin development in stored feeds for dairy cattle.

2. Materials and Methods

This study was conducted at an independent and privately owned commercial feed mill over a period of three months (March, April and May). The material used in the trial was commercial compound feed for dairy cattle in mash form, produced in the same factory with 2790 kcal/kg metabolizable energy (ME) and 21% crude protein content (Table 1).

Table 1. Nutrient composition of the commercial compound feed*

Nutritional composition	%
Crude ash	7.75
Starch	25.5
Crude fiber	Max 9
ADF	11
NDF	22
Lignin	2
Crude protein	21
ME (kcal/kg)	2790
NE_L (kcal/kg)	1813.5

*Ingredients= Wheat bran, corn, corn DDGS, sunflower seed meal, full fat soybean, rapeseed meal, molasses, limestone, salt, vitamin & mineral premixes, zeolite

With a view to examining the effects of incremental doses of organic acid blend (OA) on the mycotoxin toxicity of the compound feed, a total of 5 treatment groups were formed: A control group with no addition whatsoever, another group to account for the effect of water addition (10 lt without OA), and three treatment groups with 10 lt water plus 0.2, 0.3 and 0.4 kg/ton OA addition respectively. The OA doses used were determined by taking into account the company's recommendation. The trial was planned in a 3x5 factorial model considering the effect of groups and storage duration in months. OA (Fylax Forte-HC liquid) used in the trial was sourced from Trouw Nutrition TR Gıda Tarım Hayvancılık San. ve Tic. A.Ş., Türkiye. The said OA product consisted of a mixture of 80% organic acids (sorbic, formic, acetic, lactic, propionic acids and ammonium propionate) and 20% 1,2-Propanediol as surfactant. Throughout the trial, 10 liters of water was added to all groups, except for the control, in order to ensure moisture optimization of the compound feed and to improve production performance. The specified OA doses were added to that 10 liters of water and the solution thus obtained was added to the compound feed at the mixer

In order to determine at which production and storage stage the moisture loss and toxin formation occurs, three sets of 2x500 g samples were taken at the beginning of February to represent each trial month from the exits of the mixer, conditioner and cooler. The samples were placed in storage in plastic zipper bags under room conditions and sent for analysis at the beginning of March, April and May to monitor the change as a function of storage duration. Deoxynivalenol, zearalenone, aflatoxin, fumonisin B1, T-2 toxin, ochratoxin, fumonisin B2 and HT-2 analyzes were performed on those feed samples stored for one, two or three months, preparing composite laboratory samples from four different points of each monthly sample. Mycotoxin analyzes were performed following the procedures described in ISO 17125 Dutch Accreditation Council NEN-EN 17194 (2017). According to the procedure; the samples are desiccated for 4 hours at 103°C. The loss in mass is determined by weighing. Measurement is based on gravimetric method by LC-MS/MS. The mycotoxins are extracted using 79% acetonitrile with 1% formic acid. Following the addition of the internal standard, evaporation and reconstitution in mobile phase, the extract is analyzed by ultra-high performance liquid chromatography on a reversed phase column with a triple quadrupole mass spectrometer. For each mycotoxin a precursor is selected and fragmented to two daughter ions, after which the ratio is used as confirmation.

2.1. Statistical analysis

Variance analysis is conducted to establish whether there was a difference in terms of toxicity between three dosages of OA, untreated water addition and control groups after three different periods of storage (analyzed in March, April, May). In addition, Duncan's multiple comparison test was used to determine the differences between doses and months for the characteristics deemed to be significant. The following mathematical model (equation 1) was used for the variance analysis.

$$y_{ijk} = \mu + a_i + b_j + e_{ijk} \quad (1)$$

yijk : Measured value for any characteristic

μ: Expected average for the analyzed characteristic

ai : i. Dosage effect (i = Group I, Group II, Group III, Group IV and Group V)

bj : j. Storage duration effect (March, April, May)

eijk : Normally distributed error effect with a mean of zero and variance of σ_e^2

Moreover, HT 2 levels are counted as either below 10 μ/kg, or above 10 μ/kg (11 and 12 μ/kg) and Chi-squared test was performed. Since the expected values for this test were too small, the OA+Water groups were integrated to perform a 3x2 Chi-squared test with OA+Water, Control and Untreated Water rows, and below and above 10 μ/kg columns (SPSS, 2008).

3. Results

The average mycotoxin contents obtained from compound feed samples collected after mixer, conditioner and cooler are given in Table 2. The effect of treatment and storage duration was found to be significant between the groups in terms of post-mixer mycotoxin contents ($P<0.01$). The lowest post-mixing mycotoxin content of 11.7 μg/kg was observed in Group III with 0.4 kg/ton OA+water addition and was significantly to all other treatments (Table 2). The differences between Group I, untreated water and control groups in terms of post-mixing mycotoxin content were not significant ($P>0.05$). As to group II, it was found to be higher than the group III but lower than the other groups with statistical significance. In terms of post-mixing mycotoxin content, the lowest average mycotoxin content was 12.1 μg/kg in April and the highest was 12.5 μg/kg in May ($P<0.01$).

The effect of group and month on the mean mycotoxin content in compound feed at the exit of the conditioner was statistically significant ($P<0.01$). The lowest mean value was 14.5 μg/kg in Group III, which was the one with 0.4 kg/ton OA+water addition, and this value was significantly lower than the other groups ($P<0.01$). On the other hand, the differences between the other four groups were not significant ($P>0.05$). The lowest mean mycotoxin content after conditioner was observed in March and the highest in May and the differences between the three months were significant ($P<0.01$).

The effect of group on post-cooler mycotoxin content in compound feed was found to be significant ($P<0.01$), while the effect of month (storage duration) was not significant ($P>0.05$). Similar to the results of post-conditioner samples, the lowest mycotoxin content was found to be 11.7 μg/kg in the 0.4 kg/ton OA+water supplemented feed group. However, the differences between the other four groups were not significant ($P>0.05$).

The mean values observed for the analysed mycotoxin types (Deoxynivalenol, zearalenone, fumonisin B1 and aflatoxin) in compound feed samples are presented in Table 3. In terms of deoxynivalenol, group effect was found to be significant ($P<0.01$), whereas the month effect was not significant statistically ($P>0.05$). Although the highest mean deoxynivalenol content was found in the Control group, the differences between Control and Group I, were not significant, and the deoxynivalenol content was not affected by the addition of OA ($P>0.05$). In contrast, deoxynivalenol findings were lower in Group II than in the Control, and lower in Group III than both Water Group and Control ($P<0.01$). The differences observed between the trial groups in terms of zearalenone level were not found to be statistically significant ($P>0.05$). However, significant differences were found between the trial months. While there was no statistically significant difference between March and May, the mean of April was found to be 41.9 μg/kg, nearly 30% higher than the other two months ($P<0.01$).

The effects of group and month were found to be

significant for Fumonisin B1 (P<0.01). The highest mean Fumonisin B1 level was observed in the Water group and the Control group, while the lowest was in Group III and the differences between these groups were statistically significant (P<0.01). On the other hand, there was no difference between Group I and Group II, while these two groups were among the high and low groups and were found to be similar to them. The lowest mean fumonisin B1 value was found in May. While the differences between March and April were not significant

(P>0.05), the differences between these two months and May were statistically significant (P<0.01). The highest aflatoxin level was found in the Control Group at 1.45 µg/kg, with the lowest ones were found at 1.04 and 0.98 µg/kg in Groups II and III respectively. The difference between Group II and Group III was not statistically significant, but the difference between these groups and the Control group was significant with lower values (P<0.01). However, the levels of aflatoxin observed in the Water Group group and OA added groups were similar (P>0.05). The aflatoxin content of all 10 samples analyzed in March could not be determined as it was lower than 5 µg/kg. The aflatoxin content determined in May was 0.99 µg/kg, which was approximately 50% lower than in April (P<0.01).

The amounts of T-2, ochratoxin and fumonisin B2 could not be detected in all groups for the three months of trial as they were below 10 µg/kg. However, the results of the Chi-squared test for HT-2 amounts are given in Table 4. The analysis results indicated no difference between the groups in terms of HT-2 levels (P>0.05). In another way of saying, there was no difference between OA+Water, Control and water only groups in terms of HT-2 levels being below 10 µ/kg or above 10 µ/kg.

Table 2. Average mycotoxin values found in compound feed samples taken after the mixer, conditioner and cooler (µg)

Treatment	Mean Mycotoxin Values (µg/kg)		
	Post-Mixer	Post-Conditioner	Post-Cooler
Group	*	*	*
Group I (0.2 kg/t OA+Water)	12.4±0.04 ^{a}	15.3±0.09 ^{a}	12.9±0.08 ^{a}
Group II (0.3 kg/t OA+Water)	12.3±0.04 ^{b}	15.1±0.09 ^{a}	12.8±0.08 ^{a}
Group III (0.4 kg/t OA+Water)	11.7±0.04 ^{c}	14.5±0.09 ^{b}	11.7±0.08 ^{b}
Water	12.4±0.04 ^{a}	15.2±0.09 ^{a}	12.7±0.08 ^{a}
Control	12.4±0.04 ^{a}	15.2±0.09 ^{a}	12.9±0.08 ^{a}
Months (Storage Duration)	*	*	ns
March (one month)	12.2±0.03 ^{b}	14.5±0.07 ^{c}	12.6±0.07
April (two months)	12.1±0.03 ^{c}	14.9±0.07 ^{b}	12.6±0.07
May (three months)	12.5±0.03 ^{a}	15.8±0.07 ^{a}	12.6±0.07

*P<0.01 Means with different superscript within the same column differ significantly; ns= non-significant.

Table 3. Mean values of Deoxynivalenol, Zearalenone, Fumonisin B1 and Aflatoxin observed in compound feed samples (µg/kg)

Treatment	Mycotoxin Types (µg/kg)			
	Deoxynivalenol	Zearalenone	Fumonisin B1	Aflatoxin
Group	*	ns	**	*
Group I (0.2 kg/t OA+Water)	644.8±13.8 ^{abc}	35.5±2.3	265.0±8.7 ^{ab}	1.27±0.12 ^{ab}
Group II (0.3 kg/t OA+Water)	643.0±13.8 ^{bc}	33.0±2.3	262.0±8.7 ^{ab}	1.04±0.12 ^{b}
Group III (0.4 kg/t OA+Water)	622.0±13.8 ^{c}	32.3±2.3	238.7±8.7 ^{b}	0.98±0.10 ^{b}
Water	667.8±13.8 ^{ab}	37.3±2.3	281.0±8.7 ^{a}	1.32±0.16 ^{ab}
Control	686.7±13.8 ^{a}	39.5±2.3	283.7±8.7 ^{a}	1.45±0.10 ^{a}
Months (Storage Duration)	ns	**	**	**
March (one month)	645.0±10.7	34.4±1.8 ^{b}	268.9±6.8 ^{a}	-
April (two months)	654.7±10.7	41.9±1.8 ^{a}	284.4±6.8 ^{a}	1.44±0.09 ^{a}
May (three months)	658.9±10.7	30.3±1.8 ^{b}	244.9±6.8 ^{b}	0.99±0.07 ^{b}

*P<0.05, **P<0.01 Means with different superscript within the same column differ significantly; ns= non-significant.

Table 4. Chi-squared test results for HT-2

Group		Below 10 µ/kg	Above 10 µ/kg	Total
	n	16	2	18
OA+Water	Row	88.9%	11.1%	100.0%
	Column	66.7%	33.3%	60.0%
	n	4	2	6
Control	Row	66.7%	33.3%	100.0%
	Column	16.7%	33.3%	20.0%
	n	4	2	6
Water only	Row	66.7%	33.3%	100.0%
	Column	16.7%	33.3%	20.0%
	n	24	6	30
Total	Row	80.0%	20.0%	100.0%
	Column	100.0%	100.0%	100.0%

Pearson Chi-Square = 2.222. P=0.329

4. Discussion

Beneficial bacteria survive in a lower pH environment than pathogenic bacteria. The antimicrobial effect of OAs can be attributed to the decrease in pH they cause by virtue of their penetration through the bacterial cell membrane into the cytoplasm, forming protons and anions that are toxic to cells, and the subsequent death of permeabilized cell due to the energy loss (Youssef et al., 2017; Markazi et al., 2019). Therefore, the addition of OAs to feeds to lower pH inhibits, reduces or prevents the colonization of pathogenic bacteria and creates favorable conditions for the proliferation of beneficial bacteria (Qui, 2023). In the present studytrial, it was observed that 0.4 kg/ton OA+water addition to the compound feed during the production process significantly reduced feed toxicity in terms of both total mycotoxin content and individual mycotoxins (deoxynivalenol, zearalenone, fumonisin b1, aflatoxin), while mycotoxin content did not change with respect to the Control Group with the addition of water only. The average mycotoxin levels observed after the mixer, conditioner and cooler decreased significantly with the addition of 0.4 kg/ton OA + water to the compound feed, and at the same time, the addition of 0.3 kg/ton OA + water also ensured a drop in mycotoxin levels in the post-mixer samples compared to the other trial groups. It can be stated that this decrease was in line with longer storage durations. It has been suggested that organic acid blends have positive effects on the storage life of feeds, as one of the most effective feed additives in mycotoxin prevention (Jouany, 2007; Samlı et al., 2008). In contrast to the present study, it was reported that the protective effect of 0.2% propionic acid supplementation to layer breeder and broiler compound feeds stored for 15, 30, 45 and 60 days under normal room conditions decreases as the storage period lengthens, leading to a consequent increase in aflatoxin and fumonisin values (Erdoğan and Kaya, 2022). Similarly, Samlı et al. (2008) reported that mycotoxin formation was significantly reduced in protein-rich feed ingredients treated with 3 g/kg organic acid blend for 30 days of storage, and that this effect may vary depending on the nature of the feed ingredients, initial microorganism populations and components of the organic acid mixture. Since lactic and acetic acids are among organic acids having very strong antibacterial activities, it is emphasized that the use of bio-preservatives containing a blend of these organic acids as antimicrobial agents could be a potential solution approach (Hu et al., 2019; Rad et al., 2021). In a study on the antifungal properties of eight organic acids (propionic, acetic, formic, lactic, lactic, tartaric, citric, oxalic and malic acids) against the growth of four fungi species (Aspergillus flavus, Penicillium purpurogenum, Rhizopus nigricans and Fusarium oxysporum), acetic acid (10%) and lactic acid (10%) were reported to have the highest inhibitory effect on A. flavus and F. oxysporum toxins (Hassan et al., 2015). The presence of acetic acid and lactic acid in the organic acid blend used in this study may have played an active role in the reduction of toxins throughout the 3-month storage period. On the other hand, although the average mycotoxin levels decreased to a certain extent in April, they have shown an increase in May compared to March and April. This increase can be attributed to the rising temperatures of the feeds stored at room temperature during the trial which continued into warmer months. It can also be argued that the storage temperature should not exceed the maximum room temperature on average. In general, it is known that molds remain inactive if grains are stored below 20°C (Mousa et al., 2013). Seasonal weather conditions are reported to be the main variable in determining mycotoxin concentration, with a significant correlation observed between them (Qu et al., 2024). Moreover, it has been suggested that excessive moisture and high water activity in feeds are the foremost causes of mold growth as far as the feed industry is concerned, and that the moisture content of grains should not exceed 13% during storage, and the water activity of raw materials, feed mixtures and finished feeds should be kept below 0.8% (Mohapatra et al., 2017; Dijksterhuis et al., 2019). In

this study, although there was no change in the water group compared to the control, the improvement observed with organic acid supplementation can be considered as an indication that the optimum moisture balance was achieved with 10 liters of water addition. The results of the present study revealed that deoxynivalenol, fumonisin B1 and aflatoxin levels were significantly reduced by the addition of 0.4 kg/ton OA+water to the compound feed, and that the addition of 0.3 kg/ton OA+water led to lower levels of deoxynivalenol and aflatoxin compared to the control group. Humer et al. (2016) emphasized that the strongest mycotoxin reducing effect of organic acid treatment in soaked feed samples was on Deoxynivalenol and its derivatives compared to control feed samples, and that this effect was related to the duration of soaking and interaction with the organic acid. Besides, the claims of the researchers that the T2 level decreased below the detection limit at all soaking periods following organic acid treatment are congruent with the results of the present study. However, in contrast to the Zearalenone level, which was not affected by organic acid treatment in the present trial, they reported an increase in Zearalenone concentration in feed samples treated with acids for 24 hours, suggesting that this might be due to the release of masked zearalenone by the acid treatments. Nonetheless this suggestion needs to be clarified by further studies. In the present trial, aflatoxin levels in compound feeds supplemented with 0.3 or 0.4 kg/ton OA+water were found to be lower than the control group. Moon et al. (2018) reported that bacterial biocontrol agents might be suppressing aflatoxin production by means of inhibiting the expression of aflatoxin biosynthetic genes or due to increased antimicrobial activity of organic acids with longer aliphatic carbon chains such as butyric acid. The researchers also established that propionic acid completely inhibited the growth of A. flavus at a concentration of 0.5%, whereas sorbic acid, which was included in the organic acid mixture in that study, demonstrated two times stronger inhibitory activity compared to propionic acid. Hassan et al. (2012) found that organic acids can inhibit the growth of A. flavus strains, but only acetic, formic and lactic acids reduced growth and partially inhibit aflatoxin production. It has been reported that fumonisin and zearalenone, which are among the predominant toxins of fusarium species, usually infect raw materials before or immediately after harvest, while mycotoxin production of Aspergillus and Penicillium species are generally associated with foods during drying and storage (Qu et al., 2024). On the basis of these results, the positive effect of organic acids in reducing deoxynivalenol, fumonisin B1 and aflatoxin levels can be attributed to the effects of organic acids in suppressing pathogenic bacteria and enhancing the growth of beneficial ones (Pearlin et al., 2020). According to the findings of this study, the levels of Ochratoxin, Fumonisin B2, T-2 and HT-2 were relatively low in contaminated samples. Therefore, the decontamination potential of organic acid treatments on the said toxins could perhaps be better judged in more heavily contaminated feed samples. Further research is required in this field.

5. Conclusion

Organic acids are added to feeds to prevent mold or fungal growth during the feed production process, to evade decomposition of feeds by pathogenic microorganisms, to delay deterioration of feeds and to extend their storage life. Thus, by reducing bacterial toxins, the nutrient content of feed and animal health can be maintained and the quantity and quality of animal products can be improved. Safe feed raw materials for safe feed production are essential to ensure animal health/welfare and to supply safe animal products fit for human consumption. Mycotoxin formation is a major global problem that threatens animal and human health and causes substantial economic losses in the feed milling industry. Following a general evaluation of the results obtained from this study, it was concluded that 0.4 kg/ton OA+water addition to compound feed significantly reduced feed toxicity. More effective results can be achieved when multiple organic acids are used as blends. These findings suggest that OA blends can serve as an effective strategy for feed preservation and improved animal health. In addition, it was concluded that organic acids can also be effective even when compound feeds are stored for extended periods of time, but the storage ambient temperature should be kept at maximum room temperature on average. Addition of organic acid mixtures have positive effects on feed preservation in the feed storage process. However, the studies conducted on the subject to date are quite limited and new studies are needed. Currently, there is a clear need to deploy natural, environment friendly and safe solutions to reduce mycotoxin contaminations in feed. However, only a limited body of research is available on the efficacy and duration of the inhibitory power of antimicrobial organic acids, and further research is needed to evaluate their effects.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	D.B.	K.B.
C	60	40
D	100	
S	100	
DCP		100
DAI		100
L	100	
W	100	
CR	50	50
SR	100	
PM	60	40
FA	100	

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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A BIBLIOMETRIC ANALYSIS OF RESEARCH ON ARTIFICIAL INTELLIGENCE IN VETERINARY MEDICINE

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Abstract: The use of artificial intelligence in veterinary sciences has placed studies on this subject in a significant position in the literature. The increasing number of studies using artificial intelligence algorithms in the current literature shows that knowledge discovery in this field is increasing rapidly. This study aims to provide a general map of the literature on the utilization of artificial intelligence in veterinary medicine science and identify its application areas using bibliometric analysis. Web of Science database was used to reveal the current literature about artificial intelligence in veterinary medicine. The data were analyzed using the "Bibliometrix" package in the R statistical programming language and the VOSviewer program. Various research elements, including journals, article-citation counts, authors, institutes, and countries, were examined using bibliometric metrics. The number of studies on artificial intelligence in veterinary medicine from increased dramatically since 2019. According to the findings, the most influential countries identified were the USA, China, and Türkiye. Animals and Preventive Veterinary Medicine were determined as the most influential journals in the field. The findings indicated that artificial intelligence in veterinary medicine is a trending topic. The topics "deep learning", "active learning", and "computer-aided diagnosis" were estimated to be increasingly utilized soon. Rapid developments in artificial intelligence will likely attract more researchers to the field. This article, the first bibliometric study about the utilization of artificial intelligence in animal sciences, will offer researchers valuable information about the intellectual structure of the field.

Keywords: Artificial intelligence, Bibliometric analysis, Classification problem, Machine learning, Veterinary sciences

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1. Introduction

Artificial intelligence, driven by highly advanced computer systems and technology, is extensively employed across various areas and continues to evolve rapidly. Due to technological advancements, it is estimated that numerous professions can be performed through artificial intelligence shortly. Artificial intelligence is defined as software capable of executing high cognitive functions, including perception, learning, thinking, reasoning, problem-solving, communication, inference, and decision-making (Russell, 2010). The term artificial intelligence was first introduced by John McCarthy in 1956. However, despite theoretical studies on this subject, practical application only became feasible in the 2000s with the advancement of computer and software technologies (Kaul et al., 2020). Today, artificial intelligence has become a common method frequently utilized in medical and veterinary sciences. For example, in the field of veterinary medicine, the utilization of artificial intelligence for the development of diagnostic and treatment methods in animal health is becoming increasingly widespread. However, due to the recent increase in the utilization of artificial intelligence, there is only a limited number of studies in the literature (Appleby and Basran, 2022). Bibliometrics is an analytical approach aimed at reviewing, evaluating, and uncovering scientific interactions within a specific field of literature. Bibliometric analysis can be utilized for any research field to gain a general insight into the field before conducting a comprehensive study. Accordingly, popular topics in a field can be identified and potential emerging topics can be predicted (Yu et al., 2020). Bibliometrics examines the interaction between certain research elements (institutes, authors, journals, keywords, etc.) to evaluate bibliometric indicators such as h-index, g-index, m-index, citation count, and publication count (Wang et al., 2020). Thanks to recent academic journals, conferences, and other publication opportunities, the annual influx of academic materials, including articles, theses, dissertations, reports, case studies, etc., into the literature has reached millions, and this number continues to increase exponentially. This situation necessitates researchers to invest considerable time and effort in accessing the existing literature related to research on any topic. However, bibliometric analysis offers significant advantages in swiftly and easily obtaining the required information from a vast amount of

knowledge (Fırat et al., 2018).

A literature review revealed that bibliometric analysis in the veterinary field particularly focused on multidisciplinary studies as well as animal welfare and diseases. Cui et al. (2023) conducted a bibliometric analysis of the literature on farm animal welfare in China. They reported that most China-centered studies mostly focused on pig and chicken species, but the collaboration index between institutes and authors was low. Chen et al. (2022) conducted a bibliometric analysis of the publications from Veterinary Communication Education Research from 1 January 2000 to 31 December 2021 on Web of Science. They found that the highest number of publications in this field was from Guelph University and the Journal of Veterinary Medical Education. Furthermore, Yardibi et al. (2021) employed a bibliometric analysis to identify trending topics in animal science over the last five years (2015 – 2019). They identified "Genomic prediction" as the most trending topic, whereas, "Growth performance" and "Staphylococcus aureus" were identified as potential popular topics in the future. The current study aims to provide a general map of the literature on the utilization of artificial intelligence in veterinary medicine and to identify the most common application areas of artificial intelligence through bibliometric analysis. To the best of our knowledge, no study has yet conducted a bibliometric analysis on artificial intelligence studies in veterinary medicine. In this regard, this paper presents the first bibliometric analysis of articles about the use of artificial intelligence methods in veterinary sciences. The most influential journals, authors, countries, and popular topics were identified. The findings are believed to provide useful information for the researchers about the intellectual structure of the field and current research topics.

2. Materials and Methods

2.1. Research Methods

The studies incorporating artificial intelligence in the veterinary medicine field were examined using bibliometric analysis. Bibliometric analysis typically includes 2 stages, namely, performance analysis and mapping techniques. Performance analysis provides statistical metrics, whereas, mapping techniques allow visual examination of the interactions between research elements (Donthu et al., 2021). Certain indicators such as h-index, g-index, m-index, citation count, publication count, Journal Impact Factor (JIF), and JIF Quartile were used in the performance analysis. Addition-ally, Lotka's and Bradford's Laws were employed as bibliometric laws. The h-index is a performance index introduced by Jorge E. Hirsch that takes into account the number of studies and citations. Today, the h-index is one of the most prominent impact measures. However, it faces criticism for being influenced by time and having lower value for researchers with high citation and article counts (Bornmann and Daniel, 2007). As a result of the criticisms received by the h-index, the g-index was introduced by Leo Egghe. The g-index prioritizes publications with a high citation count, whereas the h-index does not (Egghe, 2006). The h-index may not be an effective measure for young researchers, as they may not have had sufficient time to receive citations. To address this issue, the m-index, calculated by dividing the h-index by the active years of the researcher, has been introduced (Harzing, 2012). Bradford's law, one of bibliometric laws, is used to determine a small group of journals covering an important section (one-third) of the literature on a given topic (Garfield, 1980). Lotka's law, on the other hand, determines the distribution of the contributions of authors to a particular field and measures scientific productivity. According to Lotka's law, 60% of the authors are expected to contribute to the field with one article, 15% with two articles, and 7% with three articles (Sudhier, 2013).

2.2. Data Sources and Statistical Analysis

To discover the current literature about artificial intelligence in the veterinary medicine field, Clarivate Analytics' database Web of Science (WoS) was employed. WoS and Elsevier's Scopus are the biggest databases. Both databases cover studies in compliance with publication ethics (Merigó and Yang, 2017). A search for artificial intelligence in the veterinary medicine field in these databases resulted in 212 and 467 results with filtering in Scopus and WoS, respectively. Accordingly, WoS was preferred since it allows for reaching a wider literature. The data query was performed on 10 November 2023. A search in WoS for the keywords "veterinary" OR "veterinary sciences" OR "animal sciences" AND "machine learning" OR "deep learning" OR "artificial intelligence" OR "data mining" resulted in 601 studies. After filtering by document type (research article) and language (English), a total of 467 open access and non-open access studies were listed. A review of these 467 articles revealed that 70 articles were included in the search results due to word similarity and did not incorporate artificial intelligence and the veterinary medicine field. Consequently, the bibliographic dataset included a total of 397 research articles that fulfilled the required criteria (Figure 1). For bibliometrics analysis, the "Bibliometrix" package in R programming language and VOSviewer with Biblioshiny interface were employed (Aria and Cuccurullo, 2017; Van Eck and Waltman, 2017). The performance analyses of academicians, journals, institutes, and countries were conducted through the package Bibliometrix. On the other hand, co-citation analyses, bibliographic matching, a network map of institutes, and keyword network analyses were performed using VOSviewer.

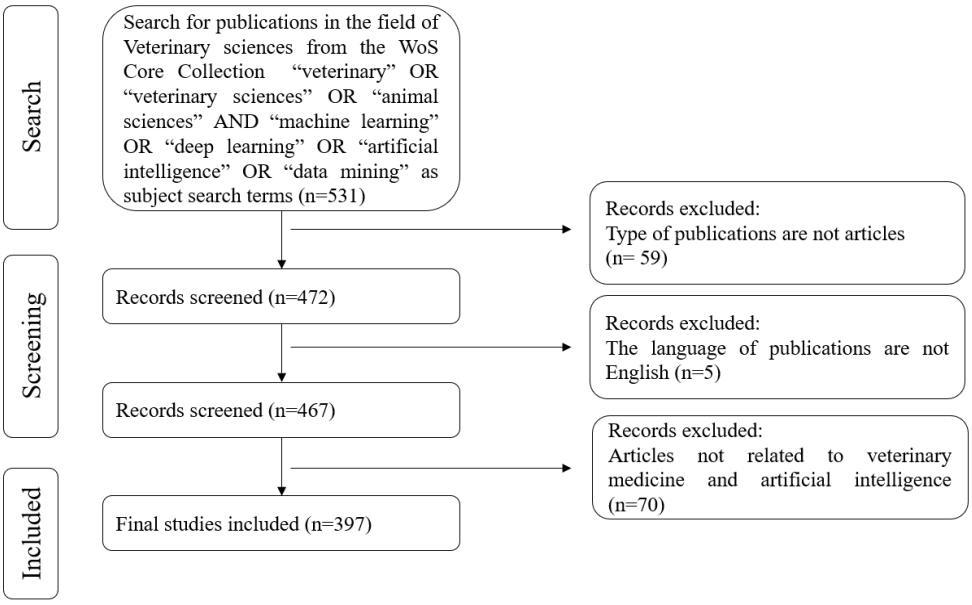

Figure 1. The publications selection process for this bibliometric analysis study.

3. Results

3.1. General Publication Trends

An overall examination of the bibliometric data showed that articles from 65 different journals in the period 1995 - 2023 were included in the analysis. Over this 28-year period, a total of 397 articles meeting the search criteria were published. The total number of researchers was 1758 with 9 single-authored articles. The citation count per article was 6.25 with a total citation count of 13958. The bibliographic data included 1127 keywords plus and 1437 author keywords. According to the analysis of author collaboration statistics, the number of articles per author was 0.23, the number of authors per article was 4.42, the number of co-authors per article was 5.45, the international co-authorship rate was 27.46% and the collaboration index was 4.53. A total of 1758 authors contributed to these studies, and their names were mentioned 2.163 times. A great portion of the articles (97.22%) was multi-authored. The author footprint index was calculated as 0.18 (see also Table 1). The trend analysis of publications on artificial intelligence in the veterinary medicine field indicates a significant increase in the number of articles from 1995 to 2023. The annual growth rate was found to be 17.88%. However, this growth did not follow a linear pattern. The publication count remained steady until 2018 but displayed a dramatic increase thereafter, and this upward trend is still ongoing. In 2018, only 7 articles were published in this field. However, this number reached to 27 in 2019, to 36 in 2020, to 70 in 2021, and increased to 105 in 2022. As of November 2023, 100 articles were published during 2023.

Table 1. Main statistics on artificial intelligence

Description	Results
Main Information About Data	
Timespan	1995:2023
Sources (journals, books, etc.)	65
Documents	397
Annual growth rate %	17.88
Document average age	2.71
Average citations per doc	6.254
References	13958
Document Types	
Article	378
Article; early access	8
Article; proceedings paper	11
Document Contents	
Keywords Plus (ID)	1127
Author's Keywords (DE)	1437
Authors	
Authors	1758
Author appearances	2163
Authors of single-authored documents	9
Authors of multi-authored documents	1749
Authors Collaboration	
Single-authored documents	11
Multi-authored documents	386
Documents per author	0.23
Authors per document	4.42
Co-Authors per documents	5.45
Collaboration index	4.53
Author footprint index	0.18
International co-authorships %	27.46

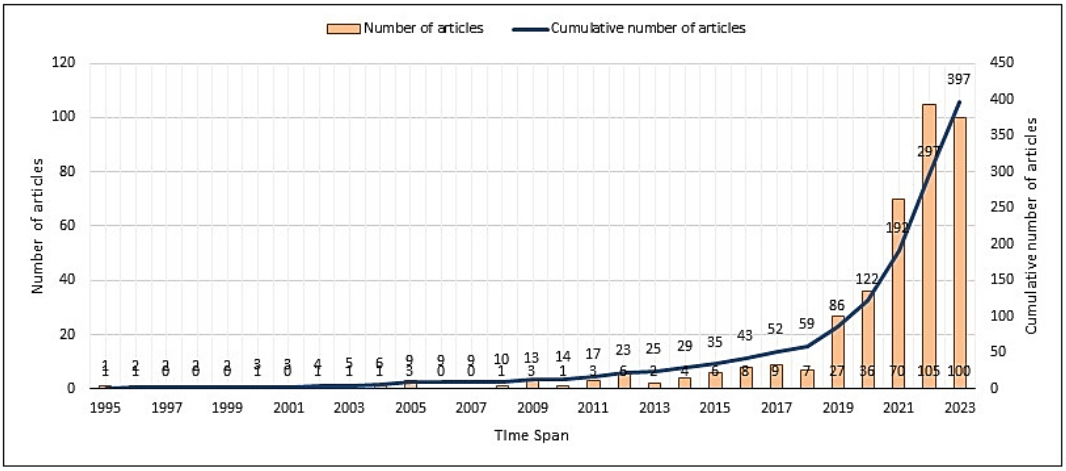

Figure 2. Annual scientific production on artificial intelligence in veterinary medicine.

The interactions between prominent sources (SO), authors (AU), and keywords (DE) are il-lustrated in Figure 3. The Animals and Frontiers in Veterinary Science journals were identified as the most influential sources. Jayon Kim and Guoming Li were the most influential authors. The most influential authors frequently used "deep learning" and "artificial intelligence" terms as keywords.

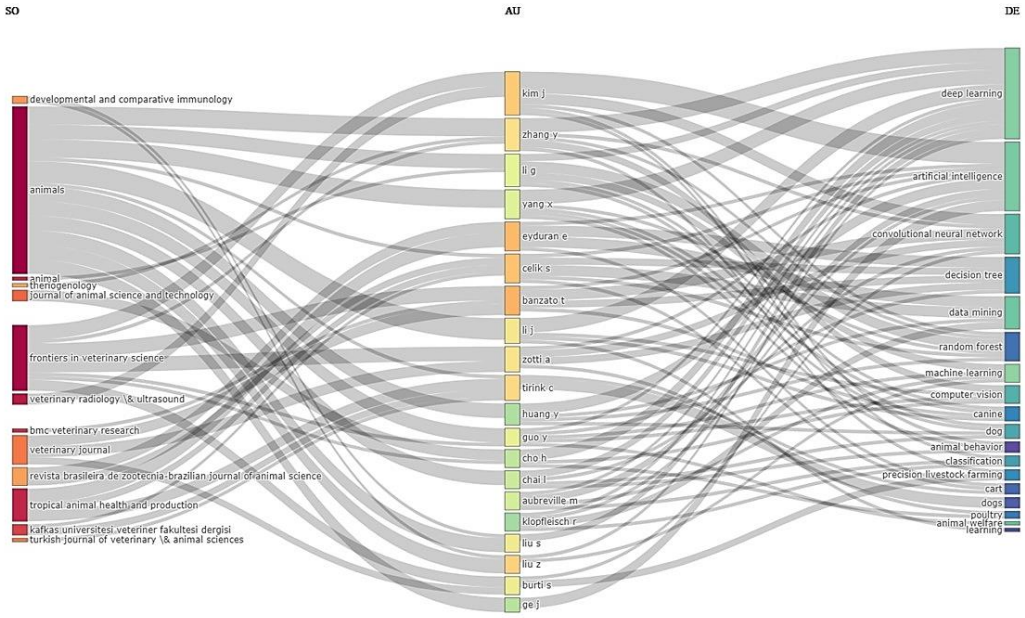

Figure 3. Artificial Intelligence three area graph, sources (left), authors (middle) and keywords (right).

3.2. Most Influential Journals

The most influential journals in artificial intelligence were evaluated by considering scientific metrics. Based on Bradford's law, the Animals and Frontiers in Veterinary Science journals were identified as the most influential and fundamental journals. According to the h-index, g-index, total publication count, and citation count, the first two journals were Animals and Preventive Veterinary Medicine. A thorough review of the purpose and scope of Animals, the top-ranking journal in the field, indicates that the journal encompasses significant studies in any field related to animals, with a particular focus on animal ethics. The review of artificial intelligence studies published in this journal indicated that studies mostly focus on animal behaviour, animal welfare, and animal tracking systems. The examination of Preventive Veterinary Medicine journal revealed that the published studies covered topics related to animal health, veterinary epidemiology, and disease control. The review of artificial intelligence studies published by this journal showed that articles mostly focus on the utilization of machine learning and deep learning in disease classification, disease detection, and out-break prediction. The journals with the highest m-index were Animals and Frontiers in Veterinary Science. According to the citation index (CI), the top two journals with the highest CI were Trans-boundary and Emerging Diseases and Veterinary Journal. Eight of the top ten most

influential journals in the field of artificial intelligence had a Category Normalized Citation Impact (CNCI) of above 1 (world average, (Gray and Price, 2020)). The analysis of the international collaboration percentages of the journals showed that the Transboundary and Emerging Diseases and Preventive Veterinary Medicine journals had the highest collaboration rates.

Table 2. The *h*-index, *g*-index, *m*-index and other scientific indices of the journals (top 10)

Source	*h* index	*g* index	*m* index	TC	NP	CI	CNCI	IC(%)	JIF	JIF Quartile	Country
Animals	11	15	2.2	405	106	3.82	1.37	32.36	3	Q1	Switzerland
Preventive Veterinary Medicine	9	14	0.474	236	32	7.38	1.38	40.89	2.6	Q1	Netherlands
Animal	6	10	0.429	109	12	9.08	1.68	38.73	3.6	Q1	United Kingdom
Frontiers in Veterinary Science	6	11	0.667	178	49	3.63	1.41	34.41	3.2	Q1	Switzerland
BMC Veterinary Research	5	10	0.313	110	10	11.00	1.33	29.52	2.6	Q1	United Kingdom
Veterinary Journal	5	6	0.227	85	6	14.17	1.13	33.22	2.2	Q2	United Kingdom
Veterinary Radiology & Ultrasound	5	9	0.455	92	16	5.75	0.86	20	1.7	Q2	USA
Journal of Veterinary Internal Medicine	4	6	0.8	37	7	5.29	1.28	29.62	2.6	Q1	USA
Kafkas University Journal of Veterinary Faculty	4	6	0.4	41	9	4.56	0.39	10.62	0.7	Q3	Türkiye
Transboundary and Emerging Diseases	4	4	0.308	58	4	14.50	1.82	43.54	4.3	Q1	Germany

NP= number of publications, TC= total citations, CI= citation impact, CNCI= category normalized citation impact, JNCI= journal normalized citation impact, IC= international collaborations, JIF= journal impact factor

3.3. Analysis of Prolific Authors

The h-index, g-index, m-index, collaborations, and other metrics of the top ten most influential authors in studies on the utilization of artificial intelligence in the veterinary medicine field (in some countries, several fields of science are integrated) according to their contribution to the literature are shown in Table 3. The authors with the highest scores for the h-index and g-index were Tommaso Banzato (Italy), Ecevit Eyduran (Türkiye), and Alessandro Zotti (Italy). On the other hand, the author with the highest scores for the m-index was Jayon Kim (South Korea) followed by Tommaso Banzato (Italy). The authors with the highest publication and citation count in this field were Ecevit Eyduran in the first place, Tommaso Banzato in the second place, and Alessandro Zotti in the third place. Regarding citation impact, the first 3 authors were Ecevit Eyduran, Lilong Chai, and Tommaso Ban-zato. According to CNCI values, 7 of the top ten authors had a CNCI value above 1. Ecevit Eyduran, Guoming Li, and Lilong Chai were identified as the most influential authors in terms of international collaboration. The only author without international collaboration was Jayon Kim. Conformity of the dataset to Lotka's law was examined accordingly, 85.7% of the authors contributed to the field with one article, 9.2% with two articles, and 3.2% with three articles. This distribution was found not to comply with Lotka's law. The reason for this result is that 85% of the articles were from the last 5 years, even though the selected period starts from 1995. With the developments in computer and software technologies, this distribution will probably change as the number of articles in this field increases in the following years. However, there may be deviations in the distribution since the studies in this field require specific fields such as veterinary medicine, software, and statistics. However, it can be argued that authors with more than 5 articles have delved deeper into the field of artificial intelligence and can be considered core authors.

Table 3. The *h*-index, *g*-index, *m*-index, and other scientific indices of the authors (top 10)

Author	h-index	g-index	m-index	TC	NP	CI	CNCI	Academic-Corporate collaboration(%)	IC(%)	Country
Banzato, Tommaso	5	8	0.833	85	8	10.63	1.32	12.2	20	Italy
Eyduran, Ecevit	4	8	0.5	96	8	12.00	1.22	0	77.78	Türkiye
Zotti, Alessandro	4	7	0.667	58	7	8.29	1.28	10.6	20.83	Italy
Brunton Lucy, A.	3	3	0.75	26	3	8.67	1.55	7.7	37.50	United Kingdom
Celik, Senol	3	7	0.375	64	7	9.14	0.87	0	25	Türkiye
Chai, Lilong	3	4	0.75	45	4	11.25	2.26	5.8	56.52	USA
Chang, Yu-Mei Ruby	3	3	0.75	26	3	8.67	0.95	7.7	25	United Kingdom
Drewe, Julian Ashley	3	3	0.75	26	3	8.67	1.64	4.2	40	United Kingdom
Kim, Jayon	3	3	1.000	13	7	1.85	0.68	0	0	South Korea
Li, Guoming	3	5	0.750	25	5	5	1.37	0	60.87	USA

NP = number of publications, TC = total citations, CI = citation impact, CNCI = category normalized citation impact, JNCI = journal normalized citation impact, IC = international collaborations

Figure 4 visualizes collaborations between the most influential authors in the field without any publication count limit. The analysis of links between authors revealed 46 authors distributed across 4 groups, with a total of 385 links. The authors with the highest citation count were Ecevit Eyduran with 96 citations, Andres Martin Perez with 78 citations, and Zhanjiang Liu with 66 citations.

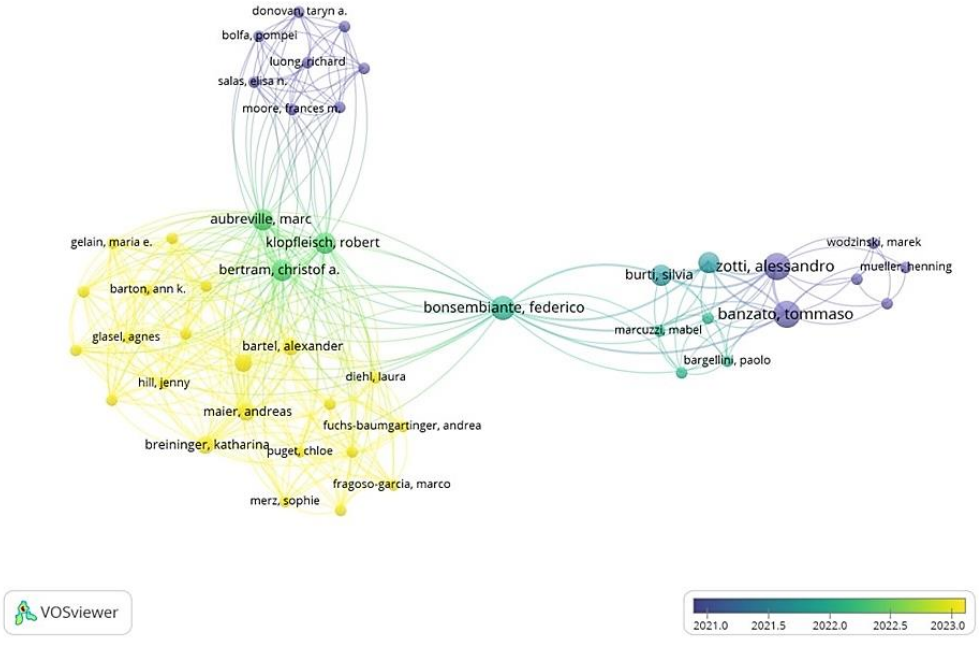

Figure 4. The network map of co-authorship related to artificial intelligence.

3.4. Contribution of Institutions

A total of 397 institutes associated with 663 articles were identified. The higher number of institutes than the number of articles can be explained by the high collaboration ratio in the field. The examination of the productivity of institutes on artificial intelligence in the veterinary medicine field showed that the institutes with the highest number of publications were UC Davis (USA), University of Padua (Italy), and China Agricultural University (China). Additionally, these institutes also held the top three positions for h-index scores. According to the CNCI values of institutes, 8 of the top 10 institutes

displayed a productivity value above 1. The examination of the international collaborations of institutes revealed that the University of Bern held the first place, the University of Padua in the second place, and the University of Guelph in the third place (Table 4).

Table 4. The *h*-index and other scientific indices of Affiliations (top 10)

Affiliation	Articles	h-index	CNCI	IC(%)	Country
University of California Davis	37	213	1.55	42.14	USA
University of Padua	32	222	1.57	52.20	Italy
China Agricultural University	30	132	1.42	31.75	China
University of Guelph	26	116	1.21	49.16	Canada
Igdir University	24	38	0.87	20.67	Türkiye
University of Georgia	19	133	1.21	32.27	USA
University of Tennessee Knoxville	17	169	1.36	41.70	USA
Kangwon National University	16	83	0.85	26.71	South Korea
University of Bern	16	233	1.74	68.26	Switzerland
University of Minnesota Duluth	16	57	1.23	38.82	USA

CNCI= category normalized citation impact, IC= international collaborations.

3.5. Contribution of Countries

The country with the highest number of publications and citations in our dataset was the USA, followed by China, and Türkiye. Furthermore, these 3 countries accounted for 41.1% of the studies about artificial intelligence in veterinary medicine globally. South Korea had the highest Category Normalized CI value. Australia, the only country to achieve a 50% Multiple-country Publication rate, displayed the highest international cooperation. The examination of the countries' CNCI values revealed that 8 of the top 10 countries exhibited a scientific productivity value above 1. According to the number of connections and total connection power, the USA, China, the United Kingdom, and Canada were determined as the most influential countries in the collaboration network (Table 5).

Table 5. Publication count and collaboration metrics of the countries (top 10).

Country	TC	NP	CI	Frequency	SCP	MCP	MCP Ratio	CNCI	Links	TLS
USA	537	72	7.46	0.181	45	27	0.375	1.29	33	75
China	276	63	4.38	0.159	51	12	0.19	1.14	14	24
Türkiye	242	28	8.64	0.071	25	3	0.107	0.86	3	5
South Korea	236	23	10.26	0.058	18	5	0.217	1.08	8	9
United Kingdom	167	23	7.26	0.058	18	5	0.217	1.47	19	40
Italy	124	19	6.53	0.048	16	3	0.158	1.33	12	18
Germany	101	18	5.61	0.045	11	7	0.389	1.29	20	42
Canada	74	15	4.93	0.038	8	7	0.467	1.39	18	28
Brazil	68	14	4.86	0.035	12	2	0.143	0.81	5	8
Australia	66	10	6.60	0.025	5	5	0.5	1.50	8	11

NP = number of publications, TC = total citations, CI = citation impact, CNCI = category normalized citation impact, SCP = single country publication, MCP = multiple countries publication, TLS = total link strength.

3.6. Keyword Analysis

An analysis of the tendency of keywords used in the field can reveal the changes in important concepts within artificial intelligence studies over the years as well as the current concepts. The examination of keywords revealed that "machine learning", "artificial intelligence", "deep learning", and "convolutional neural network" were the most frequently used terms. On the other hand, "image pro-cessing", "classification", "data mining algorithms", "active learning", and "computer-aided diagnosis" were frequently preferred terms in recent studies (Figure 5).

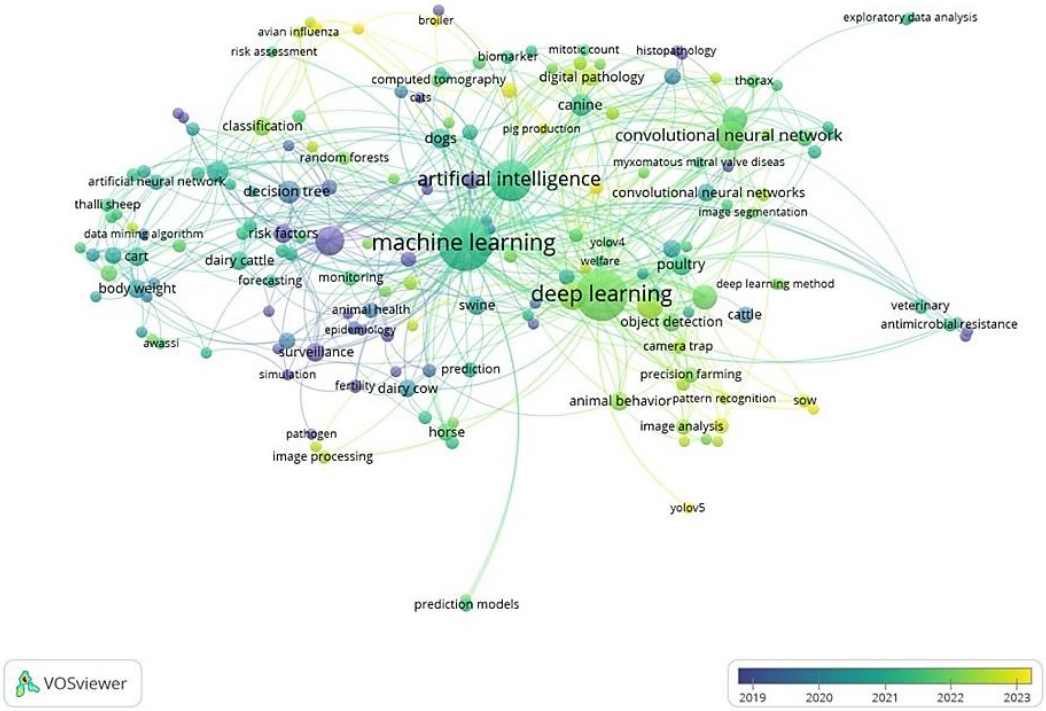

Figure 5. Network analysis of authors keywords.

3.7. Co-citation Analysis

Co-citation analysis reflects how often two documents are cited together by other sources. In the journal co-citation analysis with a minimum co-citation count of 20, 102 journals were identified. Accordingly, Computers and Electronics in Agriculture was identified as the journal with the highest co-citation count (439 co-citations, JIF 2023 = 8.3, Q1), followed by Journal of Dairy Science (412 co-citations, JIF 2023 = 3.5, Q1), and Plos One (249 co-citations, JIF 2023 = 3.7, Q2). Moreover, Scientific Reports was determined as the journal with the second highest impact factor (JIF 2023 = 4.6) and one of the most influential journals in multidisciplinary studies.

4. Discussion

Although studies on artificial intelligence in the veterinary medicine field began in 1995, no significant increase was observed until 2019. However, due to advancements in computer and software technology, the utilization of artificial intelligence algorithms in the veterinary medicine field has experienced a dramatic increase recently (Owens et al., 2023). The number of scientific studies in all disciplines has grown by an average of 3% over the last 30 years (Bornmann and Mutz, 2015). However, studies on artificial intelligence in the veterinary medicine field increased a 17.88% increase. The rapid growth in the field of artificial intelligence will likely attract more researchers. It is estimated that the linear increase in the number of publications on artificial intelligence will accelerate in the near future as new countries start to produce studies in this field. In addition to the theoretical results, studies in the field of artificial intelligence provide useful practical results for the prediction, prevention, and control of factors that threaten animal health (Kour et al., 2022). Furthermore, with the integration of artificial intelligence and advanced technological systems in animal husbandry science, human errors in labor are reduced and optimum efficiency is achieved from animals under existing conditions. Moreover, with effective features including image processing, decision-making, and prediction, such technologies offer minimum human intervention. The findings showed that the most frequently used terms in this field were “machine learning”, “artificial intelligence”, and “deep learning”. In a systematic review by Bouchemla et al. (2023) on artificial intelligence in medicine science, the keywords “machine learning”, “deep learning”, and “convolutional neural network” were used. This study is consistent with the findings obtained during the word analysis in the current study. A literature survey (Cavero et al., 2006; Ebrahimi et al., 2019; Rao et al., 2020) revealed that studies mostly addressed image processing, machine learning algorithms, disease prediction, and classification topics. This finding supports the keyword network obtained in the current study Furthermore, an examination of the keyword network based on species indicated that the studies were primarily focused on image processing in feline and canine veterinary medicine, as well as classification and prediction in poultry and ruminant medicine. Bibliometric analysis is a review of the literature. It does not suggest causality or a positive or negative conclusion. Therefore, the interpretation of the study results is the responsibility of the researcher. The studies on artificial intelligence in veterinary medicine mostly preferred the open-access

publication method. However, it should be noted that authors who cannot afford the open-access publication fee of popular journals in the field may face challenges in reaching a wider audience compared to those who can afford the open-access fees. This is one of the main reasons for the lower number of artificial intelligence studies in underdeveloped and developing countries. Publishing an original research article on artificial intelligence in the field of veterinary medicine necessitates expertise in various fields, including statistics and computer/software, in addition to veterinary medicine. Therefore, the authors of review and original research articles may differ significantly. This situation also explains why academic collaboration in this field is high. Identification of core re-searchers in a research area is important for understanding the perspectives within that area and per-forming effective consultations (Boyack et al., 2013). Ecevit Eyduran and Tommaso Banzato were identified as two core researchers in the field of artificial intelligence. These authors had an international collaboration rate above 20%. Furthermore, Alessandro Zotti and Tommaso Banzato, among the top ten influential authors, had co-authored many artificial intelligence studies. A great portion of the studies of these authors were on image processing. The collaboration between these two authors contributes to the reputation of both their institutions and their countries in this field (Banzato et al., 2021). In examining institutes supporting studies in this field, UC Davis and the University of Padua were identified as the primary publishers and institutes with the highest number of citations. Citations received by papers indicate the level of attention to a given field. In this regard, USA-centered papers achieved the highest citation count (537), followed by China (276), and Türkiye (242).

5. Conclusion

Artificial intelligence studies in the field of veterinary medicine are of great importance for facilitating rapid and accurate diagnosis and treatment of existing and potential diseases (Muehlematter et al., 2021). Accordingly, studies in the field of artificial intelligence have started to receive more attention. This bibliometrics study analyzed the WoS database over a 28-year period regarding the development of studies on artificial intelligence in veterinary medicine science, trends in different branches of veterinary medicine, as well as the most influential authors, countries, institutions, journals, and trending topics in this field. A total of 397 academic papers published between 1995 and 2023 were analyzed, and the results were statistically reported using mapping methods. By providing an overall map of the field, this study can serve as a guide for researchers in their future studies on artificial intelligence in veterinary medicine. While this quantitative research objectively reflects the structure of the field, it has some limitations. The WoS database has a vast collection of academic journals and is frequently used in bibliometric studies. However, as different databases such as Scopus and ScienceDirect produce different content, this reduces the reproducibility of the study and causes confusion among researchers. The fact that only one database was used in the study to eliminate this confusion can also be considered a limitation of the study. Since the data search phase in databases is done through keywords, the use of the same word in different languages causes language confusion. Therefore, only English studies were included in the analysis.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	H.S.	M.M.K.
C	70	30
D	50	50
S	100	
DCP	60	40
DAI	40	60
L	30	70
W	50	50
CR	20	80
SR	80	20
PM	30	70
FA	30	70

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declare no conflicts of interest associated with this manuscript.

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Ethical Consideration

Ethics committee permissions for this study were obtained from Selçuk University Faculty of Veterinary Medicine, Experimental Animal Production and Research Centre Ethics Committee and the study was carried out within the scope of the permission of this committee (Approval date: March 30, 2023 and protocol code: 2023/027).

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COMPARISON OF INOCULATION METHODS FOR SCREENING TOMATO, PEPPER, AND AUBERGINE PLANTS INOCULATED WITH *NEOSCYTALIDIUM NOVAEHOLLANDIAE*

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Abstract: *Neoscytalidium novaehollandiae* is a highly destructive fungus that causes serious yield losses in economically important crop plants. A reliable and rapid inoculation method for *Neoscytalidium novaehollandiae* was evaluated in eggplant, tomato and pepper seedlings under greenhouse conditions. Three different inoculation methods namely, cut-stem, spray, and injection were evaluated on tomato, pepper, and aubergine seedlings. The results showed that the cut-stem and injection methods were effective on tomato, pepper, and aubergine plants while the spray inoculation method was not found effective. Especially, the cut-stem inoculation method appears to be the most appropriate method for pathogenicity and screening of seedlings for *N. novaehollandiae* pathogenicity.

Keywords: *Neoscytalidium novaehollandiae*, Grafting techniques, Cut-stem, Spray, Injection

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1. Introduction

Tomato (*Solanum lycopersicum* L.), pepper (*Capsicum annum* L.) and aubergine (*Solanum melongena* L.) are among the most commonly grown and consumed crops in Türkiye as well as in the world. The production and consumption of the fruits of these plants require high demand in the market. The amount of production is 13 million tons for tomato, 3.018.775 million tons for pepper and, 781.242 tons for aubergine (TÜİK, 2024). Many diseases and pests are encountered in the production process of these vegetables during cultivation, harvesting, and storage. In recent years, global warming has played an important role and made a significant impact on plant pathogens that has not been observed previously (Jamieson et al., 2012; Elad and Pertot, 2014). The impact of plant pathogens under environmental stress conditions increased radically and has caused more devastating effects on crop plants (Félix et al., 2016; Yan et al., 2018). For example, *Lasiodiplodia theobromae* is an opportunistic pathogen of woody plants. It produces cellulase, lignocellulase, 18 chitin-binding gene families, and heat shock proteins at high temperatures. It is evident that its virulence increases along with the increase in air temperature. The most important plant pathogens for commonly consumed vegetables are *Botrytis cinerea, Clavibacter michiganensis* subsp. *michiganensis, Pseudomonas corrugata, Erwinia carotovora* subsp. *carotovora, Phytophythora infestans, Alternaria solani*, and Tomato spotted wilt, tospovirus, *Sclerotinia sclerotiorum, Phytophthora capsici,* and Cucumber mosaic virus, etc. (Fernando et al., 2004; Agrios, 2005; Höfte, 2006; Yardımcı and Çulal Kılıç, 2009; Zitikaitė and Urbanavičienė, 2010; Zhao at al., 2013; Todorović et al., 2016; Babalola et al., 2017; Hua et al., 2018; Wolters et al., 2018). In recent years, *Neoscytalidium* spp. have caused significant crop losses in vegetables and trees (Türkölmez et al., 2019a; Türkölmez et al., 2019b; Dervis et al., 2020a; Dervis et al., 2020b; Güney et al., 2022a; Güney et al., 2022b). One species of *Neoscytalidium* is *Neoscytalidium novaehollandiae* (Pavlic et al., 2008). *Neoscytalidium novaehollandiae* (Pavlic) is a dematiaceous fungal species that causes diseases in a wide range of plant hosts. This species belongs to a group of slow-growing, asexually reproducing microfungi, commonly referred to as "dark molds" or "black fungi" (Crous et al., 2006). Their damages in terms of crop quality become more prevalent. The fungal agent belongs to the *Botrtosphaeriaceae* family and forms filamentous, coiled, and irregular hyphae. The fungus mycelia develops rapidly in culture, its color is light at first and turns into brown and black over a short period of time. It can form both arthroconidia and pycnids in the same culture (Crous et al., 2006; Pavlic et al., 2008). *Neoscytalidium* species are able to infect plants through

air, soil, seed, fruit, etc. (Türkölmez et al., 2019a). They could also infect human beings through sinuses, skin, nails, wounds, etc. (Crous et al., 2006; Machouart et al., 2013; Bakhshizadeh et al., 2014; Da Silva et al., 2016). The family *Botryosphaeriaceae* contains many important plant pathogens, most of which are named opportunistic pathogens (Chethana et al., 2016). The pathogens in this family have great defensive strategies. High genetic variability among the family species gives a broad selection range for the host preferences. Many species in the *Botryosphaeriaceae* family infect a wide variety of plant hosts (Jami et al., 2014; Garcia et al., 2021). It is still under evaluation that the range of hosts could increase due to the aggressive behavior of the pathogen. Enzymes and heat shock proteins are able to protect the fungus from being digested by host-triggered enzymes and metabolites (Paolinelli-Alfanso et al., 2016). The unique genomic structure enables the adaptation of the fungus to new ecological conditions. The genes protect the fungus by detoxifying phytotoxins during the infection stage (Chen et al., 2014; Yan et al., 2018). Thus, the fungus can easily spread around and live in high temperatures and other adverse conditions and create more pathogenicity. Quite a few reasons have been attributed to increased pathogenicity such as drought, high temperature and water stress as well as plant age, growth period and inoculation methods (Jordaan et al., 2019; Khamari et al., 2019; Chaturvedi, 2021). It is, therefore, clear that opportunistic pathogens have a high capacity to adapt to stressful conditions (Kılınc, 2021).

To evaluate the behavior of the pathogen in vitro and in vivo conditions, and the responses of plants, we need to find out a quick and reliable inoculation method that would reveal the characteristic of the pathogen in a very short time. In this study, three different inoculation methods such as spraying conidia through leaves, syringe inoculation through stem, and cut-stem inoculation were made on tomato, pepper and aubergine plants with *Neoscytalidium novaehollandiae* isolated from pistachio trees in Şanlıurfa province (Kılınc,2021).

2. Materials and Methods

2.1. Growth of Fungus

Tomato (*Solanum lycopersicum* L. cv. SC2121), pepper (*Capsicum annum* L. cv. Acıburun) and aubergine (*Solanum melongena* L. cv. Diyarbakır Karası) plants were inoculated with *Neoscytalidium novaehollandiae* isolate (NCBI registration number: OL455801) at the 4-6 true leaf stage. The isolate was kindly provided by the plant pathologist Şahinmerdan Türkölmez from GAP Research Institute in Şanlıurfa. PDA medium was used for culturing of *N. novaehollandiae* isolate. The medium was poured as 10 ml into sterile plastic petri plates (10 cm in diameter) and kept until it solidified under aseptic conditions. Mycelial discs (8 mm) were taken from the ends of the fully developed 5-8 day-old *N. novaehollandiae* culture and placed in the center of newly-prepared petri plates. Petri dishes were then incubated at 25 ±1°C for 6-8 days (Fig. 1). The fungal spore suspension was adjusted to 1×10^6 conidia ml^{-1} (Kee et al., 2017).

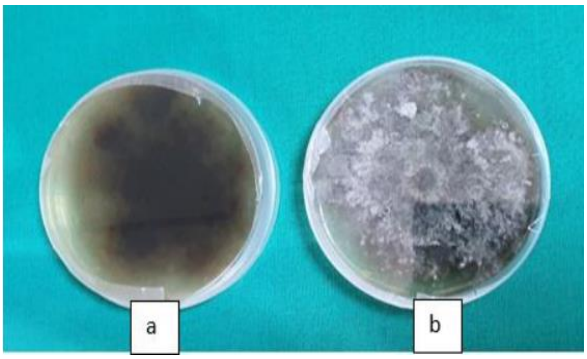

Figure 1. Appereance of mycelial growth of *N. novaehollandiae* a) back side; b) front side of the culture in PDA medium. The fungus culture gets darker during growth and development.

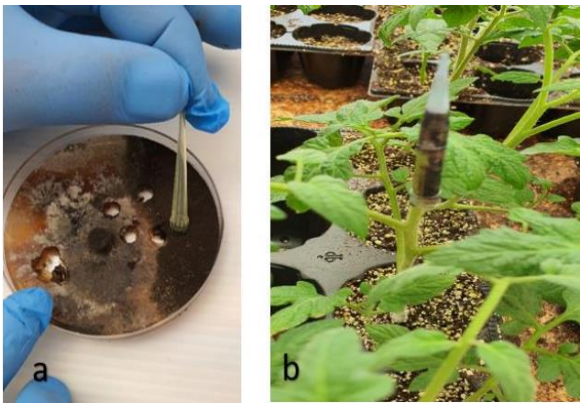

Figure 2. Cut-stem inoculation method using a 1 ml-pipette tip for the cut-surface area a) collection of mycelial discs via 1 ml-pipette tip b) pipette tip bearing fungal discs for the inoculation of cut-surface area.

2.2. Inoculation of Plants

A mixture of peat: perlite:sand (2:1:1) was prepared for the growth of tomato, pepper and aubergine plants in vials and the plants were maintained at 26 °C with 60% relative humidity at 14:10 h day: night photoperiod conditions in a greenhouse. Irrigation of the plants was made on demand.

2.2.1. Cut-stem inoculation method

The plants were cut with the help of a sterile scalpel, approximately 8 cm from the top, and the PDA discs bearing the fungus mycelia and the conidia were placed on the cut area. The cuts were protected by covering them with a 1 ml- pipette tip (Fig. 2). For the control group, sterile PDA discs were placed in these cuts and covered with a 1 ml pipette tip as shown below. A pipette tip is designed to keep the pathogen in infected tissues throughout the infection period and minimizes the pathogen escape and allows for a healthy comparison (Mengistu et al., 2007). The pipette tips were removed from the cut surface after 5 days and the progression of

the disease as brown discoloration alongside the longitudinal stem starting from the cut surface (downward) was measured for a period of 30 days at 5-day intervals. Using pipette tips for the cut-stem surface area of tomato, pepper, and aubergine plants is a new approach to increase the efficacy of mycelial or conidial inoculation. With this approach, a simulation for wounded tissues under humid conditions was created, which is one of the possible ways infection in nature (Twizeyimana et al., 2012).

2.2.2. Spray method

The fungal spore solution was prepared by pouring 10 ml sterile-distilled water onto the culture medium of *N. novaehollandiae* grown in PDA in petri plates. Then, the concentration of fungal conidia was arranged to 1×10^6 condia ml^{-1} under a microscope using a hemocytometer. The conidial suspension (5 ml) was then sprayed onto the leaves of tomato, pepper, and aubergine seedlings, Fig 3. Control plants were sprayed with sterile water only. Immediately after spray, each pot was covered fully with a transparent nylon bag and kept for 24 hours. At the end of the day, the bags were removed and the plants were evaluated symptomatologically for 30 days at 5-day intervals.

Figure 3. Spray inoculation of aubergine plants.

Figure 4. Injection of pepper plants.

Figure 5. Necrotic areas on leaflet margins.

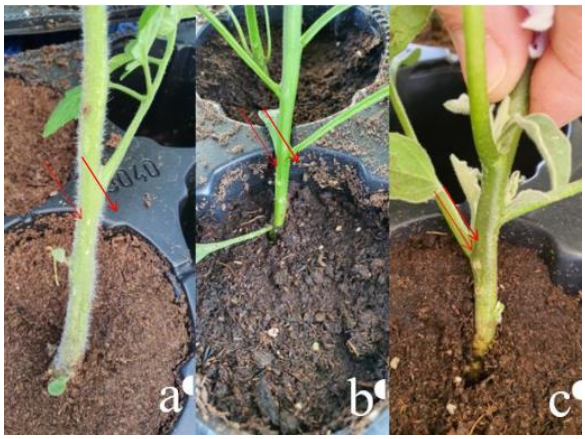

Figure 6. Necrotic spots appearing after N. novaehollandiae injecion on a) tomato b) pepper c) aubergine.

2.2.3. Injection method

The fungal spore solution (1×10^6 conidia ml^{-1}, 100 µl) was twice injected with a green-tipped syringe at 1 cm internals alongside the length of tomato, pepper, and aubergine seedlings approximately starting 3 cm above the soil surface (Fig. 4). For the control plants, 100 µl of sterile water was used (Kee et al., 2017). After the inoculations, the samples from the symptomatic plants were cut into small particles (2-5 mm) for a period of 30 days at 5-day intervals. The samples to be assessed were sterilized with 1% NaOCI solution for 45 seconds, and washed with sterile distilled water 3 times, then rinsed in 70% ethanol for 30 seconds. The samples were then dried on blotting papers. The measurements were made.

2.3. Disease Symptom Index

Plants were also scored for symptoms of a disease using the following scale (Dikilitas, 2003).

0-No symptoms

1-Trace of infection; yellowing visible symptoms at the cotyledon level,

2-Slight infection; chlorosis or yellowing patches affecting less than 50% of the leaves

3-Moderate infection; widespread symptoms such as chlorosis, wilting, necrosis, general decline in plants,

4-Severe infection; plant weak and stunted,

5-Extremely severe infection; only some green parts left in plants

6-Plants are completely dead

From these symptoms, a disease index was created and expressed with the folowing formula (i)

The number of plants showing a particular value (from 0 to 6) were multiplied by that value and the figure obtained for all plants summed and the total multipled by 100 to get % value. This value was divided by a maximum value of symtpms to get the ratio.

SI= Symptom index value
N= number of plants

$$\% SI = \frac{\sum SI}{6 \times \sum n} x100 \quad \text{(i)}$$

Table 1. The length of vascular discoloration and symptom index values of tomato plants inoculated with *N. novaehollandiae* with different inoculation methods

Incubation period (Days)	Cut-stem method Vascular discoloration (mm)/SI (%)	Spray method Vascular discoloration (mm)/SI (%)	Injection method Vascular discoloration (mm)/SI (%)
10	4 /10	0.1 /5	1 / 10
15	4/ 20	0.1 / 7	1 / 15
20	6 / 40	0.1 / 10	1 /25
25	8 / 60	0.1 / 10	1 / 30
30	12 / 65	0.1 / 10	1 / 35

SI= Symptom index value.

Table 2. The length of vascular discoloration and symptom index values of pepper plants inoculated with *N. novaehollandiae* with different inoculation methods

Incubation period (Days)	Cut-stem method Vascular discoloration (mm) / SI (%)	Spray method Vascular discoloration (mm) / SI (%)	Injection method Vascular discoloration (mm) / SI (%)
10	2 / 5	0.1 / 2	0.1 / 2
15	4 / 13	0.1 / 2	0.1 / 2
20	4 / 15	0.1 / 3	0.1 / 4
25	5 / 20	0.1 / 3	0.1 / 5
30	5 / 25	0.1 / 3	0.1 / 8

SI= Symptom index value.

Table 3. The length of vascular discoloration and symptom index values of aubergine plants inoculated with *N. novaehollandiae* with different inoculation methods

Incubation period (Days)	Cut-stem method Vascular discoloration (mm) / SI (%)	Spray method Vascular discoloration (mm) / SI (%)	Injection method Vascular discoloration (mm) / SI (%)
10	2 / 5	0.1 / 2	0.1 / 2
15	2 / 5	0.1 / 2	0.1 / 2
20	2 / 5	0.1 / 3	0.1 / 4
25	2 / 8	0.1 / 3	0.1 / 5
30	2 / 12	0.1 / 3	0.1 / 8

SI= Symptom index value.

3. Results and Discussion

Infection progress on tomato, pepper and aubergine plants was determined in terms of length (mm) in the vascular discolouration. Tomato, pepper and aubergine plants following inoculations with different methods showed that the symptoms of the disease progressed further with the increase of incubation time. However, the inoculation methods played significant roles about the progress of the disease. There were also differences in terms of disease responses among the plant species (Tables 1, 2, and 3). Infection speed in internal tissues in terms of vascular discoloration length showed that tomato plants were the most susceptible to *N. novaehollandiae* when compared to those of pepper and aubergine plants (Table 1). Inoculation after 20 days was found to be critical in plants with regard to disease progression. Infection progress in pepper and aubergine plants was prevented with the formation of callose tissues around the inoculation points and the infection area did not move on further when compared to the previous days. Aubergine plants were the most resistant plant that the infection rate remained still throughout the course of the experiment. The vascular discoloration length in thespray inoculation method remained constant at 0.1 mm for 30 days in all plants (Table 2). In some leaflets, necrotic areas and yellow patches were evident. However, this inoculation method was not found effective. Symptom index values even did not increase remarkably. When reisolation was made from those of the infected plants, the pathogenic agent was only recovered from the leaves of tomato plants exhibiting symptoms of the disease. In the injection method, the stems of the plants were more infected with 100 ml of the spore solution 1×10^6 spor (ml) through the stems. As a result, 1 mm of necrosis occurred around the inoculation site in tomato plants. However, aubergine and pepper plants formed new callus tissues around the infection sites and confound the pathogen throughout the experimental period. Reisolation was made from tomato plants while other plants gave negative results. Symptom index values significantly increased only in tomato plants. Cut-stem method was found to be the most effective method compared to those of other methods employed in this study for the determination of pathogenicity of *N. novaehollandiae* on tomato, pepper, and aubergine. This method could be suggested as a method to test the resistance level of vegetables. Although the efficient (cut-stem) method tested here was evaluated previously by other researchers on different fungi, to our knowledge no such comparison was made

on tomato, pepper, and aubergine with *N. novaehollandiae* in greenhouse conditions. Although the spray and the injection methods have been widely used in quite a few studies in different plant types in greenhouse conditions, however, callose accumulation around the infection sites following injection may retard the disease development if the germplasms are relatively/moderately tolerant or resistant to diseases. In the spray inoculation method, although a plastic bag was used to cover up the plants following inoculations, however, the low hummidity in greenhouse conditions might have prevented the disease development internally. The progress of disease symptoms in terms of internal browning has also been reflected in symptom index values taken at 5 days internally until the end of the experimental period. One of the main advantages of this technique compared to other inoculations methods used here is that a uniform amount of inoculum is placed directly onto the cut-stem paranchymatic tissues, which minimizes potential infection escapes (Ćuk et al., 2022). They stated that the method significantly revealed the resistance levels of sunflower plants to *Macrophomina phaseolina* (Ćuk et al., 2022). This method could be further evaluated with different fungi having different spore suspensions to enable precise quantitative inoculation which would help reduce experimental error. Since continuous inoculation is enabled with the cut-stem inoculation method, the resistance levels of inoculated plants could easily be determined without letting no interaction between the environmental stress agents and the pathogens. The whole experimental process could be completed over a month, in fact, first 2 weeks would indicate the resistance levels of inoculated plants. Since pathogens have no place to escape, it directly meets the paranchymatic tissues after inoculation even if variations existed in virulence levels. We could directly assess the resistance of plants as well as the virulence or pathogenicity of the fungal agent. Other environmental factors are significantly minimized. The cut-stem technique could serve as the most appropriate technique especially for new pathogens or new germplasms. Screening is going to be easier due to its ease of application. For example, Talapov (Talapov, 2020) stated that cut-stem inoculation technique was proven to be responsible on soybean plants inoculated with *Macrophomina phaseolina*. Due to easy applications of the inoculations method. Ćuk et al. (2022) suggested that the cut-stem method in sunflower plants could potentially be used in field trials and it could be a valuable tool in sunflower breeding for resistance to *M. phaseolina*. Twizeyimana et al. (2012) stated that there were several advantages of using cut-stem inoculation technique over to other inoculation methods. They reported that a uniform amount of inoculum placed onto an identical infection court via this technique minimized potential inoculum escapes. They also reported that the progress of the disease could be measured uniformly by measuring the extent of necrosis. Evaluation of this method could be completed within two weeks.

4. Conclusion

We propose that the cut-stem inoculation method applied to tomato, pepper, and aubergine is easy to apply and marks well and differentiates plant responses efficiently. However, one should notice that the rapid disease development that might result in plant death after 10-15 days of inoculation in vegetables can lead to misinterpration in germplasm studies. In this study, a preliminary investigation was made how the fungal agent *N. novaehollandiae* infected the host tissues such as tomato, pepper and aubergine plants via different routes of inoculation methods. Determination of the effectiveness of these methods could facilitate the selection of an appropriate inoculation method and screening the plant species in a very short time. For the selection of inoculation methods, we could prefer the cut-stem method over to spraying and injection methods. The cut-stem method is found the most efficient method on vegetable plants. Similar findings were also made by Talapov et al. (2021) who stated that the cut-stem method in sunflowers appeared to be the most appropriate for the pathogenicity of *Marcrophomina phaseolina* under controlled conditions. Since the fastest disease development on sunflowers was obtained with the cut-stem inoculation method. They stated that the cut-stem inoculation method yielded consistent results in pathogenicity and a variety of reaction studies. The most sensitive plant against *N. novaehollandiae* was found to be tomato and the most resistant plant was found to be aubergine. Data from this study will constitute important data for future studies, as no previous study for this pathogen has compared different inoculation methods.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	H.A.	F.D.	M.E.G.	M.D.
C	30	20	20	30
D	50			50
S	50			50
DCP	50	50		
DAI	40			60
L	60	40		
W	80			20
CR	30		20	50
SR	50		20	30
PM	20		40	40
FA		30	70	

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management. FA= funding acquisition

Conflict of Interest

The authors declare that they have no financial interests or relationships that could potentially lead to a conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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EFFECT OF DIFFERENT MIXTURE RATIOS ON SILAGE QUALITY IN SILAGE CORN AND COWPEA

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Abstract: In forage crop mixtures, the proportions of legumes and grasses should be adjusted very well. This study was conducted in Sakarya province, where pure corn (C), pure cowpea (Cp), and mixtures of 75% corn + 25% cowpea, 50% corn + 50% cowpea, and 25% corn + 75% cowpea were planted. The effect of the different mixture proportions on hay yield was then analyzed. In addition, silage was made from the obtained mixtures and the effect of the applied mixture proportions on the silage quality was determined. The field trials of the research were carried out in the trial areas of Sakarya University of Applied Sciences, Faculty of Agriculture. Silage was made in 2 kilogram containers from each plot and at the end of the ripening period; silage dry matter ratio (%), silage pH, Acid Detergent Fiber (ADF) (%), Neutral Detergent Fiber (NDF) (%), Crude Protein (CP) (%), Dry Matter Intake (DMI) (%), Digestible Dry Matter (DDM) (%) and Relative Feed Values (RFV) observations were taken as silage quality criteria. Flieg score calculation was made with physical and sensory analyzes used to determine silage quality. In the study, the highest ADF, NDF, CP, DMI, DDM and RFV were 50%C+50%Cp (%25.69) and 25%Cp+75%C (%25.71), 50%C+50%Cp (%36.75), 100%Cp (%13.59), 100%C (%0.48), 100%C (%2.79), 100%C (%0.32), 75%Cp+25%C (%0.36), respectively. According to the results of physical and sensory analyzes of the obtained silages, it was determined that all silages were very good. According to Flieg scoring, 100%C silage and 75%Cp+25%C mixtures silage were determined as good. As a result of the study, it was found that producers who wish to plant a mixture of legume and grass forage plants can obtain high-quality forage by using a 75% Cp and 25% C mixture.

Keywords: Corn, Cowpea, Silage, Quality

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1. Introduction

In Türkiye, corn, which is produced as the main product and the second product, is the grain with the largest planting area after wheat and barley. Corn is used as a raw material in both human and animal nutrition and in industry. In 2024, corn planting area for silage purposes was 505.399 ha, silage production was 27.3 million tons and average yield was 77.9 t ha^{-1} (TUIK, 2025). Corn produced of 27% in the world is used in human nutrition and 73% as animal feed. In our country, corn is used as 70% animal feed and feed raw material (Öz et al., 2017). Corn is mostly used in cattle and sheep feed and in the poultry sector. In recent years, the planting area for silage production has increased due to reasons such as the high yield obtained from the unit area of corn, its suitability for silage production and the high nutritional value and quality of corn silage. In addition, corn is the most preferred plant for silage in our country due to its easiest fermentation among water-rich roughages, its economy and its suitability for the second product (Öztürk et al., 2019). Cowpea is an important legume plant that can be used both as human food and animal feed (Debnath et al., 2018). It also enriches the soil in terms of organic matter and nitrogen (Doğan et al., 2011). Cowpeas are annual plants. Due to their general characteristics, they like warm weather. The best growing temperature is between 20-30 °C (Günay, 1992). They can grow in places with an annual rainfall of up to 600 mm without the need for irrigation (Akçin, 1988). Cow peas are used in rotation for soil improvement purposes in many countries where rice is grown. It is a plant with low soil requirements. It is a good green feed because it contains 7.5-20% crude protein and 50% digestible carbohydrates (Adeyanju et al., 2007). The ADF, NDF and crude protein content in the dry matter of cowpea varied between 27-30%, 33-36% and 17-20%, respectively (Beycioğlu, 2016). It is a delicious feed that animals love to eat as dry matter, grain feed or silage (especially with corn, sorghum and millets) (Gençkan,

1983). There are many criteria to consider when determining the species to be used in mixtures. The most important of these is that the mixtures should be arranged in a way that they contain at least one legume and one graminea. In addition, a number of factors should be taken into consideration, such as the suitability of the mixtures for grass or seed production, the compatibility of ripening times, the similarity of the tastiness and competitiveness of the species in the mixtures to be established for grazing, and the suitability of the soil and climate requirements of the species (Acar et al. 2006). Many mixed planting systems are applied in forage crop agriculture in order to increase the yield and quality obtained from the unit area. In a study where it was determined how the quality of the silage would be affected when the product obtained from the sowing of corn and some legumes alone and mixed was evaluated as silage; organoleptic analysis results and Flieg scores showed that corn and cowpea + corn had the best silage quality. It has been concluded that in case of mixed planting and silage of legumes and corn, the failure of legumes alone can be eliminated, they support each other in terms of nutrients and better quality silage can be obtained (Sarıçiçek et al., 2002). In order to determine quality roughage, the relative feed value (RFV), which is included in different quality indices, is based on the ADF and NDF contents and the estimation of the energy value that animals will provide with the potential to consume these feeds. Relative feed value is of great importance in determining the quality of feeds and their marketing (Moore, 1994). This study was conducted to determine the appropriate mixing ratios and to examine the effects of these mixing ratios on silage quality by creating a silage mixture consisting of corn as a carbohydrate source and cowpea as a protein source in the field.

2. Materials and Methods

The study was carried out on the area of Sakarya University of Applied Sciences, Faculty of Agriculture, Agricultural Sciences and Technologies Education Application and Research Center. Kale corn (*Zea mays* L.) variety and Ülkem forage cowpea (*Vigna unguiculata* L.) variety were used in the experiment. Field experiments were set up in accordance with the randomized complete block design with three replications. Parcel sizes; The planting was carried out in a way that the row length was 3 m, the row spacing was 70 cm, the distance between the rows was 17 cm, and there were 6 rows in each plot. One row at the edges and 50 cm sections from the row heads were removed as edge effect and the remaining part was evaluated. In the trial; Five different plantings were carried out: pure corn, 75% corn + 25% cowpea, 50% corn + 50% cowpea, 25% corn + 75% cowpea and pure cowpea. Seed per decare was calculated by multiplying the seed amount of the species in plain sowing by the proportions in the mixture, after a germination test was performed and seed purity was taken into consideration (Seydoşoğlu et al., 2020). Corn was shaped during the dough formation phase, and cowpeas were shaped when the lower pods were in the filling phase. Plant materials were passed through a laboratory type silage machine, chopped into 1-2 cm lengths, and silaged into 2 kilogram plastic containers. The silages were stored in the dark under laboratory conditions and opened after 60 days. Silage dry matter ratio, silage pH, crude protein ratio, ADF and NDF observations, silage physical analyzes and flieg score calculation were performed as silage quality criteria. The dry matter ratio in the silage was determined by taking approximately 150 g of silage samples from each silage after opening them, drying them at 70 °C for 48 hours and weighing them. Silage pH was measured by taking a 40 g sample from each silage container after opening it, placing it in a beaker containing 360 ml of pure water, shaking for 3 minutes and filtering, and measuring the resulting filtered medium with a pH meter (Budaklı 2009). Among the quality features, ADF, NDF, Crude Protein Ratio were determined in the ground sample using the corn silage calibration set on the NIRS (Near Infrared Reflectance Spectroscopy) device. The formulas required for relative feed value are adapted from Van Dyke and Anderson (2000). In order to calculate relative feed value, firstly digestible dry matter (DDM) is calculated from ADF value (equation 2).

$$DDM\% = 88.9 - (0.779 * ADF\%) \quad (1)$$

DMI% = 120 / NDF to calculate relative feed value, DDM and DMI values are substituted into the formula (equation 2).

$$RFV = (DDM\%) * (DMI\%) * (0.775) \quad (2)$$

(Sheaffer et al., 1995).

The relative feed value (RFV) index is based on the 100 index calculated from the ADF (41%) and NDF (53%) content of alfalfa hay in full flower. The feed quality decreases as it falls below this value, and increases as it rises. Within the scope of this classification, the RFV index value is accepted as the best quality if it is above 150, 1st quality if it is between 125-150, 2nd quality if it is between 103-124, 3rd quality if it is between 87-102, 4th quality if it is between 75-86, and 5th quality if it is below 75 (Redfearn et al., 2006; Canbolat and Karaman, 2009). At the end of the study, the plastic containers were opened and the samples taken to represent the sample were subjected to physical analysis and the scores were made according to subjective evaluations; color (0-2 points), structure (0-4 points), odor (0-14 points). For physical evaluations, the samples taken to represent the sample from each opened jar were examined by 5 experts and then the average scores for these data were calculated Silage Fleig score; The quality class of the silage feed was calculated by using the relationship between silage feed pH and dry matter content, using the Fleig score method according to the formula reported by Kılıç (1986), [Fleig Score = 220 + (2 × % Dry matter - 15) - 40 × pH] (Özata et al., 2012). As for the physical analysis of the silage, physical examinations of the samples such as odor,

structure and color were made subjectively by five experts and the scoring system developed by the German Agricultural Organization (DLG) was used in the evaluation. The results obtained in the experiment were analyzed using Jump statistical analysis package program (JMP, 2018) and the averages were examined by LSD test (Düzgüneş et al., 1987).

3. Results and Discussion

3.1. Quality values of silage mixtures

The chemical analysis of silages made from varying proportions of corn (C) and cowpea (Cp) revealed the following results: the highest ADF was observed in the 25% cowpea + 75% corn mixture (25.71%) and 50% corn + 50% cowpea mixture (25.69%). The highest NDF was recorded in the 50% corn + 50% cowpea mixture (36.75%) and 25% cowpea + 75% corn mixture (36.55%). The highest CP content was found in the 100% cowpea silage (13.59%). The highest DDM was found in the 100% corn silage (71.45%). The highest DMI was 3.70 for the 100% corn silage and 3.57 for the 25% cowpea + 75% corn mixture (Table 1). The highest RFV value was recorded in the 100% corn silage (204.93). In the study, the ADF and NDF rates of mixed silages were found to be higher than those of alone silages.

Table 1. Averages of some properties of silage mixtures

Mixture Ratios	ADF (%)	NDF (%)	CP (%)	DDM (%)	DMI (%)	RFV
100% Cp	24.01b	34.17b	13.59a	70.19b	3.51b	191.06b
100% C	22.39c	32.44c	8.16e	71.45a	3.70a	204.93a
75% Cp+25% C	23.19bc	34.28b	12.40b	70.83ab	3.50b	192.75b
50% C+50% Cp	25.69a	36.75a	10.78c	68.88c	3.26c	174.35c
25% Cp+75% C	25.71a	36.55a	8.67d	68.87c	3.57ab	190.89b
CV	3.41	2.09	1.71	0.91	2.14	3.04

There is no difference between the means shown in same letter P<0.01

Table 2. Silage pH, DM, flieg point and score

Mixture Ratios	pH	Silage DM (%)	Flieg point	Score
100% Cp	3.52	32	128.2	Very Good
100% C	4.35	37	105.0	Very Good
75% Cp+25% C	3.62	40	140.2	Very Good
50% C+50% Cp	3.88	39	127.8	Very Good
25% Cp+75% C	4.02	34	112.2	Very Good

Table 3. Silage physical analysis values, DLG points and score

Mixture Ratios	Odor	Structure	Colour	DLG	Score
100% Cp	11.74	3.32	1.57	16.63	Good
100% C	10.13	3.23	1.51	14.87	Good
75% Cp+25% C	11.91	3.14	1.49	16.54	Good
50% C+50% Cp	8.32	2.49	1.35	12.16	Medium
25% Cp+75% C	8.08	2.77	1.50	12.36	Medium

3.2. Flieg Points of Silage Mixtures

When we look at the pH and DM data for which the Flieg score is calculated, we see that the pH value varies between 3.52 and 4.35 (Table 2). Among the alone silages, the pH value of corn silage was found to be lower than that of cowpea silage. Dry matter ratios varied between 32% and 40%, and the lowest dry matter ratio was obtained from the 100% C silage. When we look at the Flieg scores calculated with dry matter and pH values, it is seen that all Flieg scores have very good values.

3.3. Physical Analysis of Silage Mixtures

Determining the quality of silo feeds; There is also a physical analysis method that is simple, low-cost and can be applied in all conditions. In the physical analysis method, it scores according to color, smell and structure and gives an idea about silage quality. The data on the physical properties of the study are given in Table 3. The color scores for the silage mixtures ranged from 1.35 to 1.57, while the odor scores varied between 8.08 and 11.91. The structure scores ranged from 2.49 to 3.32, and the total DLG scores ranged from 12.16 to 16.63. Among the mixtures, the 50% C + 50% Cp and 25% Cp + 75% C silages received medium scores, whereas the remaining mixtures were rated as good.

4. Discussion

4.1. Quality values of silage mixtures

Considering the silage quality values, the highest ADF (25.69%) and NDF (36.75%) values were obtained from the 50% C + 50% Cp mixture. Similarly, in the study conducted by Alaca and Özarslan (2017), the ADF rates of mixed silages were found to be high. In our study, the crude protein ratio was determined between 8.1% and 13.59% . Moreover, the crude protein content of silage made from cowpea, a legume forage plant, was found to be higher than that of silage made from corn. As the corn

content increased in mixed silages, the protein content decreased. Ünal (2023) determined the crude protein ratio as 18.87% in the corn-cowpea mixture in his study to determine the grass yield and quality in silage corn and some legume forage crop mixtures. 100% corn was determined to have the highest digestible dry matter ratio, which is one of the most important quality indicators of forages. When looking at the DMI value, it is seen that DMI increases as the corn ratio in the mixture increases. While the highest was detected in plain corn, this was followed by silage containing 75% corn in the mixture. Relative feed values, which are the most important criterion in determining the price of hay in the hay trade (Redfearn et al., 2012). When the results of the chemical analysis performed on silage mixtures are evaluated collectively, an ideal silage mixture is; Since the crude protein ratio is desired to be high and the ADF and NDF ratios are desired to be low, it can be said that the most suitable silage is obtained from a mixture of 75% Cp + 25% C.

4.2. Flieg Points of Silage Mixtures

Fleig point, which is an easy method to determine the qualities of silage, is calculated according to pH and dry matter ratio, and every factor affecting pH and dry matter ratio also affects the Fleig point (Woolfort, 1984). The pH value of the silage is an important parameter in determining the in-silo fermentation level. The pH value of a good silage is between 3.5 and 4.2 (Kılıç, 2006). The pH content of the silage is a critical parameter in assessing the level of fermentation inside the silo. The degree of maturity of the silage is determined by examining the pH value of the feed (Uygur, 2022). To obtain high-quality silage, an acidic environment, and therefore a low pH, is required inside the silo. After the lactic acid bacteria actively work and reduce the pH to levels between 4.2 and 4.5, the silaged material enters a stable phase (Açıkgöz, 2021). The pH values obtained in our study are within the limits expected in ideal silage and varied between 3.52 and 4.02. In their study conducted in Diyarbakır ecological conditions, they reported that the pH value varied between 3.67-3.90 (Tantekin, 2016), and in their study conducted in Tekirdağ conditions, they reported that the pH value varied between 3.58-3.66 (Kaya and Polat, 2010). The pH value obtained in the study is similar to other studies. The dry matter ratio in silage mixtures was determined to be between 32% and 40% (Table 2). In order for silage to be considered of high quality, the dry matter content must be between 25-40% (Klamem et al., 2005). A dry matter ratio of more than 40% makes it difficult to digest the feed and reduces its taste, while a low dry matter ratio reduces the carbohydrate content of the silage and causes it to spoil (Panyasak and Tumwasorn, 2015). In their study, Mut et al., (2020) determined the silage dry matter ratio between 26.50% and 36%. The dry matter ratios obtained were found to be lower than our study. The reason for this can be said to be that Mut et al., (2020) used alfalfa in the silage mixture in their study. Flieg points and scores calculated from the dry matter and pH values of silage mixtures are given in Table 2. In the trial, Flieg scores over 100 points were obtained from all silages. Flieg scores of all silages were determined as Very Good. Korkmaz et al., (2019) stated that the highest and lowest Flieg scores of different silage corn varieties grown as second crop in Çukurova ecological conditions were 122.60 and 114.53, respectively. Demirel et al. (2001) ensiled sorghum and Hungarian vetch by mixing them in different proportions and determined the fleig score as 53.93 in pure sorghum silage and as 54.69, %50S %50HV mixture. The study results are consistent with Korkmaz et al., (2019), while Demirel et al., (2001) was found to be higher. It can be said that the reason for the high finding is that the silage materials used are different and the corn used in our study is the best silage plant.

4.3. Physical Analysis of Silage Mixtures

According to the physical analysis results, 75% Cp+25% C mixture silage (16.54) and alone corn (14.87) and cowpea (16.63) silages were found to be good, while other mixtures (12.16 and 12.36) were found to be medium. Burgu and Mut, (2023) reported that physical analysis scores ranged from 9 to 20, while Kavut and Soya, (2012) found their scores to range from 18.22 to 19.06. The results obtained were similar to Burgu and Mut, (2023), but lower than Kavut and Soya, (2012). This difference may be explained by the fact that the silage mixtures are made from different types of plants.

5.Conclusion

According to the results of the chemical analysis conducted to determine the quality of silages obtained from different proportions of corn and cowpea mixtures, the highest crude protein (13.59%) is obtained from cowpea, and as the proportion of cowpea in the mixture increases, the crude protein content of the mixture also increases. Corn has been identified as the best silage crop in many studies. Similarly, in our study, corn silage appears to be the best in terms of ADF and NDF values. However, when ADF, NDF, CP, DDM, DMI, and RFV observations are evaluated collectively, it can be said that the best silage in the study is obtained from a mixture of 75% Cp and 25% C. The most important criterion for determining the quality of silage feed is its pH value. In the study, the pH values of the varieties ranged from 3.52 to 4.02. Both the Flieg scoring and the physical analysis results, including color, odor, and structure ratings, showed similar DLG values, confirming that the silage obtained from a mixture of 75% Cp and 25% C was identified as the best silage mixture, in line with the chemical analysis results. As advice to producers who plan to make legume and cereal silage, it can be said that they can obtain high-quality roughage by making silage with a mixture of 75% cowpea and 25% corn.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	M.Ö.	L.D.	B.Ö.	Ö.F.
C	25	25	25	25
D	25	25	25	25
S	100			
DCP	25	25	25	25
DAI	100			
L	25	25	25	25
W	100			
CR	100			
SR	100			
PM	25	25	25	25
FA	25	25	25	25

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to.

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EFFECT OF DIFFERENT NITROGEN DOSES AND SEED AMOUNTS ON SPIKE CHARACTERISTICS IN CULTIVATED AND WILD WHEATS

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Abstract: The study was conducted in 2022-2023 and 2023-2024 in the experimental fields of Tekirdağ Namık Kemal University, Faculty of Agriculture, Department of Field Crops. The study was conducted in 3 replications according to the split-plot experimental design using 400-450-500-550-550 seeds m^{-2} and 10-15-20 kg da^{-1} pure nitrogen dose in einkorn, mirabile wheat and NKU Zirve genotypes. In the study, spike length, number of grains per spike and grain weight per spike values of einkorn, mirabile and NKU Zirve wheats were analysed. According to the results of the analyses, it was determined that the number of grains in the spike was higher in mirabile wheat, and the values of spike length and grain weight in the spike were higher in NKU Zirve bread wheat genotypes. In spike length, the highest seed rate average was 8.06 cm in 450 seed m^{-2}, the highest nitrogen dose average was 7.95 cm in 15 kg da^{-1} pure nitrogen application and the lowest nitrogen dose average was 7.73 cm in 10 kg da^{-1} pure nitrogen dose application. In the number of grains in the spike, the highest seed rate average was 43.33 with 450 seeds m^{-2}, the lowest was 38.06 with 550 seeds m^{-2}, and the highest nitrogen dose average was 41.09 with 20 kg da^{-1}. The highest seed rate average of 400 seeds m^{-2} with 1.48 g, the highest nitrogen dose average with 1.43 g at 15 and 20 kg da^{-1}, the lowest nitrogen dose average with 1.39 g at 10 kg da^{-1} pure nitrogen dose application were determined.

Keywords: *Triticum turgidum var. mirabile*, *Triticum monococcum L.*, Sowing norm, Nitrogen dose, Spike, Local wheat

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1. Introduction

Wheat has been the main food source of the people of our country from past to present with the products obtained from wheat, especially bread. Wheat has always been considered valuable, respected and regarded as a sacred product. In our country, only 20 per cent of the existing agricultural areas can be irrigated and the remaining areas are dry farmed. This situation further emphasises the importance of field crops produced by dry farming method. Wheat has gone beyond being only a source of food and has become a strategic product on a global scale. Countries have to carry out economic and strategic international policies on wheat production and supply in order to meet the nutritional needs of their populations. The main reason for this is the vital role that wheat plays in human nutrition on a global scale. It is accepted that the relationship between wheat and humans started about 14 thousand years ago in Southeastern Anatolia, around Urfa; Einkorn, known as the ancestor of wheat, was first cultivated in Karacadağ in Urfa 12 thousand years ago and spread all over the world from there (Zaharieva and Monneveux, 2014). In a study conducted on sowing seed rate in wheat at Tekirdağ and Edirne locations, spike length, number of grains per spike and weight decreased significantly in the 600 seed m^{-2} application with the highest sowing seed rate, while spike length, number of grains per spike and weight were higher in the 300 seed m^{-2} application compared to other sowing seed rate applications (Balkan et al., 2024). In recent years, due to the increasing effect of global climate change, the effects of biotic and abiotic stresses have become more intense in many regions. Increasing temperatures cause more frequent droughts and an increase in diseases and pests. In addition, the increasing desire for healthy nutrition among consumers increases the interest and demand for natural, organic and traditional products, and there are opinions that wild and local plants should be taken into consideration more.

Most of the modern varieties, which have been bred by using different breeding techniques, can reach these yields with high input use. In addition, these modern varieties are easily affected by changing climatic conditions, which may pose risks for sustainable agriculture and sustainable food. Wild plants are genotypes that have adapted to local conditions for many years and are therefore more resistant to abiotic and biotic stresses. These plants have adapted to various climatic conditions through natural selection and

traditional agricultural practices. Increasing demands on food security and sustainability are fuelling renewed interest in local plants and local genetic diversity. Local genotypes can reduce the need for chemical and artificial fertiliser use, which increases environmental sustainability. In a study on local bread wheat populations, it was determined that local bread wheat varieties are important genetic resources in terms of quality and have significant potential for use in breeding programmes (Mut et al., 2024). In a study, pioneer farmer/consumer communities in Çanakkale, Balıkesir and Kars provinces and some community-supported agriculture groups operating in İzmir province in the dissemination of local wheat varieties and bread and other products were examined. As a result of the study, it was found that understanding the production process in consumer groups and identifying problems related to consumption through focus group studies firstly increased the consumption of local wheat products (Yıldız and Özkaya, 2024). Kaydan and Yağmur (2008) examined grain yield and yield components on 16 bread wheat varieties under Van conditions in 2005-2006 and 2006-2007 production periods for two years. They determined that grain yield was positively correlated with yield components such as number of grains per spike and spike length. In a study, yield factors such as number of spike per square metre and number of grains per spike also contributed to the increase in harvest index (Öztürk and Korkut, 2018). In wheat breeding, number of grains per spike is one of the important yield factors considered in selection. Therefore, it is of great importance to increase one or more of these yield elements in order to obtain higher yield from unit area (Toklu and Yağbasanlar, 2005). The aim of this study was to determine the effect of different sowing norms and nitrogen doses on spike traits in wild wheat varieties, mirabile wheat, einkorn wheat and NKU Zirve bread wheat variety developed by combination breeding and to reveal the potential of spike traits for high productivity in agricultural production. It is aimed that the data to be obtained as a result of the study will be the basis for future researches, interpretations, inferences and studies.

2. Materials and Methods

2.1. Materials

In the research, einkorn wheat was obtained from the Central Research Institute of Field Crops, mirabile wheat and NKU Zirve wheat were obtained from Tekirdağ Namık Kemal University, Faculty of Agriculture, Department of Field Crops. Experimental sowing was carried out on 10.12.2022 in 2022-2023 and 10.12.2023 in 2023-2024, and harvesting was carried out on 16.07.2023 and 25.07.2024. The trials, which were planned to form sub-plots of sowing densities (400, 450, 500, 550 seeds m^{-2}) and nitrogen doses (10, 15 and 20 kg da^{-1}), were carried out in two years (2022- 2023 and 2023-2024 growing period) with 3 replications according to the split plots experiment design divided in randomised blocks. In the trials, sowing was done with a plot drill on plots of 6.12 m^{-2} (6 rows of 6 metres in length and 17 cm between row spacing) and 5.1 m^{-2} area was harvested at harvest. Researches revealed that 16 kg da^{-1} pure nitrogen should be applied in wheat cultivation in Thrace Region in order to reach the potential wheat yield of the region (Güçdemir, 2006). From this point of view, 3 different nitrogen doses were applied in the experiment. 5 kg of the nitrogen doses was given to all plots as base fertiliser with 20.20.0 compound fertiliser with sowing. The remaining pure nitrogen applications were applied as top fertiliser in 4 different times by considering the growth and development periods and rainfall on the Zadoks scale (Table 1).

Table 1. Fertilization applicatipons

Applications	Sowing	Beginning of tillering (Zadoks 21)	The End of tillering (Zadoks 25)	The End of the stem elongation (Zadoks 37)
10	5 kg da^{-1} pure N	5 kg da^{-1} pure N	-	-
15	5 kg da^{-1} pure N	6 kg da^{-1} pure N	4 kg da^{-1} pure N	-
20	5 kg da^{-1} pure N	7 kg da^{-1} pure N	4 kg da^{-1} pure N	4 kg da^{-1} pure N

The first top fertilisation was applied at the beginning of tillering (Zadoks 21st period), the second top fertilisation was applied at the end of tillering (Zadoks 25th period) and the third top fertilisation was applied at the end of spike emergence (Zadoks 37th period). Herbicide application was made for weed control. No herbicide application was made on einkorn wheat. Weed control in einkorn wheat was carried out manually.

2.2. Statistical Analyses

The data obtained from the experiment were subjected to analysis of variance using TARIST computer program according to the Split Plots Experimental Design. The mean values of the traits examined in the experiment was compared using Duncan test.

3. Results

3.1. Spike Length

Analysis of variance was performed with the values related to the spike length trait of the genotypes, and when the results of the analysis of variance were analysed, the difference between the averages of year, genotypes, genotype x seed rate interaction, seed rate x nitrogen dose interaction and genotype x seed rate x nitrogen dose interaction was found statistically

significant at 0.01% level, and the difference between seed rate, nitrogen dose and genotype x nitrogen dose interaction was found statistically significant at 0.05% level. Genotype, seed rate, dose averages and significance groups for spike length are given in Table 2.

Table 2. Genotype, seed rate and dose averages and significance for spike length

Genotype	Average	Seed rate	Average	Nitrogen Dose	Average
Einkorn	5.75 b	400	7.85 ab	10	7.73 b
Mirabile Wheat	8.56 a	450	8.06 a	15	7.95 a
NKU Zirve	9.22 a	500	7.83 ab	20	7.86 ab
		550	7.64 b		

The highest spike length was measured in NKU Zirve variety with 9.22 cm, followed by mirabile wheat with 8.56 cm and einkorn wheat with 5.75 cm. Statistically, NKU Zirve and mirabile wheat were in the same group and einkorn wheat was in different statistical group. The highest spike height in different sowing rate was 450 seed m^{-2} with 8.06 cm, followed by 400 and 500 seed m^{-2} sowing seed rate with 7.85 cm and 7.83 cm. In terms of nitrogen doses, the highest spike height was measured with 15 kg da^{-1} with 7.95 cm. The lowest nitrogen dose was 7.73 cm at 10 kg da^{-1} pure nitrogen dose application. The values and significance groups of genotype x seed rate, genotype x dose, seed rate x dose and genotype x seed rate x dose interactions for spike length are given in Table 3.

Table 3. Genotype x seed rate, genotype x dose, seed rate x nitrogen dose and genotype x seed rate x nitrogen dose interaction averages and significance

Genotype	Seed rate	Nitrogen Dose			Genotype x seed rate int.
		10	15	20	
Einkorn	400	5.63 jk	6.28 ıj	6.02 jk	5.78 ef
	450	5.90 jk	6.20 j	6.00 jk	6.03 e
	500	5.40 jk	5.55 jk	5.22 k	5.39 f
	550	5.52 jk	5.70 jk	5.64 jk	5.62 ef
Genotype x Dose int.		5.61 e	5.93 d	5.72 de	
Mirabile Wheat	400	8.37 efg	8.95 c-f	9.07 b-f	8.79 bc
	450	8.73 def	9.58 a-d	9.02 b-f	9.11 abc
	500	8.41 efg	8.94 c-f	8.18 fg	8.51 c
	550	7.71 gh	7.04 hı	8.74 def	7.83 d
Genotype x Dose int.		8.75 b	8.63 b	8.30 c	
NKU Zirve	400	9.06 b-f	9.06 b-f	8.82 c-f	8.98 abc
	450	8.61 ef	8.90 c-f	9.65 abc	9.05 abc
	500	8.81 c-f	10.08 a	9.84 ab	9.57 a
	550	9.56 a-d	9.14 b-e	9.12 b-e	9.27 ab
Genotype x Dose int.		9.27 a	9.29 a	9.10 a	

Genotype x Seed Rate X Nitrogen Dose Int.	Nitrogen Dose		
Seed rate	10	15	20
400	7.69 bc	7.90 abc	7.97 ab
450	7.75 abc	8.19 a	8.22 a
500	7.88 abc	8.19 a	7.41 c
550	7.60 bc	7.83 abc	7.49 bc

In einkorn wheat, the highest spike length was measured at 450 seed m^{-2} seed rate with 6.03 cm, followed by 400 seed m^{-2} seed rate with 5.78 cm and 550 seed m^{-2} seed rate with 5.62 cm. The highest spike length of mirabile wheat genotype was measured at 450 seed m^{-2} seed rate with 9.11 cm, followed by 400 seed m^{-2} seed rate with 8.79 cm and 500 seed m^{-2} seed rate with 8.51 cm. In NKU Zirve wheat genotype, the highest spike length was measured at 500 seed m^{-2} seed rate with 9.57 cm, followed by 550 seed m^{-2} with 9.27 cm and 450 seed m^{-2} seed rate with 9.05 cm. In NKU Zirve bread wheat genotype, the highest spike height was measured at 15 kg da^{-1} pure nitrogen dose with 9.29 cm and the lowest was at 20 kg da^{-1} pure nitrogen dose with 9.10 cm. In mirabile wheat genotype, the highest spike length was 8.75 cm at 10 kg da^{-1} pure nitrogen dose and the lowest was 8.63 cm at 15 kg da^{-1} pure nitrogen dose. In einkorn wheat genotype, the highest spike length was 5.93 cm at 15 kg

da^{-1} pure nitrogen dose and the lowest was 5.61 cm at 10 kg da^{-1} pure nitrogen dose. The highest spike length of 8.22 cm was measured at 450 seed m^{-2} sowing density and 20 kg da^{-1} pure nitrogen application. The lowest spike length of 7.41 cm was measured at 500 seed m^{-2} sowing seed rate and 20 kg da^{-1} pure nitrogen application. These results obtained in the experiment show that the highest spike length is obtained in NKU Zirve bread wheat genotype when 500 seeds m^{-2} and 15 kg da^{-1} pure nitrogen application per decare. The highest spike length was obtained with 450 seeds m^{-2} and 15 kg da^{-1} pure nitrogen application in mirabile wheat, and the highest spike length was obtained with 400 seeds m^{-2} and 15 kg da^{-1} pure nitrogen application in einkorn wheat genotype. When the genotype x seed rate x nitrogen dose interactions were analysed, the highest spike height with 10.08 cm and 9.84 cm was obtained in 500 seed m^{-2} and 15 kg da^{-1} and 20 kg da^{-1} pure nitrogen applications of NKU Zirve genotype, and the lowest spike height with 8.61 cm was obtained in 450 seed m^{-2} and 10 kg da^{-1} pure nitrogen application. The highest spike length of mirabile wheat genotype was 9.58 cm in 450 seed m^{-2} and 15 kg da^{-1} pure nitrogen application, the lowest spike length was 7.04 cm in 550 seed m^{-2} and 15 kg da^{-1} pure nitrogen application. In einkorn genotype, the highest spike length was 6.28 cm in 400 seed m^{-2} and 15 kg da^{-1} pure nitrogen application and the lowest spike length was 5.22 cm in 500 seed m^{-2} and 20 kg da^{-1} pure nitrogen application plots.

3.2 Number grains per spike

The number of grains per spike, which is one of the important main yield factors in wheat, is under the influence of environmental and genetic factors. There is a positive and significant relationship between grain yield and number of grains per spike. During the development of the plant, environmental factors also affect spikelet development, pollination and fertilisation (Polat et al., 2015). It was reported that there is generally a positive interaction between the number of grains per spike and grain yield (Mutlu, 2021). Variance analysis was performed with the values related to the number of grains per spike trait of wheat genotypes, and when the results of variance analysis were analysed, year, genotypes, genotype x seed rate interaction, genotype x nitrogen dose interaction, seed rate x nitrogen dose interaction and genotype x seed rate x nitrogen dose interaction were found statistically significant at 0.01% level. Genotype, seed rate, nitrogen dose averages and significance for the number of grains per spike trait are given in Table 4.

Table 4. Genotype, seed rate and nitrogen dose averages and significance for the number of grains in the spike

Genotype	Average	Seed rate	Average	Nitrogen dose	Average
Einkorn	21.34 b	400	41.38	10	39.86
Mirabile Wheat	50.87 a	450	43.33	15	41.00
NKU Zirve	49.74 a	500	39.83	20	41.09
		550	38.06		

The highest number of grains was counted in mirabile wheat with 50.87 grains, followed by NKU Zirve genotype with 49.74 grains and einkorn wheat with 21.4 grains. The highest seed rate average was 43.33 with 450 seeds m^{-2} and the lowest was 38.06 with 550 seeds m^{-2}. The highest number of grains in the spike was measured in 20 kg da^{-1} application with 41.09 grains. The lowest nitrogen dose average was found in 10 kg da^{-1} pure nitrogen dose application with 39.86 grains. Genotype x seed rate, genotype x nitrogen dose, seed rate x nitrogen dose, seed rate x nitrogen dose and genotype x seed rate x nitrogen dose interaction averages and significance are given in Table 5. In NKU Zirve bread wheat genotype, the highest number of grains per spike was 54.18 at 500 seed m^{-2} seed rate, followed by 450 seed m^{-2} seed rate with 52.20 and 550 seed m^{-2} seed rate with 47.25. In mirabile wheat genotype, the highest number of grains per spike was at 400 seed m^{-2} seed rate with 58.36, followed by 450 seed m^{-2} seed rate with 54.73 and 550 seed m^{-2} seed rate with 45.95. In einkorn wheat genotype, the highest number of grains per spike was 23.07 at 450 seed m^{-2} seed rate, followed by 550 seed m^{-2} seed rate with 20.97 and 500 seed m^{-2} seed rate with 20.87. In NKU Zirve bread wheat genotype, the highest number of grains per spike was measured at 20 kg da^{-1} pure nitrogen dose with 50.41, followed by 15 kg da^{-1} pure nitrogen dose application with 50.38. The highest number of grains in the spike was 53.44 with 10 kg da^{-1} pure nitrogen dose, followed by 50.60 with 15 kg da^{-1} and 48.58 with 20 kg da^{-1} pure nitrogen dose for mirabile wheat. In einkorn wheat genotype, the highest number of grains in the spike was 22.02 with 15 kg da^{-1} pure nitrogen dose, followed by 21.41 with 20 kg da^{-1} pure nitrogen dose. When the genotype x seed rate x nitrogen dose interaction was analysed, the highest number of grains in the spike was obtained with 63.89 grains in 400 seed m^{-2} and 15 kg da^{-1} pure nitrogen application of mirabile wheat. This was followed by 450 and 400 seeds m^{-2} and 20 kg da^{-1} and 10 kg da^{-1} pure nitrogen application with 58.76 and 57.97. The lowest number of grains per spike was 18.64 in 400 seed m^{-2} and 15 kg da^{-1} pure nitrogen application of einkorn wheat, followed by 19.90 grains per spike obtained in 500 seed m^{-2} and 10 kg da^{-1} pure nitrogen application of einkorn wheat. The highest number of grains per spike was 63.89 in 400 seeds m^{-2} and 15 kg da^{-1} pure nitrogen application, followed by 58.76 450 seed m^{-2} and 57.97 in 400 seeds m^{-2} and 20 kg da^{-1} and 10 kg da^{-1} pure nitrogen application. The lowest

number of grains in the spike was 35.76 in 550 seed m^{-2} application with 15 kg da^{-1} pure nitrogen in mirabile wheat.

In einkorn wheat genotype, the highest number of grains per spike was 24.75 in 450 seed m^{-2} and 15 kg da^{-1} pure nitrogen application, followed by the value obtained in 550 seed m^{-2} and 15 kg da^{-1} pure nitrogen application with 22.52. The lowest number of grains in the spike was 18.64 grains in the spike obtained from 400 seeds m^{-2} and 15 kg da^{-1} pure nitrogen. In NKU Zirve bread wheat genotype, the highest number of grains per spike was 58.38 at 500 seeds m^{-2} and 10 kg da^{-1} pure nitrogen application, followed by 56.83 and 55.70 at 500 and 450 seeds m^{-2} and 15 and 20 kg da^{-1} pure nitrogen application. The lowest number of grains in the spike was 44.08 with 400 seeds m^{-2} and 15 kg da^{-1} pure nitrogen application.

Table 5. Genotype x seed rate, genotype x nitrogen dose, seed rate x dose and genotype x seed rate x nitrogen dose interaction averages and significance

Genotype	Seed rate	Nitrogen Dose			Genotype x seed rate int.
		10	15	20	
Einkorn	400	20.32 ı	18.64 ı	22.43 ı	20.46 d
	450	22.15 ı	24.75 ı	22.32 ı	23.07 d
	500	19.90 ı	22.16 ı	20.57 ı	20.87 d
	550	20.04 ı	22.52 ı	20.34 ı	20.97 d
Genotype x Dose int.		20.60 c	22.02 c	21.41 c	
Mirabile Wheat	400	57.97 ab	63.89 a	53.23 bcd	58.36 a
	450	55.66 bc	56.40 bc	58.76 ab	54.73 ab
	500	37.35 gh	46.35 def	52.13 b-e	44.44 c
	550	43.34 fg	35.76 h	49.62 c-f	45.95 bc
Genotype x Dose int.		53.44 a	50.60 ab	48.58 b	
NKU Zirve	400	46.54 def	44.08 f	45.34 ef	45.32 bc
	450	50.52 c-f	50.38 c-f	55.70 bc	52.20 abc
	500	58.38 ab	56.83 bc	47.33 def	54.18 abc
	550	46.21 def	50.21 c-f	45.33 ef	47.25 bc
Genotype x Dose int.		48.42 b	50.38 ab	50.41 ab	

Genotype x Seed Rate X Nitrogen Dose Int.	Nitrogen Dose		
Seed rate	10	15	20
400	41.61 abc	42.20 abc	40.33 a-d
450	42.77 ab	43.84 a	43.38 a
500	38.54 cde	41.78 abc	39.17 b-e
550	36.53 de	36.16 e	41.48 abc

3.3. Grain Weight in the Spike

Grain weight in spike, which is the result of many yield components that occur in early developmental stages of wheat, is one of the main yield factors affecting yield. Grain weight per spike plays an important role in yield formation as it directly affects the harvest index. Grain weight per plant is a direct indicator of the efficient utilisation of nutrients and their transport to the storage parts of the plant (Borojevic, 1983). Since grain yield is a combined quantitative trait under the influence of many traits and environmental conditions, grain weight per spike should be increased in order to achieve high grain yield (Syme, 1972). Variance analysis was carried out with the values related to grain weight in spike of wheat genotypes and when the results of variance analysis were analysed, the difference between year, genotypes, genotype x seed rate x nitrogen dose interactions and genotype x seed rate interactions were found statistically significant at 0.01% level. The difference between seed rates was statistically significant at 0,05% level. Genotype, seed rate, nitrogen dose averages for grain weight in spike trait are given in Table 6.

The highest spike grain weight was measured in NKU Zirve genotype with 2.08 g, followed by mirabile wheat with 1.64 g and einkorn wheat with 0.53 g. The highest spike grain weight was measured in 400 seed m^{-2} with 1.48 g, followed by 450 and 500 seed m^{-2} sowing seed rate with 1.46 g and 1.45 g, respectively. The highest average grain weight in the spike was 1.43 g at 15 and 20 kg da^{-1} pure nitrogen dose treatments. The lowest nitrogen dose average was 1.39 g in 10 kg da^{-1} pure nitrogen dose application. Genotype x seed rate, genotype x nitrogen dose, seed rate x nitrogen dose and seed rate x dose interaction averages and significance for

grain weight in spike are given in Table 7.

Table 6. Genotype, seed rate, nitrogen dose averages and significance for grain weight in the spike

Genotype	Average	Seed rate	Average	Nitrogen Dose	Average
Einkorn	0.53 b	400	1.48 a	10	1.39
Mirabile Wheat	1.64 a	450	1.46 a	15	1.43
NKU Zirve	2.08 a	500	1.45 a	20	1.43
		550	1.28 b		

Table 7. Seed rate x nitrogen dose and genotype x seed rate x nitrogen dose interaction averages and significance

Genotype	Seed rate	Nitrogen Dose			Genotype x Seed rate
		10	15	20	
Einkorn	400	0.58 ı	0.43 ı	0.61 ı	0.54 f
	450	0.59 ı	0.64 ı	0.58 ı	0.61 f
	500	0.43 ı	0.54 ı	0.49 ı	0.49 f
	550	0.51 ı	0.56 ı	0.42 ı	0.49 f
Genotype x Dose int.		0.53	0.54	0.53	
Mirabile Wheat	400	2.12 bcd	2.17 a-d	1.84 c-f	2.05 ab
	450	1.62 efg	1.91 c-f	1.51 fg	1.68 cd
	500	1.28 gh	1.57 efg	1.65 efg	1.50 de
	550	1.33 gh	1.07 h	1.59 efg	1.33 e
Genotype x Dose int.		1.59	1.68	1.65	
NKU Zirve	400	1.83 c-f	1.75 def	1.97 cde	1.85 bc
	450	1.93 cde	1.95 cde	2.40 ab	2.09 ab
	500	2.53 ab	2.38 ab	2.19 abc	2.37 a
	550	1.93 cde	2.13 a-d	1.94 cde	2.00 bc
Genotype x Dose int.		2.05	2.05	2.12	

Genotype x Seed Rate x Nitrogen Dose Int.	Nitrogen Dose		
Seed rate	10	15	20
400	1.51	1.45	1.48
450	1.38	1.50	1.50
500	1.41	1.49	1.45
550	1.26	1.26	1.31

In NKU Zirve bread wheat genotype, the highest spike grain weight was measured at 500 seed m^{-2} seed rate with 2.37 g, followed by 450 seed m^{-2} with 2.09 and 550 seed m^{-2} seed rate with 2.00. In mirabile wheat genotype, the highest spike grain weight was measured at 400 seed m^{-2} seed rate with 2.05 g, followed by 450 seed m^{-2} seed rate with 1.68 g and 500 seed m^{-2} seed rate with 1.50 g. In einkorn wheat genotype, the highest spike grain weight was measured at 450 seed m^{-2} seed rate with 0.61 g, followed by 400 seed m^{-2} with 0.54 g, 550 and 500 seed m^{-2} seed rate with 0.49 g. In NKU Zirve bread wheat genotype, the highest spike grain weight was measured at 20 kg da^{-1} pure nitrogen dose with 2.12 g, followed by 10 and 15 kg da^{-1} pure nitrogen dose treatments with 2.05 g. The highest spike grain weight was measured at 15 kg da^{-1} pure nitrogen dose with 1.68 g in mirabile wheat genotype and the highest spike grain weight was measured at 15 kg da^{-1} pure nitrogen dose with 0.54 g in einkorn wheat. When these results obtained in the experiment were analysed, the highest grain weight in spike was obtained in NKU Zirve bread wheat genotype when 500 seeds m^{-2} and 20 kg da^{-1} pure nitrogen was applied. Decreases in nitrogen dose caused a decrease in grain weight in spike in NKU Zirve wheat genotype. It was determined that the grain weight in the spike decreased due to the long plant height of mirabile wheat at 500 seeds m^{-2} and above, and the grain weight in the spike did not increase when a fertiliser application above 15 kg pure nitrogen was applied per decare. It is seen that 400 seed m^{-2} density and 15 kg da^{-1} pure nitrogen

application is sufficient for mirabile wheat genotype. In einkorn wheat genotype, the highest spike grain weight was obtained at 450 seed m^{-2} and 15 kg da^{-1} pure nitrogen application. When the genotype x seed rate x nitrogen dose interaction was analysed, the highest grain weight in the spike with 2.53 g and 2.40 g was obtained in NKU Zirve genotype with 500 and 450 seeds m^{-2}, 10 and 20 kg da^{-1} pure nitrogen applications. These were followed by 500 seeds m^{-2} and 15 kg da^{-1} pure nitrogen applications of NKU Zirve genotype with 2.38 g in the same statistical group. The lowest spike grain weight of NKU Zirve genotype was 1.75 g in 400 seed m^{-2} and 15 kg da^{-1} pure nitrogen application. This was followed by the grain weight of NKU Zirve genotype with 1.83 g obtained in 400 seed m^{-2} and 10 kg da^{-1} pure nitrogen application. The highest grain weight of mirabile wheat genotype was 2.17 g in 400 seeds m^{-2} and 15 kg da^{-1} pure nitrogen application, followed by 2.12 g and 1.91 g in 400 and 450 seeds m^{-2} and 10 kg da^{-1} and 15 kg da^{-1} pure nitrogen application. The lowest spike grain weight was obtained from 550 seeds m^{-2} with 1.07 g in 15 kg da^{-1} pure nitrogen application. In einkorn wheat genotype, the highest spike grain weight was 0.64 g in 450 seed m^{-2} and 15 kg da^{-1} pure nitrogen application, followed by the values obtained in 400 seed m^{-2} and 20 kg da^{-1} pure nitrogen application with 0.61 g. The lowest spike grain weight was obtained from 550 seeds m^{-2} and 20 kg da^{-1} pure nitrogen application with 0.42 g.

4. Discussion

Spike length is one of the important morphological traits affecting the yield potential of wheat. Long spike length is generally associated with higher grain number and thus higher yield potential, while short spike length is generally associated with lower grain number and yield. The long spike length of NKU Zirve genotype makes this genotype advantageous in terms of grain number and therefore yield potential. The short spike length of einkorn wheat shows that this genotype has a limited structure in terms of grain number and yield potential. Short spike length is generally associated with low grain number and therefore low yield. Grain number is an important parameter that directly affects the yield potential of wheat. High grain number is generally associated with higher yield, while low grain number indicates that yield potential is limited. In addition to the high number of grains per spike in mirabile wheat, the effect of grain number on yield also depends on other factors such as grain weight and fullness. If the grains are not small and plump, high grain number may not always mean high yield. Mirabile wheat genotype can be included in breeding studies due to its high grain number. The low number of grains per spike of einkorn wheat shows that this genotype has a limited structure in terms of yield potential. Grain weight in the spike is an important parameter that directly affects the yield potential and quality characteristics of wheat. High grain weight is generally associated with plump and high-quality grains, while low grain weight causes a limited potential in terms of yield and quality. The high grain weight per spike of NKU Zirve genotype indicates that this genotype produces plump and heavy grains. Although the number of grains per spike is high in spelt wheat, the grain weight is not at the desired level, which is due to the insufficient development of the grains. The low grain weight of einkorn wheat shows that this genotype should be preferred for more quality-oriented production and can be evaluated in terms of its adaptation to traditional and low-input agricultural systems. In a study conducted on 4 bread wheat cultivars in Bangladesh during the 2004-2005 growing period, it was shown that nitrogen applications made by division had a significant effect on spike length and number of grains per spike (Ferdous et al., 2005). In a study aiming to determine some morphological and biological traits in einkorn wheat depending on nitrogen fertilisation level, it was found that nitrogen fertilisation had significant effects on spike length, number of grains in spike and spike grain weight in einkorn wheat. In addition, it is understood that there is a positive relationship between different nitrogen levels and plant growth (Kirchev and Semkova, 2016). In a study conducted in Pakistan in 2015-2016 growing period, the effects of applying nitrogen doses at different times on spike length and spike weight were found statistically significant (Shahzad and Akmal, 2017).

5. Conclusion

The highest spike length was measured in the NKU Zirve genotype with 9.22 cm, and the einkorn wheat genotype with 5.75 cm was the genotype with the lowest spike length among the wheat genotypes tested. The fact that the spike length of the NKU Zirve bread wheat genotype is higher than that of the einkorn and mirabile wheat genotypes shows that it has a high potential in terms of grain number and yield potential in the spike, while the einkorn wheat genotype with a short spike length has a limited structure in terms of grain number and yield potential. It was determined that the number of grains per spike of the mirabile wheat was higher than that of the NKU Zirve bread wheat genotype. It was determined that the number of grains per spike decreased when the mirabile wheat genotype reached 400 seeds m-2 and above due to its long plant height. The highest grain number was counted in the mirabile wheat with 50.87 grains, and the lowest grain number per spike was obtained from the einkorn wheat genotype with 21.34. It has been determined that genotypes with high grain counts may provide an advantage in terms of yield potential, but other factors such as grain fullness and weight should also be taken into consideration, as in mirabile wheat. It has been determined that the grain weight measured in the spike in the NKU Zirve wheat genotype is 4 times higher than the einkorn genotype.

Decreases in nitrogen dose caused a decrease in the grain weight in the spike in the NKU Zirve wheat genotype. It has been determined that the grain weight in the spike decreases when the mirabile wheat genotype reaches 500 seeds m-2 and above due to its long plant height. It is observed that genotypes with high grain weight provide an advantage in terms of yield potential, but other factors should also be taken into consideration.

Author Contributions

The percentages of the author' contributions are presented below. All author reviewed and approved the final version of the manuscript.

	M.A.	İ.B.
C	50	50
D	50	50
S	50	50
DCP	50	50
DAI	50	50
L	50	50
W	50	50
CR	50	50
SR	50	50
PM	50	50
FA	50	50

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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PERFORMANCE ANALYSIS OF AGRICULTURE, LIVESTOCK AND HUNTING SECTOR FIRMS USING PIOTROSKI F-SCORE METHOD

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Abstract: The agricultural sector is critical in providing nutrition, which is one of the basic needs of the world population. The financial performance of the industry, which has been affected by global climate changes in recent years and is sensitive to social, economic, and technological risks, is a point that should be emphasized for investors and countries in terms of sectoral development. This study will discuss the financial performance of agriculture, livestock and hunting sector firms traded in Borsa Istanbul (BIST) between 2020-2023 through the Piotroski F Score. Piotroski F Score calculations used nine financial indicators showing the firms' profitability, operating efficiency, leverage and liquidity status. In the research on four firms listed on the BIST, averages were calculated and grouped by scoring from 9 to 1. As a result of the Piotroski F Score method, agricultural sector firms differed yearly. In general, according to the Piotroski F scores of the firms, their financial performance was at a medium level, and they were worth investing in. It can be said that only one of the two firms has a strong financial position in two years and the other in one year, while the financial position of the other firm is at a medium level in all years during the analysis period. In the analysis, it was determined that the top three financial indicators of the firms according to the F score method are the return on assets, the change in return on assets and the change in the current ratio. Conversely, earnings quality and the change in leverage ratios are the least successful indicators. Also, their asset turnover rates are low.

Keywords: Financial performance, Agriculture sector, Piotroski F-score, Borsa İstanbul

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1. Introduction

Agriculture is one of the most important sectors that maintains its importance worldwide. Although it is a sector supported and protected by all countries to meet food needs, it has a relatively low share compared to the industry and services sector in the world. According to the 2024 Statistical Yearbook published by the Food and Agriculture Organisation of the United Nations (FAO), although the global agricultural value has increased by 89% in real terms in the last two decades and reached USD 3.8 trillion in 2022, the contribution of agriculture to global economic output has remained relatively constant. The proportion of the worldwide workforce employed in agriculture has decreased from 40% in 2000 to 26% in 2022 (www.fao.org). According to the report, further investment in agri-food systems is considered necessary to ensure malnutrition and food security, which continue to be a problem affecting a significant portion of the world's population. The agricultural sector also contributes to employment and a substantial portion of national income (Özbay, 2024). Therefore, to survive efficiently, it needs to be developed and financed by more investors than it has. The Gross Domestic Product (GDP) ratios of the agriculture, forestry, and fisheries sectors in Türkiye stated that the agricultural sector's contribution to GDP was 6.7% in 2020, which decreased to 5.5% in 2021, 6.5% in 2022, and 6.2% in 2023. It is seen that the share of the agricultural sector in the Turkish economy has decreased over the years. The agricultural sector's growth figures, while the industry's growth rate was 5.8% in 2020, decreased to -3% in 2021, 1.3% in 2022 and -0.2% in 2023 (www.tarımorman.gov.tr.). It can be said that there is a similar decline in employment figures in the agricultural sector. While the 2020 agricultural sector employment rate was 17.8%, this figure declined over the years and decreased to 14.8% in 2023 (TURKSTAT, 2023). On the other hand, The World Bank supports the financing of the agricultural sector and attaches importance to agricultural loans among its loans. In 2022, the Bank provided Türkiye with a loan of 341.27 million USD to support the sustainable agrarian sector (tarimorman.gov.tr). In the agricultural sector, reasons such as high costs and long production periods, being rapidly affected by seasonal fluctuations and loss of income reduce the capital turnover rate. This situation may cause an increase in firms' short-term debt. Although the sector is supported, access to finance, industry development, provision of funds, and continuity of production are problems for firms (Şahin, 2025). Financial systems in developing countries are insufficient

to finance vital changes such as the transition to sustainable agriculture. Banks, microfinance institutions, and institutional investors provide limited resources to the sector. Agricultural loans and investment portfolios are disproportionately lower than agriculture's GDP share (www.worldbank.org). Risks specific to the agricultural industry, limited effective demand for financing, and lack of expertise of financial institutions in managing agricultural loan portfolios limit the source of investors and funds in the sector. Assessing financial performance and predicting failures is crucial for business owners, managers, investors, and lenders. Therefore, this study aims to analyze the financial performance of firms with service activities related to Agriculture, Livestock, and Hunting listed in Borsa Istanbul (BIST) in Türkiye. For this purpose, financial analysis was carried out using the Piotroski F-score method with the help of annual data obtained from the Public Disclosure Platform (PDP) between 2020-2023 and various ratios. In this way, the sector's financial performance will be revealed, and firms worth investing in will be identified. The study's significant limitation is the data limitation due to the small number of agricultural sector firms traded on the BIST. Financial performance is an indicator that shows the effectiveness of a firm's activities. Business performance is considered when estimating the firm's current investments and the sources of funds provided for investments, capital structure, and competitiveness in investment decisions. Predicting the financial performance and strength of the firms and making investment decisions or determining investment strategies are factors that should be considered by managers, shareholders, and potential investors. Analyzing the information presented in the firm's financial statements provides information about the firm, such as its profitability, liquidity, borrowing structure, activities, and cash flow to those inside and outside the firm. As a result of the ratio analyses made with this information, existing / potential investors who have information about the firm's financial performance take action. Although accounting-based financial ratio calculations provide information about the firm's financial soundness, the combined evaluation of accounting data and stock performance is among the methods used. Piotroski F-Score, developed by Joseph Piotroski (2000), is a score that measures the financial condition of a firm. Based on accounting signals, this scoring distinguishes between firms with good and bad fundamental scores. Piotroski argued that the information in financial statements is useful for selecting good companies for reasons such as the tendency of firms to be ignored by analysts, the low credibility of companies given the poor recent information they announce to the market, and the tendency of firms to be in financial distress (Piotroski, 2000). In the calculation made over nine indicator variables that examine the firm's financial performance, the firm is scored from 0 to 9. Nine (zero) indicates a business with more (less) good signals and attractive investment opportunities (Piotroski, 2000).

2. Materials and Methods

2.1.Materials

In this study, the financial performance of 4 firms in the Agriculture, Livestock, and Hunting sectors traded in Borsa Istanbul for 2020-2023 were estimated using the data in the annual balance sheet, income statement, and cash flow statement. Financial statement data were obtained from the Public Disclosure Platform (www.kap.org.tr). Instead of using the names of the analyzed firms, it would be legally more appropriate to use abbreviations such as ENT (enterprise) -1, ENT-2, ENT-3, and ENT-4 in the following parts of the study. For these reasons, the results and evaluations regarding the firms in the following parts of the study will continue with these codes.

2.2. Method

Piotroski's F-score provides the opportunity to evaluate and compare the financial performance of firms based on nine different indicators. These nine indicators are categorized into three groups. In scoring as 1 or 0 for each indicator, 0 indicates the firms with the lowest financial performance, and 9 indicates the firms with the highest financial performance. In other words, as the F score approaches nine, the company's strength increases (Gökten et al., 2017). Table 1 shows the criteria for the method. The model that is formed by the indicators shown in Table 1 is presented below (Piotroski, 2000):

F-Score= ROA + CFO + EQ + ΔROA + ΔLEV + ΔCR + ΔPIC + ΔMARGIN + ΔAT Nine criteria can be categorized into three groups: profitability criteria, operating efficiency criteria, and change in solvency/liquidity criteria. The profitability criteria have four indicators: ROA is calculated as net profit divided by total assets. CFO is calculated by dividing the cash flows from the company's principal operating activities by the total assets. Change in ROA and EQ (earning quality is the difference between CFO and ROA). The operating efficiency criteria have two indicators: Change in gross margin (Gross profit margin is calculated by dividing gross profit by net revenue.) and change in asset turnover.

The change in solvency/liquidity criteria has three indicators: A change in leverage ratio (leverage ratio is calculated by dividing total debts by total assets), a change in current ratio (current ratio is calculated by dividing current assets by total short-term debt), and a change in paid-in capital.

Table 1. Piotroski F-score Evaluation Criteria*

No	Variable	Criteria
1	Return On Asset (ROA)	If ROA is positive, the score is 1
2	Cash Flow From Operations (CFO)	If CFO is positive, the score is 1
3	Earnings Quality (EQ)	If the difference between CFO and ROA is positive, the score is 1
4	Change in Return on Assets (ΔROA)	If ROA is greater than the previous year's ROA, the score is 1
5	Change in Leverage Ratio (ΔLEV)	If the ratio of total debt to assets is less than the previous year, the score is 1 (scoring 1 is also given if the company does not have debt even though the assets are increasing)
6	Change in Current Ratio (ΔCR)	If the current ratio increases compared to the previous year, the score is 1
7	Change in Paid-in Capital (ΔPIC)	If the amount of paid-in capital is more than the previous year, the score is 1
8	Change in Gross Margin (ΔMARGIN)	If the gross margin is higher than the previous year, the score is 1
9	Change in Asset Turnover (ΔAT)	If the asset turnover is higher than the previous year, the score is 1

*Source: Asmadi et al. (2021).

3. Results

In the analysis, calculations related to nine financial indicators between 2020 and 2023 in 4 firms listed in the agricultural sector in Borsa Istanbul were carried out by applying them in an Excel environment.

Each firm's F-score was calculated according to the criteria shown in Table 1. Table 2 shows the firms' F-score results. Table 2 shows that the F-score value of ENT-1 in 2021 and 2022 was 8. The firm's financial condition is good and highly worth considering for investment. However, the F-score of the firm in 2023 decreased to 3, and it can be said that its financial conditions have been weak in the recent period. The Piotroski F-scores of the ENT-2 in 2020 and 2023 were calculated as 6. It can be stated that the firm's financial condition is at a medium level and worthy of investment consideration (Asmadi et al.,2021). The lowest f-score value was determined as 3 in 2021, and it can be said that the firm's financial strength is weak and unsuccessful.

Tablo 2. Piotroski F-score of Agriculture Sector Firms

Indicator	ENT-1				ENT-2				ENT-3				ENT-4			
	2023	2022	2021	2020	2023	2022	2021	2020	2023	2022	2021	2020	2023	2022	2021	2020
ROA	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1
CFO	0	1	1	1	1	1	1	0	1	0	1	0	0	1	0	0
EQ	0	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0
ΔROA	0	1	1	0	1	1	0	1	1	0	1	0	1	1	1	1
ΔLEV	0	1	0	1	1	0	1	1	0	0	1	0	0	0	0	1
ΔCR	1	1	1	0	1	1	0	1	1	1	0	0	1	1	1	1
ΔPIC	1	1	1	0	0	1	0	1	1	0	1	0	0	0	1	0
ΔMARGIN	0	1	1	0	1	0	0	1	1	0	0	0	1	1	1	1
ΔAT	0	0	1	0	0	0	0	1	1	1	1	1	0	0	1	0
F SCORE	3	8	8	4	6	5	3	6	8	3	6	2	4	5	6	5

The ENT-3 had the highest F score (8) in 2023, and it was determined that it is financially strong and highly preferable for investors, while in 2020 and 2022, it had a low f score, and its financial situation was unsuccessful. Finally, it was observed that the F score values of the ENT-4 were 4-6 for the periods included in the analysis; in other words, the financial strength was moderately acceptable. When analyzed for financial indicators, the ROA of ENT-1, ENT-3, and ENT-4 were calculated as 1 in all analysis periods. On the other hand, the ROA ratio of ENT-2 was calculated as 0 in all periods. The CFO and EQ of ENT-1 took the value of 1 in all periods except 2023, the change in the current ratio and the change in paid-in capital ratios took the value of 1 in all years except 2020. On the other hand, the change in asset turnover was calculated as 0 in all periods except 2021. It is determined that ENT-1 has the highest F score value (8) for the years 2021 and 2022. In other words, according to the model, its financial situation is the strongest and the most investable years for investors. ENT-2, another

firm within the scope of the analysis, determined that the CFO and EQ indicators were calculated as 1 in all periods except for 2020. The indicators of change in return on assets, change in current ratio and change in leverage were also calculated as 1. On the other hand, the most unsuccessful indicator of ENT-2 was the change in asset turnover rate (ΔAT). In general, it was found that ENT-2 had a maximum F-score value of 6 in 2020 and 2023. It was determined that the firm had a moderate financial performance and was feasible for investment. The year 2021 had the lowest F-score value for ENT-2, and it can be said that the firm failed financially during this period. ENT-3, another company included in the analysis, was analyzed; the indicator that received a value of 1 for all periods was the change in asset turnover (ΔAT). The model found the EQ, ΔLEV, and ΔMARGIN indicators to be the most unsuccessful ENT-3 had the highest F-score value (8) in 2023; in other words, according to the model, its financial situation was the strongest and the most investable year for investors. On the other hand, in 2020 and 2022, it had the lowest F-score value. It was determined to be financially weak during these periods. The last company, ENT -4, was analyzed; the EQ indicator was calculated as 0 in all analysis periods. On the other hand, ΔROA, ΔCR, and ΔMARGIN were calculated as 1 in all periods. The firm's F-score values between 4 and 6 were observed. According to the model, the firm's performance was stated to have a moderate financial condition and was feasible for investment.

4. Discussion

This study analyzed the financial performances of four firms in the Agriculture, Livestock, and Hunting sectors traded on BIST between 2020 and 2023 using the Piotroski F-score method. Based on financial reports, the model calculates the firms' financial status using nine indicators collected under three headings. When the literature is examined, the Piotroski f-Score method is not found among the studies conducted to evaluate financial performance in the agricultural sector. The studies conducted to evaluate the sector's financial performance and those that include the method used in the research are given below. In the studies conducted in the Agriculture, Livestock, and Hunting sectors, Koç et al. (2016) analyzed the financial performance of 3 agricultural sector firms registered in BIST between 2010 and 2015. They conducted panel data analysis by calculating seven different ratios that they thought would be effective on Return on Assets (ROA) and Return on Equity (ROE) ratios. The results show that the change in asset turnover rate positively affects ROA and ROE compared to other ratios. According to the study, it is possible to increase the performance of agricultural sector firms in Türkiye using an effective asset turnover ratio. The results obtained from the study support this research. Similarly, Şahin (2025) made a financial analysis of the agricultural sector and a publicly traded sector enterprise in 2016-2022. In the study, in which the Central Bank of Türkiye's agriculture, forestry, and fisheries sector data were used, many ratios such as profitability, borrowing, activity, liquidity, cash flow, and growth rates were calculated. According to the study results, agricultural firms remained dependent on short-term borrowing due to low asset turnover, operational profitability, and difficulties in obtaining long-term financing. Besides, some studies have documented the financial structure of the agricultural sector. According to Ağızan and Bayramoğlu (2023), the liquidity and financial structures of the firms are strong and competitive, while their profitability is not at a sufficient level. Tiryaki and Kandil Göker (2021) examined the riskiness and financial structure of the sector with the ratios obtained from Central Bank annual financial reports between 2009 and 2019. They stated that short-term borrowing is high, creating a liquidity squeeze. It was noted that the agricultural sector has a low capital turnover rate and that seasonal conditions and lack of savings will create short-term financing needs. There are different studies on the factors affecting the financial performance of the agricultural sector. For instance, Odalo et al. (2016) examined the effect of firm size on agribusinesses' financial performance in the Nairobi Stock Exchange in Kenya, using ROA, ROE, and earnings per share ratios to measure financial performance. It was concluded that firm size has a positive effect on financial performance. Pokharel et al. (2019) compared financially stressed and non-stressed co-operatives using financial ratios to identify the causes of financial stress in 583 agricultural co-operatives in the United States and to make management-based recommendations to their shareholders, dividing financial stress between return on assets, leverage and interest rate issues and determining that the primary source of stress is a low real return on assets. Intan et al. (2024) examined the effects of endogenous and exogenous factors on the financial performance of 17 agribusinesses listed on the Indonesia Stock Exchange; ROA was used to measure financial performance. As a result, it was stated that the debt-to-asset ratio and exchange rate ratio are negatively related to ROA and that the ratio of production costs should be reduced. Previous studies suggested that the Piotorski F-score model performs well with different sectors. For instance, Duran-Vazguez et al. (2014) examined 63 firms in Mexico between 2005 and 2011 with Piotroski F-score management. As a result of the study in which the Ohlson Model was added to the scores obtained, it was determined that there were asymmetric signs in Piotroski score variables, and the Ohlson model provided explanatory power for Mexican data. Sasikala (2021) examined the risk of bankruptcy with the Altman Z-score, business trend distress with the Piotroski F-score, and earnings manipulation risk with Messod Beneish's M-score models between 2008 and 2017 for a single firm and stated that Piotroski's F-score can be used to detect the distress of industrial enterprises. Asmadi et al. (2021) analyzed the financial performance of 30 Sharia stocks in

Indonesia between 2017 and 2018. They found that a few stocks were worth investing in due to their excellent financial condition, while others had moderate financial conditions. Karadeniz and İskenderoğlu (2024a) analyzed the financial performance of health enterprises operating in BIST with the Piotroski F-score method. According to the study's results, which included four firms, the most substantial financial indicators were ROA and cash flows from operating activities. However, all companies failed regarding earnings quality indicators, and cash flows were lower than net profit. Abdioğlu and Aytekin (2024) revealed the financial determinants of manufacturing enterprises with the Piotroski F-score. The study found that Altmaz's Z-score, return on investment, market/book value ratio, and Tobin's Q ratio significantly affect the Piotroski score. Karadeniz and İskenderoğlu (2024b), examined the financial performance of football clubs using the Piotroski F-Score method. As a result of the research, clubs exhibit moderate financial strength, and the most successful indicators are changes in paid-up capital, earnings quality, and cash flows from operating activities; on the other hand, ROA, changes in leverage, and changes in MARGIN are the poorest financial indicators of the model. The Piotoroski F-score results of this study show that ENT 1 (2021-2022) and ENT-3 (2023) scored 8, indicating strong financial performance and investment potential. Therefore, it has been determined that these firms are suitable for investment and financially excellent. The Piotroski F score value of the ENT-4 was moderate for all analysis periods. This indicates that it has medium financial conditions and is worthy of consideration for investment. However, it can be stated that ENT-3 in 2020 and 2022, ENT-2 in 2021 and AGROT in 2023 are unsuccessful for investment due to their weak financial conditions. The weak financial performance of the firms in 2020 and 2021 may be due to the negative effects of the Covid 19 pandemic. In particular, ENT-1 was found to have the lowest F score value in 2023, unlike other firms in the sector. Although the change in the firm's ability to pay its short-term debts is high, indicators such as the change in return on assets, change in leverage ratio, and change in asset turnover rate are low. Low asset turnover, operating profitability, and insufficient long-term funding sources in the agricultural sector increase the sector's financial risk by leading to dependence on short-term borrowing (Şahin, 2025: 396).Generally, the firms listed in the BIST agriculture, livestock, and hunting sectors have medium-level financial performance and are worth evaluating for investment activities (Asmadi et al.,2021; Karadeniz, İskenderoğlu, 2024a; Karadeniz ve İskenderoğlu,2024b). Regarding the F Score value, ENT-4 shows the least fluctuation, while ENT-1 and ENT-3 show the highest fluctuation. According to the scope of the analysis, the top three financial indicators that all firms of this study are the strongest in, according to the F-score method, are ROA, change in the current ratio, and change in ROA. On the other hand, changes in earnings quality, leverage, and asset turnover ratio are found to be the three least effective financial indicators. It can be said that firms in the agricultural sector utilize their assets efficiently. The financing support provided to the sector, which does not have much difficulty paying its short-term debts, especially the loans provided by the World Bank, relieves the firms in the short term. Although it has been found in previous studies that the increase in the short-term borrowing rate increases the Piotoroski F Score value (Tepeli and Kahraman, 2023), the low change in the borrowing rate in the study indicates that firms in the agricultural sector resort to short-term borrowing due to the high use of foreign resources and limited access to long-term financing opportunities in the sector (Tiryaki and Göker, 2021; Şahin, 2025: 395). The results show that the decrease in asset turnover rate is not due to the firm's inability to use its assets effectively. The fact that the sector is adversely affected by seasonal movements, cost increases, loss of income and the decrease in growth over the years may negatively affect the change in asset turnover rate. Effective asset management, agricultural policies, and incentives are recommended to help the sector provide the resources it needs to finance assets and overcome the difficulties arising from financing.

5. Conclusion

Investors seeking stable returns should prioritize firms with consistently high Piotroski F-Scores (7-9). These firms exhibit strong profitability, liquidity, and operational efficiency. Moreover, monitoring changes in asset turnover and leverage ratios can help identify firms that are improving their financial health. For risk-averse investors, companies with profitability may also be worth considering. Diversifying across multiple agricultural firms can further mitigate sector-specific risks.

Author Contributions

Percentages of the author' contributions are present below. The author reviewed and approved final version of the manuscript.

	N.Y.E.
C	100
D	100
S	100
DCP	100
DAI	100
L	100
W	100
CR	100
SR	100
PM	100
FA	100

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The author declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because there was no study on animals or humans.

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PROPOLIS SUPPLEMENTATION IN BROILER BREEDER CHICKENS: ENHANCING MEAT QUALITY

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Abstract: This study aimed to evaluate the effects of dietary propolis supplementation on the meat quality of 35-week-old broiler breeder chickens. A total of 100 Ross 308 chickens were divided into five groups and supplemented with 0, 100, 200, 400, and 800 ppm of propolis in their diet. The study measured various meat quality parameters, including pH, color (L*, a*, b*), cooking loss, and drip loss over three storage periods (1, 4, and 7 days). Results showed that propolis supplementation influenced pH, with the P800 group exhibiting the highest pH value by day 7. Color parameters, particularly yellowness (b*), were significantly reduced in thigh meat in the 200 ppm group. Propolis supplementation, especially at 800 ppm, significantly reduced drip loss and cooking loss, improving water retention and texture in both breast and thigh meat. These findings suggest that propolis supplementation, particularly at 800 ppm, can improve meat quality by enhancing water-holding capacity and influencing color stability. The improved meat quality in terms of water retention and texture, makes propolis supplementation a promising option for enhancing the quality of processed poultry products, such as salami and sausages. Further research is needed to optimize the appropriate dosage for maximizing the benefits of propolis in poultry meat.

Keywords: Propolis, Meat quality, Drip loss, Cooking loss, Poultry

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1. Introduction

In recent years, the use of natural feed additives, particularly propolis, has gained significant attention due to its promising effects on animal health and meat quality. Propolis, a biologically active substance produced by bees from plant resins, is widely utilized in animal nutrition for its strong antimicrobial, antioxidant, and immunomodulatory properties. Numerous studies have investigated its effects on growth performance, immune function, and meat quality in broiler chickens (Zulhendri et al., 2021; Abdel-Maksoud et al., 2023, Sahin and Ozturk, 2018). However, while most research has focused on younger broilers (Mahmoud et al., 2016; Prakatur et al., 2020), the impact of propolis on the meat quality of broiler breeders, particularly at 35 weeks of age, remains largely unexplored.

Propolis is composed of flavonoids, aromatic acids, and phenolic compounds such as galangin and pinocembrin (Koohsar et al., 2018). These bioactive compounds play a crucial role in reducing oxidative stress in muscle tissue and preventing lipid oxidation (Kaewsatuan et al., 2023; Mujica et al., 2017), which can contribute to extending meat shelf life while preserving freshness. Oxidative stability is a key factor influencing meat quality parameters such as color (particularly the red hue associated with a* values) and flavor (Sabuncular et al., 2021). As muscle pigmentation changes with age, evaluating the effects of propolis on this process is critical for meat processing and marketing.

Meat quality traits such as pH, color (L*, a*, b*), water-holding capacity, drip loss, and cooking loss directly impact consumer preferences and market value. Age-related changes, including increased connective tissue and variations in intramuscular fat distribution, significantly affect meat texture and chewiness (Yalcın et al., 2014). Compared to beef or pork, poultry meat is more susceptible to spoilage, making the monitoring of quality losses during storage essential for maintaining freshness and ensuring quality assurance. Effective control of meat quality is critical for enhancing sensory attributes, minimizing economic losses, and improving the efficiency of the poultry meat industry.

Understanding meat quality changes in older broilers is important for both scientific research and commercial applications. The breast and thigh meat from older broilers may exhibit differences in flavor, aroma, texture, and nutrient composition compared to younger broilers (Komiyama et al., 2010). These age-related changes provide a valuable opportunity to examine the effects of bioactive feed additives such as propolis on meat characteristics. Evaluating propolis's impact on muscle pH, water-holding capacity, and color in 35-week-old broiler breeders could provide crucial insights into its role

in improving meat quality.

In certain markets, meat from older broilers is preferred for processed products such as sausages and salami. Since muscle glycogen levels and connective tissue content change with age, variations in pH, water-holding capacity, and tenderness can significantly influence meat processing characteristics (Khan et al., 2019). Investigating 35-week-old broilers can yield valuable data on the quality of breeder meat and support the development of high-quality processed poultry products.

This study investigates the effects of dietary supplementation with different levels of propolis extract (100, 200, 400, and 800 ppm) on meat quality parameters, including pH, color, drip loss, and cooking loss, in 35-week-old broiler breeders. The use of propolis as a natural feed additive has the potential to enhance meat production quality. However, further research is needed on the optimal dosage and economic sustainability of propolis to improve the applicability of broiler meat in processed products. The findings of this study will provide insights into the impact of propolis on meat quality and contribute to understanding the market potential of more mature broilers, ultimately supporting the production of healthier and higher-quality meat.

2. Materials and Methods

2.1. Animals, Diet, and Experimental Design

This study was conducted on 35 week-old 100 Ross 308 female broiler breeder chickens, which were randomly allocated into five groups (each group consisted of four replicates, with five chickens per replicate, resulting in a total of 20 chickens per group). The experimental groups included a control group (C) and four treatment groups supplemented with propolis at varying concentrations (P100, P200, P400, and P800). The broilers were housed on wood shaving under standardized conditions throughout the experimental period (for four weeks), following the management guidelines for Ross 308 hybrids (AVIAGEN, 2014).

Broilers had ad libitum access to water and were fed a standard basal diet at an average of 155 g per day. The composition and nutritional profiles of the diets used during the chick, pullet, and breeder stages are presented in Table 1.

2.2. Propolis Extraction and Characterization

Raw propolis used in this study was obtained from a local beekeeper in Giresun, Türkiye, who collected the material from a consistent floral source. Upon collection, the propolis was stored at –20 °C until extraction, following the protocol described by Choi et al. (2006). For extraction, the hardened propolis was ground into a fine powder using a mortar and pestle. Ethanol extraction was performed as outlined by Shalmany and Shivazad (2006), and the resulting extracts were stored in dark-colored glass jars at room temperature until use (Cetin et al., 2010).

The chemical composition of the extract was determined using gas chromatography-mass spectrometry (GC-MS). The analysis confirmed the presence of key bioactive compounds, including phenolic acids and flavonoids, which are known for their antioxidant and antimicrobial properties. These findings were consistent with the results reported by Konanç and Ozturk (2025).

The doses of propolis extract have been determined based on the recommended dose ranges in the literature (Irawan et al., 2021). Propolis extract supplementation was performed by spraying onto the feed, and the treatment groups received ethanol-extracted propolis for four weeks. The control group was fed a basal diet without any additives (Table 2).

2.3. Sample Collection and Measurements

At 35 weeks of age, four broiler (one broiler each replicate) from each group were randomly selected and slaughtered following a 10-hour feed withdrawal period. The broilers were euthanized by cervical dislocation and exsanguinated for two minutes. Carcasses were manually defeathered, eviscerated, and immediately processed without prior chilling.

Carcass body weight was recorded using an electronic scale with a precision of 0.01 g. Carcass yield was calculated as the percentage of live weight converted into carcass weight. The technological meat quality parameters were assessed on breast and thigh muscles, including pH, drip loss, cooking loss, and color measurements.

2.4. pH Measurement

The pH of breast and thigh muscles was measured at three post-mortem time points: 1 day (45 minutes after slaughter), 4 days, and 7 days. The initial pH was recorded 45 minutes after slaughter using a calibrated pH meter (Thermo 205) with a solid probe at ambient temperature. The electrode, immersed in the muscle samples, was kept in place until the pH meter reading stabilized. The measurement was then recorded once the value on the screen became constant. The measurements were repeated for samples stored at +4°C for 4 and 7 days. Each measurement was performed three times, and the average value was recorded.

2.5. Drip Loss Measurement

Breast and thigh muscle samples (~10 g; 3 cm in length, 2 cm in diameter) were placed in polyethylene bags and suspended in a refrigerator at +4°C for 24 hours. The samples were weighed before and after refrigeration. Drip loss was determined using the following formula (equation 1):

$$\text{Drip Loss (\%)} = [(\text{Initial Weight} - \text{Final Weight}) / \text{Initial Weight}] \times 100 \quad (1)$$

where initial sample weight refers to the weight of the fresh sample before storage, and final sample weight represents the weight after a specific storage period at refrigeration temperature. The process was repeated for samples stored for 4 and 7 days.

Table 1. The composition of the feed mixture, for chicks (1-28 days old)[1], for pullets (28-154 days old)[2], for broiler breeders (155+ days old)[3]

Ingredients	%			Nutrient composition	%		
	1	2	3		1	2	3
Corn	547.00	619.00	687.28	Crude protein	19.000	15.000	15.000
SBM*	229.24	109.24	154.16	Eher extract	3.050	3.000	3.370
Wheat bran	72.97	109.15	13.03	Crude Fiber	4.080	4.860	3.550
Wheat meal	60.00	60.00	60.00	Total Ash	5.930	5.200	10.700
SFM**	50.00	100.00	50.00	Total P	0.763	0749	0.583
Limestone powder	11.33	10.14	70.18	Av. Phospho.	0.450	0.420	0.350
MCP***	9.41	7.75	6.79	Calcium	1.000	0.900	3.000
Vegetable oil	5.00	1.81	6.57	DL-Methionine	0.423	0.320	0.300
Salmonella inhibit	3.00	3.00	2.00	Lysine	1.010	0.740	0.740
Broiler mix****	3.00	3.00	3.00	Tryptophan	0.236	0.175	0.175
Salt	2.09	2.20	2.20	Threonine	0.724	0.565	0.567
DL-methio. (99%)	1.18	0.47	0.39	İsoleucine	0.798	0.585	0.624
Toxin binder	1.00	1.00	1.00	Histidine	0.520	0.403	0.416
Vitamin D3	1.00	1.00	0.50	Valine	0.901	0.700	0.715
$NaHCO_3$	1.00	0.71	1.06	Leucine	1.573	1.244	1.335
Organic minerals	1.00	1.00	1.00	Arginine	1.293	0.969	0.995
Probiotics	1.00	0.50	-	Phenylalanine	0.922	0.692	0.732
Lysine (99%)	0.68	1.67	0.33	Clor	0.167	0.202	0.160
Threonine	0.11	0.27		Sodium	0.160	0.160	0.160
Vitamin E	-	-	0.50	Potassium	0.814	0.634	0.600
				Linoleic acid	1.387	1.325	1.552
				Cholin mg/kg	0.311	0.285	0.286
Total	1000.00	1000.00	1000.00	ME*****	2800	2800	2800

SBM*; Soybean meal (46 %CP), SFM**; Sunflower meal (36 %CP), MCP (%22.7 Ca)***; Monocalcium fosfat, Broiler mix****; V+M+E=Vitamin +Mineral+Enzyme, ME*****; Metabolisable Energy (Kcal/kg).

Table 2. Feeding protocol for groups

Groups	Number of chickens	Feeding Protocol
Control (C)	20	Feed mixture
P100	20	Feed mixture+100 ppm propolis extract
P200	20	Feed mixture+200 ppm propolis extract
P400	20	Feed mixture+400 ppm propolis extract
P800	20	Feed mixture+800 ppm propolis extract

2.6. Cooking Loss Measurement

Approximately 10 g of minced breast and thigh meat from each sample was sealed in a polyethylene bags and heated in a water bath at 80°C for 20 minutes. The samples were then cooled to room temperature, dried with a paper towel, and reweighed. Cooking loss was calculated using the following formula(equation 2):

$$\text{Cooking Loss (\%)} = [(\text{Initial Weight} - \text{Final Weight}) / \text{Initial Weight}] \times 100 \quad (2)$$

where pre-cooking weight represents the initial sample weight before cooking, and post-cooking weight refers to the final weight after cooking. The process was repeated for samples stored for 4 and 7 days.

2.7. Color Measurement

Breast and thigh muscle color was evaluated after 24 hours of refrigeration at +4°C. Color measurements were performed using a Minolta CR 300 Chroma Meter (Minolta Camera Co., Osaka, Japan) after making a fresh vertical incision in the middle of the muscle. The color parameters were recorded in the CIE-Lab* system, measuring lightness (L*), redness (a*), and yellowness (b*) at three points on the skinless left thigh and breast muscles (Hunt et al., 1991). Each measurement was performed three times, and the average value was recorded.

2.8. Statistical Analysis

The effect of propolis supplementation on measured parameters was analyzed using one-way analysis of variance (ANOVA), considering treatment groups as the main factor. Means were compared using Duncan's multiple range test within the GLM procedure SPSS v26 (IBM Inc.). Data were expressed as mean ± standard deviation (SD). Statistical significance was set at P< 0.05.

3. Results

3.1. Body weight

The average carcass body weight and yield values are presented in Table 3. The lowest live weight was observed in the 100 ppm group (3552.86 g), while the highest was in the 400 ppm group (3812.17 g), with no statistically significant differences among groups (P=0.217). Similarly, carcass body weight ranged from 2714.17 g (200 ppm) to 3118.00 g (400 ppm), and carcass yield varied between 75.94% (200 ppm) and 81.59% (400 ppm), but these differences were not significant (P>0.05). These findings indicate that propolis supplementation did not affect live weight, carcass weight, or yield.

Table 3. Estimated average ± SD of live weight (g), carcass body weight (g) and carcass yield (%) according to the groups

Groups/ Parameters	Live weight (g)	Carcass body weight (g)	Carcass yield (%)
Control	3719.500±197.234	2948.250±199.587	79.216±1.488
P100	3552.857±379.768	2835.142±409.023	79.595±1.378
P200	3556.000±342.015	2714.166±412.787	75.941±1.488
P400	3812.166±295.655	3118.000±367.262	81.589±1.488
P800	3680.000±340.073	2934.000±371927	79.575±1.630

Means within rows without common superscripts differ significantly according to ANOVA (P<0.05).

3.2. pH values

The pH values of thigh and breast meat over postmortem days 1, 4, and 7 are shown in Table 4. Overall, pH values increased over time. On day 1, thigh pH ranged from 5.87 to 5.99, reaching 6.89–7.25 by day 7. Breast pH followed a similar pattern, increasing from 5.68–5.76 on day 1 to 6.12–6.53 by day 7. On day 4, the 800 ppm group had the lowest thigh pH (6.03), significantly lower than the control group (6.46) (P<0.05). On day 7, the highest thigh pH was in the 800 ppm group (7.25), but all groups were statistically similar (P=0.359). For breast meat, significant differences were observed on day 7, with the 100 ppm group having the highest pH (6.53) and the 400 ppm group the lowest (6.12) (P<0.05).

3.3. L*, a*, b* values

Table 5 summarizes the color parameters. Lightness (L*) values did not differ significantly among groups for thigh (P=0.873) or breast meat (P=0.243). Redness (a*) values were also not significantly affected by propolis supplementation in either meat type (P>0.05). However, yellowness (b*) showed significant variation, with the 800 ppm group having the highest thigh meat b* value (9.206) and the 200 ppm group the lowest (6.558) (P<0.05). A similar trend was noted in breast meat, where the 200 ppm group exhibited significantly lower yellowness compared to the control (P<0.05).

3.4. Drip loss

Drip loss values over days 1, 4, and 7 are presented in Table 6. Propolis supplementation, particularly at 800 ppm, reduced drip loss in both thigh and breast meat. The control group had the highest drip loss values across all time points, while the 800 ppm group consistently showed the lowest values. Significant reductions were observed on days 4 and 7 for both meat types, with the P800 group exhibiting the lowest values (P<0.05).

Table 4. Estimated average ± SD of pH values according to the groups on thigh and breast meat

	pH values	1.day	4.day	7.day	P-value
Thigh meat	Control	5.866±0.088^{Ac}	6.460±0.333 ^{Ab}	6.966±0.321 ^{Aa}	0.001
	P100	5.994±0.185 ^{Ac}	6.334±0.173 ^{ABb}	7.067±0.391 ^{Aa}	0.001
	P200	5.981±0.128 ^{Ab}	6.358±0.327 ^{ABb}	6.888±0.409 ^{Aa}	0.001
	P400	5.938±0.148 ^{Ac}	6.246±0.238 ^{ABb}	6.931±0.106 ^{Aa}	0.001
	P800	5.930±0.119 ^{Ab}	6.028±0.195 ^{Bb}	7.246±0.123 ^{Aa}	0.001
	P-value	0.538	0.114	0.359	-
Breast meat	Control	5.681±0.024 ^{Ab}	6.005±0.096 ^{Aa}	6.260±0.357 ^{ABa}	0.001
	P100	5.731±0.063 ^{Ac}	6.064±0.208 ^{Ab}	6.534±0.255 ^{Aa}	0.001
	P200	5.748±0.113 ^{Ab}	6.156±0.246 ^{Aa}	6.403±0.295 ^{ABa}	0.001
	P400	5.710±0.072 ^{Ab}	6.093±0.193 ^{Aa}	6.123±0.115 ^{Ba}	0.001
	P800	5.756±0.087 ^{Ab}	6.096±0.206 ^{Aab}	6.446±0.382 ^{ABa}	0.005
	P-value	0.491	0.769	0.136	-

Means within rows without common superscripts (a-c) are significantly different according to ANOVA (P<0.05), indicating differences between groups at the same time point. Similarly, means within columns without common superscripts (A-B) are significantly different (P<0.05), representing differences between time points within the same group. Groups sharing the same superscripts do not show statistically significant differences.

Table 5. Estimated average ± SD of color values of chicken thigh and breast meat expressed as L*a*b* according to the groups

	L*a*b* values	L	a	b
Thigh meat	Control	53.885±1.893^{Aa}	23.053±2.430 ^{Aa}	8.273±2.158 ^{ABa}
	P100	53.583±3.866 ^{Aa}	20.269±4.155 ^{Aa}	7.294±2.116 ^{ABa}
	P200	52.565±0.826 ^{Aa}	21.867±1.428 ^{Aa}	6.558±1.065 ^{Ba}
	P400	53.697±2.993 ^{Aa}	21.415±2.739 ^{Aa}	7.887±2.012 ^{ABa}
	P800	52.580±3.069 ^{Aa}	23.997±2.828 ^{Aa}	9.206±1.839 ^{Aa}
	P-value	0.873	0.244	0.217
Breast meat	Control	59.390±2.802 ^{Aa}	4.255±1.908 ^{Aa}	4.310±1.527 ^{Aa}
	P100	59.530±4.537 ^{Aa}	4.715±0.907 ^{Aa}	3.697±1.378 ^{ABa}
	P200	59.285±3.038 ^{Aa}	3.964±0.843 ^{Aa}	2.279±1.482 ^{Ba}
	P400	56.818±2.132 ^{Aa}	5.439±1.703 ^{Aa}	3.123±1.483 ^{ABa}
	P800	56.249±1.808 ^{Aa}	4.570±0.774 ^{Aa}	4.020±1.073 ^{ABa}
	P-value	0.243	0.393	0.137

Means within rows without common superscripts (a-c) are significantly different according to ANOVA (P<0.05), indicating differences between groups at the same time point. Similarly, means within columns without common superscripts (A-B) are significantly different (P<0.05), representing differences between time points within the same group. Groups sharing the same superscripts do not show statistically significant differences. lightness [L*], redness [a*], and yellowness [b*]

Table 6. Estimated average ± SD of drip loss (%) values according to the groups on thigh and breast meat

	Drip loss (%)	1.day	4.day	7.day	P-value
Thigh meat	Control	3.692±1.453^{Ac}	7.161±1.779^{Ab}	11.272±3.010^{Aa}	0.001
	P100	2.216±1.409^{Ac}	5.831±3.011^{Ab}	9.306±2.998^{ABa}	0.001
	P200	2.128±1.496^{Ab}	5.254±3.349^{Aab}	8.083±3.486^{ABa}	0.011
	P400	2.379±0.760^{Ac}	5.339±1.353^{Ab}	9.023±3.248^{ABa}	0.001
	P800	2.471±1.568^{Ab}	4.222±1.656^{Aab}	5.992±1.543^{Ba}	0.015
	P-value	0.286	0.381	0.088	-
Breast meat	Control	3.102±1.308^{Ab}	9.474±5.849^{Aa}	10.931±4.505^{Aa}	0.016
	P100	2.145±1.086^{Ac}	5.305±1.415^{Bb}	8.464±1.996^{ABa}	0.001
	P200	2.890±1.936^{Ab}	6.828±3.551^{ABa}	7.875±0.866^{ABa}	0.006
	P400	2.077±0.595^{Ac}	4.955±0.943^{Bb}	7.418±1.520^{Ba}	0.001
	P800	2.142±0.928^{Ac}	4.285±1.083^{Bb}	6.548±1.918^{Ba}	0.001
	P-value	0.483	0.073	0.066	-

Means within rows without common superscripts (a-c) are significantly different according to ANOVA (P<0.05), indicating differences between groups at the same time point. Similarly, means within columns without common superscripts (A-B) are significantly different (P<0.05), representing differences between time points within the same group. Groups sharing the same superscripts do not show statistically significant differences.

3.5. Cooking loss

Table 7 shows the cooking loss results. While propolis supplementation reduced cooking loss, the effect was statistically significant only on day 7 for breast meat, where the 800 ppm group had the lowest loss (19.30%) compared to the control (P=0.049). No significant differences were found on days 1 and 4 (P>0.05), though numerical reductions were noted, suggesting a potential long-term benefit of propolis in improving water retention.

Table 7. Estimated average ± SD of cooking loss (%) values according to the groups on thigh and breast meat

	Cooking loss (%)	1.day	4.day	7.day	P-value
Thigh meat	Control	26.330±6.110^{Aa}	22.921±5.242^{Aa}	24.988±3.271^{Aa}	0.511
	P100	25.951±4.262^{Aa}	25.091±3.713^{Aa}	23.997±6.744^{Aa}	0.880
	P200	27.336±1.531^{Aa}	22.551±5.150^{Aab}	21.295±4.471^{Ab}	0.048
	P400	26.991±3.884^{Aa}	24.465±3.881^{Aa}	23.055±2.679^{Aa}	0.181
	P800	27.526±4.518^{Aa}	26.453±4.014^{Aa}	23.371±1.093^{Aa}	0.194
	P-value	0.878	0.671	0.573	-
Breast meat	Control	22.925±1.946^{Aa}	22.778±1.685^{Aa}	22.845±1.108^{Aa}	0.988
	P100	23.477±1.820^{Aa}	22.373±1.909^{Aa}	21.864±2.130^{ABa}	0.312
	P200	21.253±2.045^{Aa}	22.298±1.079^{Aa}	20.058±2.578^{Ba}	0.186
	P400	21.353±1.166^{Aa}	22.340±2.591^{Aa}	20.930±2.010^{ABa}	0.477
	P800	21.888±1.708^{Aa}	20.286±5.382^{Aa}	19.306±1.929^{Ba}	0.509
	P-value	0.129	0.623	0.049	-

Means within rows without common superscripts (a-c) are significantly different according to ANOVA (P<0.05), indicating differences between groups at the same time point. Similarly, means within columns without common superscripts (A-B) are significantly different (P<0.05), representing differences between time points within the same group. Groups sharing the same superscripts do not show statistically significant differences.

4. Discussion

The use of propolis in broiler chickens, particularly at higher doses (P400 and P800), has been shown to improve meat quality and enhance water-holding capacity. These effects could contribute to the enhancement of the quality of processed meat products at an industrial level. However, considering the potential higher cost of propolis compared to traditional feed additives, its economic sustainability requires careful evaluation. The efficacy of higher doses may increase production costs, yet the long-term health benefits and potential reduction in antibiotic use could enhance the economic value. The widespread availability of propolis is also closely tied to the supply chain and beekeeping production, making it essential to assess the conditions under which propolis can be reliably sourced.

The results of this study highlight the significant effects of propolis supplementation on broiler meat quality. Propolis, rich in phenolic compounds and flavonoids, reduces oxidative stress and inhibits lipid oxidation, contributing to meat freshness and shelf life. Oxidative stability influences key quality parameters such as color, water-holding capacity, and flavor (Huang and Ahn, 2019). Propolis supplementation, particularly at higher doses, improved meat color and water-holding capacity, with notable effects in the P800 group.

No statistically significant differences were observed in live weight, carcass weight, or yield among the treatment groups. The P400 group had the highest live and carcass weight, but differences were not significant. Previous studies suggest dietary supplements, including propolis, can influence poultry growth (Prakatur et al., 2020). However, Haščík et al. (2015) reported that lower doses improve growth performance, while higher doses may have variable effects. The non-significant reduction in live and carcass weights in the P200 group may be dose-dependent. The P400 group yielded the best results in carcass weight, suggesting that this dose positively influences the overall growth and development of broiler chickens, leading to improved carcass yield. The ability of this dose of propolis to support muscle development and enhance body composition may explain the observed increase in carcass weight. On the other hand, the P800 group demonstrated a more significant improvement in water-holding capacity, indicating that the biochemical effects of higher doses of propolis on water retention in meat are more pronounced. It can be hypothesized that higher doses of propolis may enhance the meat's water-holding capacity, leading to reduced dehydration and extended shelf life. However, the impact of P800 on carcass weight may be more limited, as the increased water retention could potentially influence other structural parameters of the meat, which may lead to differing outcomes in carcass weight. While propolis did not significantly affect body weight or carcass yield, its benefits for meat quality remain evident. Mahmoud et al. (2013) found that high propolis doses reduced body weight gain ($P<0.05$). This suggests higher doses might limit growth. Our results indicate that 400 ppm propolis may enhance carcass yield, but further research is needed to determine the optimal dosage.

The pH values of broiler meat were within expected ranges and increased during storage, consistent with postmortem glycolysis. Values were slightly lower than those reported by Barbut et al. (2005) and Swatland (2008), possibly due to differences in breed and diet. The postmortem pH increase was more prominent in thigh meat, especially in the P800 group on day 7, suggesting a potential metabolic influence of propolis. Similar findings were reported by Šulcerová et al. (2011).

Meat color, assessed via L* (lightness), a* (redness), and b* (yellowness) values, was not significantly affected by propolis supplementation. However, the P800 group had the highest a* and b* values, indicating a possible trend toward increased redness and yellowness, potentially due to the antioxidant properties of propolis. This aligns with findings by Lee et al. (2022), suggesting natural antioxidants help retain meat color by reducing oxidative changes.

Propolis supplementation significantly improved water-holding capacity, particularly in the P800 group, which exhibited a 47% and 40% reduction in drip loss for thigh and breast meat, respectively, on day 7. Increased pH levels in this group further support its role in enhancing water retention (Türkyılmaz et al., 2021). The P200 group also showed a significant reduction in drip loss ($P=0.011$). These findings align with research highlighting the benefits of natural supplements on meat quality (Lee et al., 2022; Prakatur et al., 2020).

Cooking loss showed no significant differences among groups ($P=0.573$ for breast and $P=0.511$ for thigh), but a trend toward lower values in the P200 group was observed. The P200 group exhibited a decrease in cooking loss from 27.34% on day 1 to 21.30% on day 7 ($P=0.048$), indicating a possible improvement in meat texture. This is consistent with Tan et al. (2022), who reported similar cooking loss values for poultry meat.

Propolis supplementation, particularly at 800 ppm, significantly enhanced broiler meat quality by improving water retention and reducing drip loss, resulting in juicier and more tender meat. While the impact on color parameters was minimal, higher doses exhibited a trend toward increased pH levels and enhanced antioxidant effects, which may contribute to an extended shelf life. These findings highlight propolis as a promising natural supplement for improving poultry meat quality, with potential applications in processed products.

Consistent with the current results, the use of propolis at different doses in broiler diets has shown potential to improve meat quality, enhance water-holding capacity, and optimize carcass yield. Notably, the higher doses (P400 and P800) were particularly effective in increasing water-holding capacity, and positive effects were observed on carcass weight. However, the absence of sensory testing is a significant limitation of this study. Organoleptic attributes such as flavor, texture, and

tenderness are crucial in determining consumer acceptability, especially for industrial applications. Therefore, future studies should incorporate sensory analysis to provide a more comprehensive evaluation of propolis's impact on meat quality.

Economically, while propolis may help reduce health-related costs as a natural alternative to antibiotics, its commercial use faces certain limitations. The seasonal and geographical constraints of propolis production, along with the high costs of standardization and quality control, can impact its economic feasibility. Particularly in regions where beekeeping is not widespread, propolis might need to be imported, increasing production costs.

Moreover, the chemical composition of propolis varies according to its botanical and geographical origin, as well as the season of harvest and extraction methods (Kasote et al., 2022). This variability may influence its consistency and efficacy as a feed additive. Thus, the development of standardized guidelines for propolis characterization and its use in poultry nutrition is essential.

From a food safety perspective, while propolis is generally considered safe, the potential accumulation of bioactive compounds in edible tissues over long-term use warrants attention (Tumbarski et al., 2022). Currently, there are no established maximum residue limits (MRLs) for propolis-derived substances in meat or egg products. This highlights the need for further toxicological and regulatory studies to ensure its safe use in food animal production systems.

In this context, it is important to consider the balance between the beneficial effects of propolis and its economic and regulatory feasibility. Although its antioxidant and immune-supportive effects make it a strong candidate as a natural additive, its variable composition, cost of standardization, and lack of residue regulations present challenges to routine use (Wojtacka, 2022). Therefore, broader studies investigating not only the physiological responses in poultry but also the economic, compositional, and safety-related aspects of propolis are essential for its integration into industrial-scale poultry nutrition.

5. Conclusion

In summary, propolis shows great promise as a natural feed additive to improve broiler meat quality, particularly in terms of water-holding capacity and carcass yield. However, to fully establish its industrial applicability, future studies should focus on determining the optimal dosage by evaluating intermediate concentrations, conducting sensory analyses to assess organoleptic qualities, performing toxicological assessments and residue analyses to address food safety concerns, and developing cost-effective extraction and standardization methods for broader commercial use. Such research will be essential to clarify the role of propolis in poultry nutrition and its potential as a safe, sustainable, and effective alternative in modern poultry production.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	K.K.	E.Ö
C	70	30
D	100	
S		100
DCP	70	30
DAI	70	30
L	70	30
W	70	30
CR	70	30
SR	70	30
PM	70	30

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management.

Conflict of Interest

The authors declare no conflict of interest.

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Ethical Consideration

The experimental procedures were approved by the Animal Experiments Local Ethics Committee of Ondokuz Mayıs University (Approve date: October 10, 2013 and protocol code: 2013-10).

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INNOVATIVE APPROACHES TO INCREASE GRAFTING SUCCESS RATES IN WALNUT SEEDLING PRODUCTION: GRAFTING CLIP AND TONGUE GRAFTING MACHINE

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Abstract: Walnut seedling production is essential in terms of sustainability and commercial value of the agricultural output. However, traditional grafting methods are time-consuming and labor-intensive and have problems such as high cost and low efficiency. This study tested the most preferred method of grafting walnut propagation, the tongue-and-groove graft grafting method. It aimed to accelerate the grafting process, increase efficiency, and reduce production costs. The performance of prototype clips was evaluated using field trials in the study. A portable tongue-and-groove graft machine and manual labor methods were used in the grafting processes. The study results show that the new grafting clip significantly shortens the grafting time compared to traditional methods and increases graft retention rates. In the plastic graft tie application, 75.6% of the grafts made by hand and 62.2% produced by the grafting machine were successful. In the clip application, 86.7% of the grafts made by hand and 68.9% of the grafts made by the grafting machine were successful. Compared with the traditional and modern grafting methods in the literature, it was revealed that the developed clip reduces the need for labor and offers an innovative product with commercialization potential. The study aims to contribute to a more efficient and economical grafting process in walnut cultivation and sapling production.

Keywords: Tongue grafting, Grafting clip, Grafting machine, Walnut, Sapling

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1. Introduction

Walnut (*Juglans regia* L.) is an agricultural product with high economic and nutritional value worldwide. Walnut cultivation is becoming increasingly important due to its high nutritional content, health benefits, and value in global trade. Professional and amateur gardens established in Turkiye have provided significant production increases in recent years. According to 2023 data, 360,000 tons of walnut production was achieved in Turkiye (FAO, 2025). However, the seedling production process is one of the biggest challenges encountered in walnut production. Grafting techniques, in particular, are one of the most critical factors determining seedling quality and productivity. The success of grafting varies depending on the method used, seasonal conditions, quality of the grafting material, and application process (Zobel, 1984; Hartmann et al., 2011). Walnut is an important plant not only in terms of agricultural production but also due to its ecological and economic benefits. Its use in rehabilitating forested areas is critical for environmental sustainability due to its carbon sequestration potential and contributions to soil fertility (Vahdati and Lotfi, 2013; Preece, 2005; Reighard and Loreti, 2008). In addition, the global walnut trade is growing and is becoming a strategic export product, especially for countries such as Turkiye (FAO, 2021a). This situation increases the need for more efficient and economical grafting techniques. Grafted seedlings have become widespread in walnut cultivation, both for preventing juvenile infertility and for cultivation with standard varieties. Traditional grafting methods, especially tongue-and-groove grafting, are widely used in walnut cultivation. Although this method is preferred due to its high retention rates, the time-consuming application process and the high labor costs during grafting are among the important disadvantages (Chandel et al., 2006). The tongue-and-groove grafting method is a method that requires more dexterity and labor compared to other grafting processes. Technological developments to optimize the grafting process have attracted attention in recent years. For example, automatic and semi-automatic grafting machines play an important role in increasing the efficiency of the grafting process (Özkan, 2001). However, these machines' high costs and ineffectiveness in all conditions require developing more practical and economical solutions.

Developing grafting techniques in walnut cultivation is important for increasing sectoral efficiency. Various studies have focused on different methods to increase grafting success rates. Ahmed et al. (2016) examined the

effects of different grafting methods on walnut seedlings' growth and development performance and stated that some techniques provide higher success under certain conditions. Similarly, Sharma et al. (2022) evaluated the effects of the materials and binding techniques used during grafting on graft retention rates and emphasized the importance of technical improvements. Rezaee, et al. (2008) they achieved over 80% success in their work in the open field. In a similar study, Achim and Botu (1999) achieved a success rate of over 80% with the tongue-and-groove method. This study focuses on developing a new grafting clip and machine to make the walnut grafting process faster, more efficient, and less costly. The aim is for the developed clip and grafting machine to optimize the grafting process by reducing labor requirements and providing high graft retention rates. The effectiveness of the clip and the grafting machine was evaluated through field trials conducted within the scope of the study. The study results will provide innovative and applicable solutions in walnut cultivation and sapling production.

2. Materials and Methods

2.1. Plant material

One-year-old seedlings produced from *Juglans regia* L. seeds were used in grafting operations. The seedlings were supplied by a private company and stored at Tokat Gaziosmanpaşa University Agricultural Application and Research Center until the grafting date. The grafts used in grafting operations were supplied from the walnut parcel established within Tokat Gaziosmanpaşa University Agricultural Application and Research Center. The scions were prepared for grafting operations by cutting them from one-year-old branches with at least three buds. Grafting operations were carried out in the Tokat Gaziosmanpaşa University Agricultural Application and Research Center greenhouse.

2.2. Graft clips

The graft clips designed and produced by the project team are manufactured from ABS material on a 3D printer for field trials. The graft clip (Figure 1.) is formed by the union of two symmetrical parts. The two semicircular parts are joined with a reciprocal gear system to provide opening and closing features. Both parts have the same dimensions and features and are joined symmetrically. It is made of 3 mm thick hard plastic material and has an inflexible structure. The inner diameter of the hard outer surface is 3 cm, and its height is 7 cm. The locking system is easy to open and close and can be used with one hand. It is an important feature in terms of performing operations quickly and practically. It has an adjustable feature thanks to its locking system design. It is suitable for rootstocks and scions of different thicknesses. The inner material of the clip has a flexible structure, and soft polyethylene material is used. It is attached and fixed on the hard surface. Its wall thickness is 1 cm and covers the entire inner surface. The gap in the middle fully grasps the graft area. Its flexible feature ensures it surrounds the graft area and does not leave an air gap. Soft polyethylene material is long-lasting and economical.

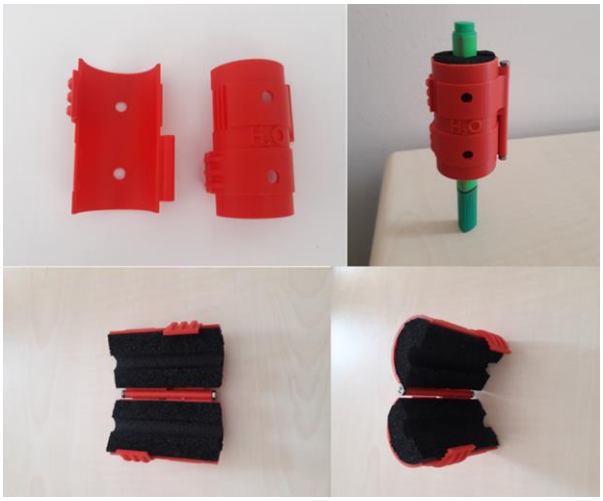

Figure 1. Grafting clip (original).

2.3. Grafting Machine

The developed grafting machine is designed to ensure that the tongue-shaped scion grafting is carried out quickly, precisely, and in a standard manner. The design and production of the machine belong to the project team and were developed to minimize the time loss and labor costs in the manual grafting process. The grafting machine is designed as a portable and easy-to-use rechargeable system. Thanks to its rechargeable structure, it can be easily used in field conditions, thus allowing mobile grafting operations without needing electricity. The machine's body is light and durable, offering an ergonomic experience for long-term use. The cutting blade of the machine (Figure 2) is specially designed and manufactured for the tongue-shaped graft technique.

Figure 2. Grafting knife design (original).

The cutting mechanism is optimized to create a precise and symmetric tongue angle. Since it is important for the rootstock and the scion to fit together perfectly in a tongue-shaped graft, the cutting angle of the blade is adjusted to create a standard and smooth surface. One of the most important components of the grafting machine is

the sensor-controlled cutting mechanism. The sensors inside the machine complete the cutting process by moving the blade back and forth in the cutting area. This way, a standard and error-free tongue cut can be made in a single move without the operator manually cutting (Figure 3).

Figure 3. Grafting machine (original).

In the traditional tongue-and-groove technique, one of the most significant advantages of this developed machine is that cutting errors and time losses that vary depending on the operator's manual dexterity are prevented.

2.4. Method

The grafting operations were carried out using the standard tongue-and-groove graft grafting method. Since ensuring the diameter compatibility of the rootstock and grafts is a critical factor in successful graft retention rates, seedlings and grafts with similar thickness (8-12 mm diameter) were selected in both methods. Hand grafting: The traditional tongue-and-groove graft method was applied manually. The grafts were carefully shaped with a sharp grafting knife and placed on the rootstock.

Machine grafting: A tongue-and-groove cut was made in a single move using a grafting machine, and the graft was placed on the rootstock. The grafting area has a cutting area of approximately 4-5 cm. The graft clip was designed to be 7 cm long to ensure complete graft closure. After the grafting operation was completed, the operator closed the clip's locking system with one hand and performed the binding process. The graft clip's soft inner texture allowed it to adapt to rootstocks fully and grafts with different diameters without leaving any air gaps.

The trial was designed in a randomized plot design with three replications according to the factorial design. Three hundred seedlings were used, with 50 seedlings in each replication. All grafting operations were carried out on the same day and under similar environmental conditions, and the operations were carried out in the greenhouse to minimize the effect of external factors.

2.5. Data Collection and Analysis Method

The following parameters were measured to evaluate the effectiveness of the grafting operations:

- Grafting time: The time spent for manual and machine grafting operations was compared in seconds.
- Tie-up time (seconds): The duration of the tie-up operations with plastic grafting tie and grafting clip was measured.
- Graft efficiency (%): Graft retention rates were recorded by observing specific periods after grafting.
- Graft awakening times (in days): The effect of different tying methods was evaluated by considering the time when the first bud burst occurred.

In this study, the differences in success rates between two different methods (hand grafting and machine grafting) and two different fixation materials (graft tie and clips) used in walnut grafting were statistically analyzed using the chi-square test.

3. Results

Within the scope of the study, grafting, tying time, grafting efficiency, and grafting awakening times were evaluated to determine the grafting clip and the grafting machine's grafting performance.

Grafting and Tying Times: In the study, while the traditional manual grafting process took an average of 140 seconds, this time was reduced to 33 seconds with the developed grafting machine (Table 1). In the traditional method, the cutting time was measured as 118.87 ± 2.10 seconds and the tying time as 23.30 ± 0.88 seconds. In contrast, the cutting time with the grafting machine was determined as 21.03 ± 0.83 seconds and the tying time as 12.60 ± 0.41 seconds. These findings reveal that the grafting machine significantly accelerated the cutting and tying processes.

Table 1. Cutting times according to cutting methods

Graft Cutting Method	Cutting Time (s)	Tying Time (s)	Total (s)
Hand cutting	118.87 ± 2.10	23.30 ± 0.88	142.85
Machine cutting	21.03 ± 0.83	12.60 ± 0.41	33.63

Grafting efficiency: As a result of the chi square analysis, it was determined that there was no significant interaction between the grafting method and the grafting tie application. In the samples where graft ties were used, there was no statistically significant difference between the success rates of hand grafting and machine grafting (χ^2 = 1.30, P=0.255). This result shows that both methods have similar success rates with graft ties.

In the samples where clips were used, the hand grafting method showed higher retention success. The p-value

obtained as a result of the chi-square test was 0.076, and although this value was not significant at the 5% significance level, it was at a level that could be considered significant at the 10% level (χ^2 = 3.15). This situation suggests that the hand grafting method may be more successful than the machine method in grafting with clips. In both the plastic grafting tie and clip application, a higher retention rate was obtained from the grafting done by hand than the grafting done by the grafting machine. In the plastic graft tie application, 75.6% of the grafts made by hand and 62.2% of the grafts made by the grafting machine were successful. In the clip application, 86.7% of the grafts made by hand and 68.9% of the grafts made by the grafting machine were successful. In other words, in both tying methods, grafts made by hand gave better results than grafts made by the grafting machine. When the average values are taken into account, 81.1% of the grafts made by hand and 65.6% of the grafts made by the grafting machine were successful. When the general success of the methods was evaluated, a significant difference was found between hand vaccination and machine. The hand vaccination method showed statistically higher retention success than the machine method (χ^2 = 4.80, P=0.028). This difference is significant at 5% significance level and shows the general effectiveness of hand vaccination. (Table 2).

Table 2. Grafting success rates of saplings tied with grafting ties and grafting clips

	Chi-Square Value (χ^2)	Degrees of Freedom (df)	P-value	
Grafting Tie	1.30	1	0.255	Not significant
Grafting Clip	3.15	1	0.076	Significant at 10%
Hand Cutting vs Machine Cutting	4.80	1	0.028	Significant

***=** A statistically significant difference exists between the groups (P<0.05).

Graft awakening times: After the grafting operations, the grafting areas were closed with a classic grafting tie and clip (Figure 4). Depending on the binding materials used, a difference of approximately nine days was observed between the first bud burst dates. An average of nine days of late awakening was detected in seedlings using grafting clips. Late awakening did not negatively affect the graft take rates. This situation shows that the protective effect of the clip on the grafting area compensates for the delay in awakening times. In addition, the delay in awakening times may have contributed to the more robust and durable development of the grafted seedlings. This finding reveals that using clips plays both a protective and supportive role in the grafting processes.

Figure 4. Seedling grown with grafting clip (original).

4. Discussion

This study evaluated the effectiveness of a new grafting clip and machine developed to optimize grafting processes in walnut (*Juglans regia* L.) cultivation. The findings show that significant time savings can be achieved in grafting processes, and graft retention rates can be increased. The findings obtained in the study revealed that traditional grafting processes performed manually took an average of 140 seconds, while grafting processes performed with the developed grafting machine took an average of 33 seconds. It is stated that traditional grafting methods are labor and time-intensive processes (Gandev, 2007; Hartmann et al., 2011). In particular, the tongue grafting method requires precise cutting of the grafting area and appropriate binding materials (Polat and Ordek, 2008). This result shows that a significant increase in efficiency is provided in grafting processes. Many studies indicate that automatic and semi-automatic grafting machines shorten grafting times. In their studies, Akça and Palazoğlu (2022) achieved success with the semi-automatic Akça vaccination machine (93.00%). In March, the highest vaccination success rate (100.00%) was determined in the tongue vaccination and Akça vaccination machine method (90.00%) in the plastic greenhouse environment. The omega vaccination success rate in the plastic greenhouse environment varied between 15.00% and 25.00%. Özkan (2001) stated that automatic grafting machines reduce grafting times by 50-60%. However, the 33-second time obtained in this study is relatively low compared to other studies in the literature. Yilmaz et al. (2020) reported that the average grafting time with a similar machine was 45 seconds. A study by Zhang et al. (2017) determined that automatic grafting machines accelerated the grafting process by compared to traditional hand grafting and increased labor

efficiency. This difference can be explained by the cutting blade's special design and the developed machine's sensor technology. In addition, a significant decrease in tying times was observed. While the tying time was 23.30 seconds on average when a classic plastic grafting tie was used, this time decreased to 12.60 seconds with the grafting clip. This finding shows that the practical use of the clip accelerates the grafting process. Similarly, Demirtas et al. (2019) examined the effect of different tying techniques on grafting times and stated that flexible tying materials can be applied faster. The time savings provided by the grafting machine can provide a significant advantage, especially in large-scale seedling production facilities. In traditional methods, the grafting process is both time-consuming and labor-intensive. This can negatively affect production capacity, especially when seasonal conditions are limited. The use of a grafting machine accelerates this process and allows more seedlings to be grafted in a short time.

Graft retention rates are one of the most critical parameters in evaluating grafting success. In this study, the retention rate of hand-made grafting was 81.1%, while the retention rate of grafting with a grafting machine was 65.6%. These results show that traditional hand-made grafting has a higher success rate. Studies have shown that hand-made grafting can succeed with the correct technique and applications. However, the physical pressure created by mechanized systems in the grafting area and differences in cutting sensitivity can reduce success rates (Gandev, 2019).

However, the retention rates of grafting with a grafting machine are higher than other automatic grafting machines in the literature. Gürcan et al. (2017) reported that the retention rates of grafts made with automatic grafting machines were 50-60%. The retention rate of 65.6% obtained in this study demonstrates the success of the developed machine. Manual grafts provided higher retention rates in walnut grafting, but automatic systems were advantageous regarding time-saving. The findings of this study support this view.

When plastic graft tie was used as the graft binding material, a success rate of 68.9% was obtained, and when the developed graft clip was used, a success rate of 77.8% was obtained. Among the graft binding methods, clips provided a retention rate of approximately 9% higher than the plastic graft tie. This finding reveals that the graft clip provided a higher success rate than traditional binding materials. It is stated in the literature that binding materials are effective in graft success (Vahdati and Lotfi, 2013; Vahdati et al., 2019). Similarly, Gandev (2019) examined the effect of different binding techniques on graft retention rates and stated that flexible binding materials provided higher success rates. The flexible internal structure of the clip used in this study supports these results. In addition, Vahdati and Lotfi (2013) stated that air exposure of the graft site may negatively affect graft success. The structure of the clip that limits air circulation may have provided an advantage in this respect.

Graft awakening times are another important indicator of grafting success. This study observed an average of nine days of late awakening in seedlings using grafting clips. This may be associated with the clip covering the grafting site completely and limiting air circulation. However, this late awakening did not negatively affect graft take-up rates. Although similar findings are not found in the literature, Hartmann et al. (2011) stated that air exposure to the grafting site may negatively affect graft success. Therefore, the structure of the clip that limits air circulation may increase graft take-up rates while extending the awakening times. In addition, Yılmaz et al. (2020) stated that graft awakening times may vary depending on the grafting method and environmental conditions. In this study, the fact that clip use extended the awakening times supports this view. It is thought that this delay resulting from the use of clip material will reduce the risk of damage from late spring frosts that may occur after the grafting process is carried out in open fields. The findings of this study, when compared to other studies in the literature, reveal both similarities and differences. Soleimani et al. (2010), and Hartmann et al. (2011) stated that the methods and materials used in the grafting processes significantly affect the success of the graft. Similarly, this study's grafting method and the binding material significantly affected graft retention rates. However, the grafting clip and machine developed in this study provided higher success rates than other automatic grafting systems in the literature. In addition, Mır and Kumar (2011) emphasized that the development of grafting techniques in walnut cultivation plays a critical role in increasing sectoral efficiency. The study presented findings supporting this view. The developed grafting clip and machine significantly contributed to walnut cultivation by accelerating the grafting processes and increasing the retention rates. In addition, FAO (2021b) stated that the global walnut trade is growing, and countries need innovative solutions to compete in this field. This study offers a practical and applicable solution for this need.

5.Conclusion

This study evaluated the effectiveness of a new grafting clip and machine developed to improve grafting processes in walnut cultivation. The findings show a significant decrease in grafting times, and increased graft retention rates were achieved. While the grafting clip provided higher retention rates than the plastic grafting tie, the grafting machine significantly accelerated the grafting process. Although the machine's efficiency is 15% lower, it is thought to be advantageous thanks to the time and labor savings obtained. These results provide innovative and applicable solutions in walnut cultivation and seedling production. In future studies, it is recommended that the clip and machine be tested in different ecological conditions and larger-scale trials be conducted.

Author Contributions

Percentages of the authors' contributions are present below. All authors reviewed and approved final version of the manuscript.

	H.K.	H.P.	O.S.
C	33	34	33
D	33	34	33
S	33	34	33
DCP	33	34	33
DAI	33	34	33
L	33	34	33
W	33	34	33
CR	33	34	33
SR	33	34	33
PM	33	34	33
FA	33	34	33

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because there was no study on animals or humans.

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Review
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BOVINE GENETIC DISEASES - OLD SYNDROMES IN A NEW PERSPECTIVE

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Abstract: Congenital malformations have always fascinated humans but it was not until recently that deeper knowledge on their causes have become more evident. The development within genomic analysis has until now resolved the molecular cause of almost 200 bovine genetic diseases. However, the cause of certain well-known congenital syndromes in cattle have remained unexplained. The Danish Bovine Genetic Disease Program was in its current form established in 1989 and many genetic diseases have been investigated. Lately, we have focused on some well-known congenital disorders: schistosoma reflexum, congenital syndromic Chiari-like malformation and the bulldog calf syndrome. Genetic analyses of such cases sampled as part of the bovine genetic disease surveillance in Denmark have confirmed their genetic etiology. Investigations into a fourth congenital syndrome, perosomus elumbis, are underway.

Keywords: Schistosoma reflexum, Spina bifida, Bulldog calf, Chiari malformation; Perosomus elumbis

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1. Introduction

Congenital malformations have throughout history always puzzled and fascinated people. In the Middle Ages, occurrence of malformed animals was considered supernatural, while during the Enlightenment (17th and 18th centuries), scientists tried to understand why congenital malformations occurred. However, it was only after Gregor Johann Mendel's (1822-1884) description of the laws of heredity that a deeper understanding of the importance of genetics for the development of malformed fetuses grew. Still there was a long way to go to understand the causes themselves. In the 1920s, the first hereditary malformations in cattle were identified based on the Mendelian laws, but the actual understanding of why malformations develop had to await the breakthrough in genetic technology that enabled the identification of mutations, and thereby an understanding of the molecular mechanisms that lead to abnormal fetal development.

The number of recognized genetic disorders in cattle has raised steadily since Lerner in 1944 published a list of 44 lethal or sub-lethal conditions in cattle until today (July 26, 2024), where 270 diseases with Mendelian inheritance are recognized and of which at least one likely causal variant is known for 189 (OMIA, 2024).

The development within genetic technology is growing fast, which has made identification of mutations and other chromosomal abnormalities easier, more rapid and less costly.

Surveillance for genetic diseases in cattle was in Denmark formally established in 1989, which has led to the recognition and eradication of several genetic conditions. During recent years, we have tried to solve the riddle of bovine congenital syndromes that have been known for many decades or even longer. Here, an introduction to our recent research on a number of these classical bovine syndromes is given.

2. Schistosoma Reflexum

Schistosoma reflexum (SR) is a congenital syndrome characterized by dorsal retroflexion of the spine. The spine will thereby get an almost U-shaped form with the consequence that the hindlimbs point in the direction of the head. The retroflexion of the spine, hinders ventral midline closure of the fetal body cavities and therefore their contents (intestines, etc.) protrude (Figure 1).

A fetus affected by SR is born alive at the end of a normal length gestation period, but die immediately due to impaired respiration. SR is associated with dystocia and affected fetuses must be released by cesarean section or fetotomy unless the size of the fetus/the size of the maternal birth canal allow assisted vaginal delivering.

Figure 1. A typical case of schistosoma reflexum in a Holstein calf.

SR occurs sporadically in cattle but its etiology has not been known. Recessive inheritance has been proposed as some Holstein cases were genetically related (Citek, 2012), but a genetic cause has not been proven. Genetic relationship in cattle breeding as evidence of inheritance should be interpreted with caution as some breeding lines have been widely used and therefore some sires may be present in the pedigree of most cattle of certain breeds.

We investigated DNA of 23 SR cases, mostly Holsteins (n=20) and when available, also their parents. Evidence of recessive inheritance was not found, but we were able to detected frameshift and missense variants in 12 cases. These genes belonged to the class of haploinsufficient loss-of-function genes, are involved in embryonic and pre-weaning lethality or are known to be associated with severe malformation syndromes in other species and therefore considered causal for development of SR. Study details have been published in Jacinto et al. (2024a).

3. Congenital Syndromic Chiari-Like Malformation

Congenital syndromic Chiari-like malformation (CSCM) is a sporadically occurring malformation that is often associated with dystocia (breech presentation). The most striking lesion is an almost bilateral symmetric arthrogryposis of the hindlimbs associated with hypo-/dysplasia of the associated muscles.

Figure 2. A case of congenital syndromic Chiari-like malformation. Notice the bilateral symmetric hindlimb arthrogryposis, lumbar lordosis and the flattened neurocranium. Reprinted from Jacinto et al. (2024b).

In many cases, a closer examination reveals a hairless skin lesion in the dorsal midline of the lumbar spine. This represents spina bifida, i.e. a lesion where the vertebral arch is not complete so the spinal cord is exposed. The spina bifida is sometimes covered by skin (a so-called spina bifida occulta).

The rostral aspect of neurocranium is often flattened. This causes compression of the developing brain and due to insufficient space, parts of the brain is dislocated caudally and some parts even protrude through the foramen magnum into the vertebral canal, a so-called Chiari type II like malformation.

Figure 3. Chiari type II-like malformation. Notice that the occipital lobes are dislocated towards the foramen magnum and the cerebellum and parts of the brain stem protrudes into the vertebral canal.

We performed a genomic analysis of 14 cases of CSCM (mainly Holsteins) and when available, also their parents and discovered that CSCM is due to extensive genetic heterogeneity, including both possible recessive alleles and dominant acting *de novo* mutations. The two recessive missense variants were identified in the genes *SHC4* and *WDR45B*, respectively. Analysis of 1209 sires included in the 1000 Bull Genomes Project (Hayes and Daetwyler, 2019) revealed the presence of carriers of both variants. The *SHC4* variant may originate from the sire Wapa Arlinda Conductor (born 1970) or one of his parents, while the *WDR45B* variant could be traced to the sire Mascol (born 2000). Partial monosomy of chromosome 2 was identified in two other cases. Study details have been published in Jacinto et al. (2024b).

4. Perosomus Elumbis

Perosomus elumbis (PE) is a congenital syndrome characterized by lack of development of the lumbar, sacral and coccygeal spinal segments (Figure 4 and 5). The spine and the spinal cord end near the thoraco-lumbar junction and the caudal part of the body therefore consists of a hypoplastic pelvis, hypoplastic hindlimbs with arthrogryposis, and an abdominal wall sac-like structure that encloses the abdominal organs (Agerholm et al., 2014).

This syndrome occurs sporadically and is associated with dystocia as the fetus is often in breech presentation.

We have recorded several cases in the Danish surveillance program for bovine genetic diseases. Some have occurred sporadically, but we have also encountered a cluster of cases after a Belgian Blue sire used for crossbreeding. Multiple cases after a single sire indicates that a dominant paternal germline mutation is the cause of PE in this particular family and that the sire is mosaic for a causal mutation, but as found for SR and CSCM, PE can probably develop due to a range of genetic scenarios.

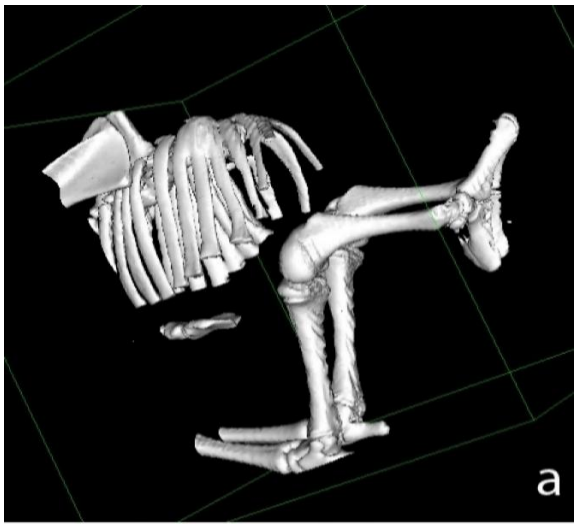

Figure 4. Surface rendered computed tomography image of perosomus elumbis. The lack of spinal development caudal to the thorax is visualized. Reprinted from Agerholm et al. (2014).

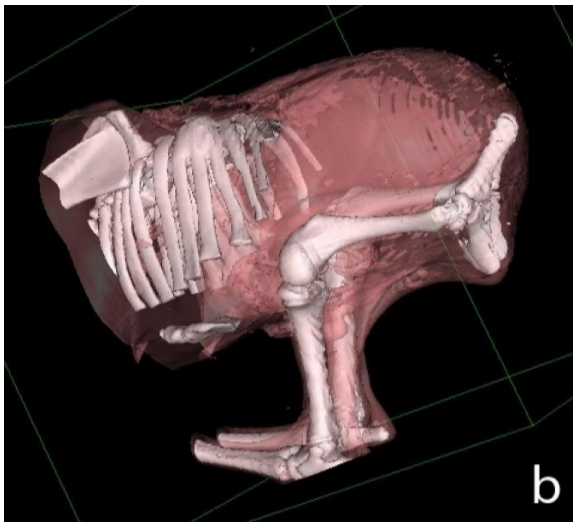

Figure 5. Surface rendered computed tomography image of perosomus elumbis The skin surface of the calf is visualized. Reprinted from Agerholm et al. (2014).

5. Bulldog Calf Syndrome

The bulldog calf syndrome (BCS) or bovine congenital generalized chondrodysplasia was the first hereditary disorder to be reported in cattle (Seligmann, 1904) and was therefore given the number A1 on Lerner's list from 1944. BCS was originally reported in the Dexter breed, but BCS has turned out to consist of a very heterogeneous group of skeletal malformations that have disturbed endochondral ossification in common (Agerholm, 2007). The phenotype and mode of inheritance varies across different cases.

The "prototype" of BCS has dysplasia of the viscerocranium, a doomed neurocranium, a short compact body and short compact limbs (Figure 6). The tongue is often protruding because of the short viscerocranium and eventration of parts of the abdominal organs is a common finding, which is due to the short body that reduces the size of the body cavities.

Figure 6. Typical case of the bulldog calf syndrome. Holstein fetus. Reprinted from Jacinto et al. (2020).

Genetic analyses of BCS cases have in many cases identified a causal mutation in the *collagen type II alpha 1 chain* gene (*COL2A1*) as reviewed by Jacinto et al. (2020). Some cases have occurred in clusters related to a single phenotypically normal sire, who had then been mosaic for the causal mutation and carried the dominant mutation for BCS at a certain level in his spermatozoa (1-21%) (Daetwyler et al. 2014; Bourneuf et al. 2017). Other cases have been due to a *de novo* mutation occurring in the developing embryo. Study details have been published in Jacinto et al. (2020).

6. Conclusions

Identifying the cause of a congenital syndrome in cattle is important to prevent spread of genetic disorders, to increase the breeders' economy by reducing loss of offspring and future breeding animals, and to reduce the critical animal welfare issues related to birth of defective offspring. However, as many important congenital syndromes share a common morphology (disease prototype), detailed genetic investigations are needed to differentiate between inherited and *de novo* mutations that have occurred in the developing embryo. While the former may require implementation of breeding restrictions, lethal mutations occurring in the developing embryo are not transmitted to the next generation.

Author Contributions

The percentages of the author' contributions are presented below. The author reviewed and approved the final version of the manuscript.

	J.S.A.
C	100
D	100
S	100
L	100
W	100
CR	100
SR	100

C= concept, D= design, S= supervision, L= literature search, W= writing, CR= critical review, SR= submission and revision.

Conflict of Interest

The author declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because there was no study on animals or humans.

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Review Article
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EVALUATION OF WATER QUALITY MONITORING STUDIES: CURRENT STATUS AND PRACTICES

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Abstract: Water quality monitoring studies are of great importance for the sustainable use and protection of water resources. These studies entail the periodic measurement of physical, chemical, and biological parameters in water resources. These assessments are particularly crucial for the identification of alterations in surface and groundwater, and for the regulation of biological, chemical, and physical parameters within water bodies. This systematic observation process facilitates the implementation of effective management decisions by providing a comprehensive assessment of the current status of water bodies. The Water Framework Directive is widely acknowledged as a foundational document for the preservation and management of water resources within the European Union. It has played a pivotal role in the development of methodological approaches for the monitoring of water quality and the enhancement of data networks. The directive enables the precise evaluation of water resources contamination and environmental hazards. Water quality monitoring is also crucial for the early detection of water pollution and the timely implementation of interventions. These monitoring processes also reveal the impacts of agricultural, urban, and industrial activities on water resources. The objective of this study is to provide a comparative evaluation of water quality monitoring activities conducted under the European Union Water Framework Directive and those carried out in Türkiye. In this context, the institutional and legal infrastructure in Türkiye has been examined, and the compatibility of existing structures with the Water Framework Directive has been analyzed. In Türkiye, the responsibility for water quality monitoring is divided among various institutional bodies. It is evident that establishing precise responsibilities and operational domains for these institutions will enable more expeditious and effective access to the requisite data. The study also offers solution proposals and policy recommendations for improving water quality monitoring systems in Türkiye.

Keywords: Water quality, Water quality monitoring, European Union water framework directive

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1. Introduction

The global population is increasing, which is expected to result in increased demand for food and water on a global scale. However, in light of the repercussions of climate change on water resources, it is imperative to employ water resources in an efficient manner.

The escalating demand for water has precipitated a series of substantial challenges. This includes the depletion of groundwater resources, the pollution and degradation of aquatic ecosystems, and the contamination of water resources due to various environmental problems. While water is considered a renewable natural resource, there are concerns that its status as a renewable resource may be compromised in certain areas, which could lead to a highly hazardous scenario. This has led to a situation where the provision and development of new water resources are not only increasingly expensive but also appear to be impractical. Consequently, there is an increasing imperative for the preservation of existing water resources in terms of both quantity and quality (Yurtseven et al., 2010).

The preponderance of global food demand is met by irrigated regions. The production of plant and animal products is entirely dependent on water, and the utilization of water in agriculture is paramount to global food security. Water resources management can be defined as the planned development, distribution, and utilization of water resources. Prior to the development of water resources, it is essential to ascertain the type, location, quantity, and quality of existing water resources. Beyond the realm of water management, ensuring the sustainable utilization of water resources is paramount. It is equally crucial to safeguard water quantity and quality, and to implement measures to avert water quality deterioration, such as pollution prevention strategies (Gökalp and Çakmak, 2016).

The most salient challenge confronting contemporary agriculture is the sustainable production of sufficient, healthy food for a growing population while preserving soil and water resources. Inadequate management, operation, monitoring, and evaluation activities in irrigation networks result in the suboptimal performance of irrigation systems. Monitoring and evaluation stand as pivotal components in enhancing water efficiency in

agriculture. The enhancement of water use efficiency in agriculture is contingent upon the advancement of soil and water management, the technology employed, and the efficacy of the monitoring and evaluation system (Torun and Çakmak, 2024).

Globally, water resources that are both safe and suitable for domestic, agricultural, and industrial use account for a mere 2.5% of the total water resources available on the planet (SKYB, 2018). Given the uneven distribution of water resources globally, the ramifications of water utilization on national policies are unavoidable. According to projections by the Turkish Statistical Institute, the population of Türkiye is expected to reach 93 million by the year 2050 (TÜİK Population Projections, 2023-2100). Preserving existing soil and water resources will ensure the availability of 1,069 m^3 of usable water per capita in 2050. This value places Türkiye in proximity to the category of water-scarce countries. Furthermore, this projection indicates that the annual availability of water may increase, potentially leading to heightened water consumption across various sectors due to the country's economic growth. This phenomenon is expected to exert mounting pressures on water resources. Consequently, enhancing water efficiency and conserving and utilizing water resources emerge as pivotal strategies to ensure the sustainability of water resources (SYGM, 2021).

It is anticipated that the issue of global food security will be exacerbated by the lack of access to clean and potable water. Ensuring food security is inherently linked to ensuring water security. According to recent estimates, approximately 2.1 billion individuals worldwide lack access to clean drinking water, while 4.4 billion people are confronted with inadequate sanitation. The interconnection between water scarcity, pollution, and limited access to water is well-documented, and the ramifications of these issues are profound. For instance, food production is vulnerable, and migration is a potential consequence (Çakmak and Torun, 2023).

Water resources management is an all-encompassing activity that necessitates the supervision of not only the quantity of water but also its quality. This multifaceted endeavor necessitates the establishment of a multidimensional organizational structure, thereby ensuring the active involvement of a diverse array of stakeholders. In this regard, endeavors aimed at safeguarding water quality assume a pivotal role in the broader context of water management. On a global scale, the "Integrated Water Resources Management" approach has emerged as a primary response to the looming threat of water scarcity. The European Union has adopted this approach, implementing the basin-based management model outlined in the "Water Framework Directive" (2000/60/EC). This directive aims to protect and control the water resources within the EU borders in terms of quantity and quality. The Directive establishes a comprehensive policy framework for Member States, mandating the protection and management of their water resources in accordance with a uniform standard.

The Sustainable Development Goals encompass a range of water-related targets, including food, energy, ecosystem, and climate change. Conversely, projections indicate that regions encompassing Türkiye will encounter significant challenges in accessing sufficient and reliable water resources in terms of quality and quantity in the imminent future. Consequently, the regular monitoring and evaluation of water resources in terms of quantity and quality has been identified as a critical measure to avert a water crisis and ensure the sustainable utilization of water resources (Gültaş et al.,2025).

In Türkiye, the primary consumers of water resources are as follows: municipalities (35.14%), thermal power plants (46.36%), manufacturing industry workplaces (14.75%), villages (2.05%), organized industrial zones (0.74%), and mining enterprises (0.95%). This data is derived from TÜİK (2022). A pivotal legislative instrument employed for the safeguarding of water resources in Türkiye is the "Water Pollution Control Regulation" (SKKY). This regulatory framework encompasses a comprehensive set of principles and prohibitions aimed at safeguarding water resources, guidelines for wastewater discharge, provisions for wastewater infrastructure facilities, and the mechanisms and principles of monitoring and inspection to prevent water pollution. In our country, the assessment of water resources is limited to physico-chemical parameters, and water quality is classified according to these parameters. However, this approach is inadequate in terms of ensuring the protection and enhancement of water resources and hinders the implementation of effective planning measures.

In this study, the current situation and practices in monitoring water quality studies in Türkiye were evaluated.

2. The Implementation of Institutional and Legal Infrastructure in Water Quality Monitoring

Water management, as defined by the United States Environmental Protection Agency (USEPA), encompasses the deliberate planning, allocation, and utilization of water resources. A critical component of this multifaceted definition pertains to the preservation of water quantity and quality, along with the assurance of its availability for future generations. In this regard, the design and operation of monitoring and evaluation mechanisms in water management should be guided by the objective of fulfilling all data requirements associated with this definition. These data sets should encompass evaluations of existing water resources and available water potential, water quality and its various qualities in terms of impact, efficiency, and sustainability. These evaluations should be conducted by comparing the data sets with the needs and consumption of different sectors.

In Türkiye, the management of water resources is a multifaceted endeavor, entrusted to a diverse array of institutions and organizations that possess a range of roles and functions. The multi-purpose use of water resources (domestic, industrial, irrigation, energy, etc.) and the fact that services related to the provision of water require different areas of expertise cause many institutions to be involved in the water management process. However, despite the efforts of various public institutions to conduct water quality monitoring studies within the scope of their legal authorities, the effectiveness of these studies is constrained by factors such as ambiguity in authority, a lack of coordination and cooperation among institutions, and the resulting duplication of efforts.

In the context of water quality monitoring studies conducted by various institutions and organizations within our nation, a duplicated monitoring approach is employed at consistent locations, with a focus on the assessment of analogous parameters. An evaluation of the laboratory infrastructures reveals that each institution possesses laboratories at the provincial and regional levels. Despite the utilization of analogous devices and analytical methodologies across these entities, inconsistencies emerge in the data obtained through analyses executed with varying methodologies and standards.

The General Directorate of State Hydraulic Works (DSİ) has been conducting water quality monitoring studies since 1978 and implementing Water Framework Directive-compliant monitoring programs since 2015. These programs ensure the monitoring of parameters in accordance with the legislation, taking into account the pressures and impacts on water bodies, and the evaluation of the results within the framework of River Basin Management Plans (RBMP). However, due to a paucity of technical infrastructure, monitoring can only be carried out in rivers. In 2018, the responsibility for water quality monitoring was transferred to the General Directorate of Water Management, and Basin Monitoring Programs are now being developed with consideration for the pressures on water resources and other pertinent factors. These programs offer a comprehensive set of parameters for assessing water quality, delineating the parameters to be monitored, the monitoring points, and the monitoring frequencies. The institutions and organizations responsible for conducting water quality monitoring studies in Türkiye are enumerated in Table 1.

3. Monitoring Water Quality in The European Union Water Framework Directive

The European Union Water Framework Directive (WFD), adopted in 2000, was developed to establish a comprehensive framework for the protection, improvement, and sustainable management of water resources in Member States. The overarching objective of the Directive is to enhance the ecological status of surface and groundwater bodies and to safeguard water quality. The SCD aims to prevent the degradation of water by natural and anthropogenic influences, establishing specific standards and monitoring programs to evaluate water quality. The monitoring of water bodies is instrumental in determining their ecological status, facilitating the identification of pollutants, biodiversity, and adverse impacts on aquatic ecosystems.

The European Union Water Framework Directive (SCD) acknowledges water quality monitoring as a pivotal element in the preservation and administration of water resources. The Directive delineates water quality monitoring as a mechanism for determining the ecological status of water bodies, detecting pollutants, and achieving environmental objectives. Water quality monitoring is a process that involves the regular assessment of surface and groundwater quality, the evaluation of ecosystem health, and the measurement of biological, chemical, and physical parameters in water bodies. This systematic observation provides precise data on the current state of water bodies, facilitating the implementation of appropriate management decisions.

According to the Water Framework Directive, water quality monitoring activities are classified under two main headings: Surveillance Monitoring and Operational Monitoring (European Commission, 2015). Surveillance monitoring is defined as the long-term observation of natural changes and anthropogenic impacts in surface waters. This type of monitoring is used to assess biodiversity and habitat quality, while determining the ecological and chemical status of water bodies (European Commission, 2015). Conversely, operational monitoring is implemented in regions where there is a potential for pollutants to enter water bodies. This monitoring is particularly crucial in areas with high pollutant loads, as it enables the rapid detection of changes in water quality and the prediction of deleterious effects (Hering et al., 2010).

The Water Framework Directive (WFD) stipulates the intensification of water quality monitoring in designated areas, including protected areas and sensitive water bodies. The Directive stipulates the monitoring of sensitive areas, including drinking water sources, habitat and species protection areas. The necessity of such monitoring is paramount for the preservation of ecosystem integrity and the maintenance of water quality for its manifold uses. Additionally, monitoring is mandatory for bathing water, water bodies utilized for recreational purposes, and the protection of economically significant aquatic species. This comprehensive monitoring framework is instrumental in developing regional water management plans and ensuring the sustainability of water resources.

Table 1. Institutions and organizations conducting water quality monitoring studies in Türkiye

Ministry	Type	Name of Institution
Ministry of Agriculture and Forestry	General Directorate	General Directorate of Water Management
		General Directorate of Nature Conservation and National Parks
		General Directorate of Combating Desertification and Erosion
		General Directorate of Food and Control
		General Directorate of Fisheries and Aquaculture
		General Directorate of Agricultural Reform
	Department Directorate	Department of information Technologies
	Research Institution	TAGEM (General Directorate of Agricultural Research and Policie)
	Subsidiary Organization	DSi (General Directorate of State Hydraulic Works)
		MGM (General Directorate of Meteorology)
		OGM (General Directorate of Forestry)
		SUEN (Water Institute)
Ministry of Environment, Urbanization and Climate Change	General Directorate	General Directorate of Spatial Planning
		General Directorate of Protection of Natural Assets
		General Directorate of Environmental Management
		General Directorate of EIA (Environmental Impact Assessment)
		General Directorate of Construction Works
		General Directorate of Infrastructure Services
		General Directorate of GIS (Geographical Information Systems)
	Subsidiary Organization	iLBANK
Ministry of Energy and Natural Resources	General Directorate	General Directorate of Renewable Energy
		General Directorate of Energy Affairs
	Subsidiary Organization	MTA (General Directorate of Mineral Research and Exploration)
		EMRA (Energy Market Regulatory Authority)
	Regional Directorates	MTA Regional Directorates
Ministry of Health	General Directorate	General Directorate of Public Health
	Provincial Agency	Community Health Centers
		Public Health Directorates
Ministry of Culture and Tourism	General Directorate	General Directorate of Investments and Enterprises
Ministry of Foreign Affairs	General Directorate	General Directorate of Energy, Water and Environmental Affairs
	Subsidiary Organization	Directorate for European Union Affairs

In accordance with the stipulations outlined in the Water Framework Directive, the findings derived from the periodic evaluation of water quality must be meticulously documented and disseminated. This information serves as a crucial foundation for the formulation of water management strategies and the implementation of targeted measures. The analysis of monitoring data facilitates the identification of measures aimed at preventing deterioration, as they emerge during the course of water quality monitoring. Moreover, EU Member States collaborate and formulate common strategies at both regional and national levels to accomplish the objectives established for the monitoring and management of water quality. This collaborative effort fosters the establishment of an effective management mechanism, thereby ensuring the long-term preservation of water bodies.

The European Union's Water Framework Directive (WFD) establishes the identification and regular monitoring of various parameters for the assessment of water quality. These parameters are used to assess the ecological status, chemical quality, and biodiversity of water bodies (European Commission, 2000). Accurate identification of the factors affecting water quality is instrumental in the development of sustainable water management and conservation strategies.

3.1. Physicochemical Parameters

- pH level
- Dissolved oxygen
- Temperature

- Electrical conductivity (EC)Total dissolved solids (TDS)
- Nutrients such as ammonia, nitrate and phosphate
- Turbidity
- Chemical oxygen demand (COD) and biological oxygen demand (BOD)

These physicochemical parameters are monitored to assess the basic chemical properties of water and its pollution levels. Of particular relevance are the levels of organic matter, nutrients, and oxygen, which directly impact the biological health of the water.

3.2. Biological Parameters

- Phytoplankton (algae)
- Zooplankton (aquatic animals)
- Benthic organisms (organisms living on the water floor)
- Secondary producers and biodiversity of ecosystems

Biological parameters are critical indicators that assess the ecological health and biodiversity of a water body. These parameters are instrumental in the ongoing monitoring of the health and sustainability of aquatic ecosystems.

3.3. Hydromorphological Parameters

- Water flow velocity and direction
- Water levels
- Physical structure of the water body (e.g. channel depth and width)
- Morphological structure of ecosystems and condition of habitats

Hydromorphological parameters are instrumental in the monitoring of the physical characteristics of a water body. These parameters assess the naturalness of water flow and the physical effects of water, including flood risk.

3.4. Contaminants (Chemical Pollutants)

- Heavy metals (e.g. mercury, lead, arsenic)
- Pesticides and other chemical pollutants
- Industrial waste and toxic substances

The parameters in question are monitored for two primary reasons: first, to detect the entry of pollutants into water bodies; and second, to determine the levels of chemical contamination of water. The presence of pollutants in water bodies can have deleterious effects on water quality and the integrity of ecosystems.

3.5. Nutrients

- Nitrogen compounds (ammonium, nitrate, nitrite)
- Phosphorus compounds

Nutrients can enter water bodies, particularly as a result of agricultural activities and wastewater discharges. Conducting regular monitoring of these parameters is instrumental in identifying issues such as eutrophication, a phenomenon characterized by an excess of nutrients leading to adverse environmental consequences. The Water Framework Directive requires monitoring of these parameters in order to provide comprehensive information on the ecological status of water bodies. Regular and systematic monitoring of these parameters provides the necessary data for the protection and improvement of water resources.

4. Water Quality Monitoring Studies in Türkiye

Legislators have established legal frameworks to establish the legal and technical principles necessary for the effective and efficient management of surface water resources (i.e., rivers, lakes, streams, reservoirs, coastal, and transitional waters) and groundwater. These frameworks include monitoring, quality classification, and determination in line with sustainable development goals, with the aim of protecting and improving water quality. In this regard, a range of legislative measures have been instituted to safeguard water resources within our nation. Türkiye's legislative harmonization process with the EU Water Framework Directive is presented chronologically in Table 2.

The Regulation on Water Pollution Control is the most significant legislative instrument employed for the protection of water resources. This regulatory framework encompasses a comprehensive set of principles and prohibitions aimed at safeguarding water resources, guidelines for the discharge of wastewater, principles for the issuance of permits for wastewater discharge, concerns pertaining to wastewater infrastructure facilities, and the methodologies and principles underlying monitoring and inspection activities aimed at preventing water pollution. In Türkiye, the current monitoring regime is limited to physico-chemical parameters, and water quality is classified according to these parameters. However, this approach is inadequate for ensuring the protection and enhancement of water resources and represents a significant impediment to effective planning. To address this gap, it is imperative to establish environmental objectives for each water source, encompassing a comprehensive range of quality elements, including chemical, physico-chemical, biological, and hydromorphological aspects.

The purpose of the By-Law on Surface Water Quality Management is to determine, classify, and monitor the biological, chemical, physico-chemical, and hydromorphological quality of surface water resources and coastal and transitional waters. This regulatory framework is designed to safeguard water resources in accordance with the principles of sustainable development, thereby ensuring the attainment of optimal water quality. The by-law encompasses a multifaceted approach, addressing the assessment of existing water quality, identification of measures for enhancement, and the administration of water resources.

Achieving optimal water quality and quantity is paramount for maintaining good water status. However, the current state of water quality monitoring in Türkiye does not fully align with the stipulations outlined in the

Water Framework Directive. Consequently, the establishment of a National Monitoring Network is imperative to ensure comprehensive and systematic water quality monitoring. Moreover, in order to safeguard surface water resources against the pernicious effects of diffuse and point source pollution, it is imperative to establish receiving environment standards and implement protective measures in accordance with these standards.

Table 2. Progress of Türkiye's legislative alignment with the EU water framework directive: A chronological overview (1999–2023)

Year	Development / Legislative Step	Description
1999	Start of EU Accession Process	Türkiye initiated full membership negotiations with the EU. Harmonization with EU environmental legislation was targeted.
2000	Start of EU Environmental Negotiations	Türkiye began aligning with EU environmental acquis.
2003	Accession Partnership Document and National Program	Harmonization with EU environmental acquis was targeted. Water sector identified as a priority area.
2004	Ministry of Environment and Forestry – EU Alignment Studies	Initiated legal infrastructure work compatible with the EU WFD in the water sector.
2006	Preliminary preparations for Water Management Department	Institutional structuring process began.
2012	Establishment of General Directorate of Water Management (GDWM)	A central authority was established for harmonization with the EU WFD. Assigned duties include basin and water quality management.
2013	Publication of Basin Protection Action Plans and Monitoring Regulation	Studies aligned with the monitoring component of the Directive were carried out.
2014	Preparation of River Basin Management Plans (RBMPs) began	One of the main requirements of the WFD was initiated.
2016	Draft Turkish Water Law prepared	Aimed to create a fully WFD-compliant legal framework, but not enacted.
2017	Strengthening of basin-based monitoring networks	Biological, chemical, and hydromorphological monitoring systems were developed in line with WFD requirements.
2019	RBMPs completed (some basins)	First plans were completed in Gediz, Büyük Menderes, and Meriç-Ergene basins.
2021	Development of Integrated Water Information System (IWIS)	Digital infrastructure was established to align with the EU's principle of knowledge-based management.
2023	Environmental Agency of Türkiye and digital monitoring	Aimed to align with EU environmental data policies through integrated digital systems.

The monitoring and evaluation of groundwater is of significant importance. This involves the continuous observation of the quality and quantity of groundwater, as well as the implementation of measures aimed at preventing and remediating pollution. The deterioration of the chemical status of groundwater, the identification of significant and increasing pollution trends, and the implementation of improvement works in this regard are critical for the protection of water resources. Water contamination resulting from the utilization of plant protection products constitutes a salient concern. The active ingredients present in these products, utilized during agricultural practices, have the potential to adversely affect the environment, thereby contributing to water contamination. Consequently, there is a need for research and development initiatives aimed at determining the presence of these substances in receiving environments. Such initiatives also aim to develop environmental quality standards and to prevent diffuse water pollution.

In Türkiye, the General Directorate of Water Management is responsible for conducting water quality monitoring studies and determining water bodies. Since 1979, the General Directorate of State Hydraulic Works has been conducting water quality monitoring studies in 25 river basins, adopting a comprehensive approach to water quality monitoring. These studies persist with the objective of identifying water bodies and establishing monitoring programs.

The effective management of the quantity and quality of groundwater is imperative for meeting the European Union's (EU) requirements for groundwater

management. In this regard, the imperative for addressing groundwater contamination and degradation, sustaining its current state of quality, and enhancing its condition where necessary, has been underscored. Consequently, the regulatory framework has been reinforced through the establishment of the "Regulation on the Protection of Groundwater against Pollution and Degradation," thereby ensuring the effective management of this vital resource.

Regular monitoring of both water quality and quantity, as well as the implementation of appropriate measures, are critical for the effective implementation of groundwater management.

The General Directorate of State Hydraulic Works has been conducting water quality monitoring in 25 river basins since 1979 in accordance with Law No. 6200. The most recent regulation, Presidential Decree No. 4 dated 15.07.2018, has expanded the scope of the General Directorate's responsibilities to include the monitoring of surface and groundwater quality. These studies are conducted by the Department of Studies, Planning and Allocations, and the water quality monitoring network has been augmented with approximately 1,375 monitoring points.

General-purpose monitoring stations are equipped with instruments capable of measuring 25 mandatory parameters, including physical and chemical parameters such as biological oxygen demand (BOD), calcium, chloride, dissolved oxygen, electrical conductivity, ammonium, nitrate, nitrite, sulfate, suspended solids, temperature, pH, total dissolved matter, organic matter, and phosphorus. In stations dedicated to the monitoring of drinking water, the measurement of 36 parameters is conducted, encompassing heavy metals, pesticides, hydrocarbons, and other pollutants.

In the context of Türkiye's EU accession process, the environment chapter was initiated for negotiations on December 21, 2009. Concurrently, the General Directorate of Water Management was established within the Ministry of Forestry and Water Affairs in 2011, thereby initiating pivotal reforms in the domain of water management. In 2014, the Regulation on the Monitoring of Surface Waters and Groundwater was established, thereby establishing the principles and guidelines for the development of monitoring programs. Concurrently, the EU Project on Capacity Building on Water Quality Monitoring facilitated the identification of water bodies and the establishment of monitoring programs in six basins (Büyük Menderes, Akarçay, Ergene, Susurluk, Sakarya and Konya Closed). In 2017, the programs underwent a revision that included the addition of protected area monitoring points, which were designed to represent sensitive water bodies (SYGM, 2025).

In 2018, the General Directorate of Water Management finalized the River Basin Management Plans for the Susurluk, Büyük Menderes, Meric-Ergene, Gediz, and Konya-Kapalı basins. In 2019, the successful completion of water quality monitoring projects in Akarçay, Western Mediterranean, Yeşilırmak and Sakarya basins was documented. In 2020, the scope of these initiatives expanded to encompass the Antalya, Western Black Sea, Eastern Black Sea, Marmara, Kızılırmak and Eastern Mediterranean basins. These projects underscore a comprehensive approach to water quality monitoring (SYGM, 2025).

The General Directorate of State Hydraulic Works has been conducting water quality monitoring activities in 25 river basins since 1979. The most recent regulation has transferred the responsibility for monitoring the quality of surface and groundwater to the Presidential Decree dated July 15, 2018. The network has expanded significantly, reaching approximately 1,375 points. However, challenges such as the absence of biological monitoring, the unability to sample from lakes, and the inadequacy of laboratory infrastructure hinder the effectiveness of water quality monitoring. To address these challenges, it is imperative to expand the scope of biological monitoring, enhance laboratory infrastructure, and augment the number of trained personnel. The overarching framework for the management of groundwater in the region has been delineated by the Regulation on the Protection of Groundwater against Pollution and Degradation, and significant strides are being made to align with the EU's stipulated requirements for groundwater management.

Among the most salient issues encountered in water quality monitoring studies in Türkiye are the dearth of biological monitoring, the inability to take samples from lakes, the inadequacy of laboratories and equipment, and personnel shortages. These obstacles impede the effective monitoring of water quality, resulting in delays in implementing necessary measures. These challenges include difficulties in sampling from water bodies such as lakes and rivers, inability to monitor biological parameters, and limited laboratory capacity. These factors collectively negatively affect the effectiveness of the monitoring network. To address these challenges, it is imperative to enhance the water quality monitoring network, expand the scope of biological monitoring, upgrade the laboratory infrastructure, and augment the number of trained personnel.

The European Union Water Framework Directive is widely acknowledged as a foundational policy document throughout Europe, aimed at safeguarding and collectively managing water resources. In the context of Türkiye's accession process to the European Union, it is imperative to assimilate the principles and requirements of the Directive into national legislation and practices to ensure the sustainable management of water resources. In this regard, a range of legal and institutional frameworks have been established in our nation, accompanied by numerous studies conducted for the implementation of these frameworks. Presently, the development and harmonization activities for these studies are ongoing. As illustrated in Table 3, a

comparative analysis is presented of the fundamental requirements of the European Union Water Framework Directive and the studies carried out in Türkiye in this context.

Table 3. EU water framework directive requirements and Türkiye's legislative compliance

Field	EU WFD Requirement	Türkiye's Legislative Compliance Status
River Basin Management	Preparation of river basin management plans	✓ Plans prepared for 25 basins, regulations published
Water Quality Monitoring	Monitoring of physical, chemical, and biological parameters	✓ A system compliant with the Surface Water Quality Regulation (2012, 2018) established
Pollution Control	Monitoring of hazardous substances and point sources	✓ Regulation on the Control of Hazardous Substances (2016) enacted
Public Participation	Public involvement in planning processes	⚠ Limited implementation, legal framework exists
Economic Analysis	Recovery of water service costs	⚠ Partially compliant, implementation insufficient
Legal Framework (Water Law)	National water legislation	✕ Water Law still in draft stage
Integrated Water Policy	Policy integrated with all relevant directives	⚠ Fragmented legislation, lack of inter-agency coordination

5. Conclusions and Recommendations

A number of issues have been identified with regard to the quality assurance and evaluation of water resources in Türkiye. Among these issues, institutional challenges, legal inadequacies, staff shortages and the unavailability of adequate technical measurement networks are particularly salient. These deficiencies pose a significant threat to the sustainable management of water resources. Consequently, there is an urgent need to implement fundamental measures to enhance the effectiveness and efficiency of water quality monitoring in Türkiye.

First and foremost, it is imperative to ensure that the institutions and organizations responsible for water quality monitoring and assessment activities operate in a more coordinated manner. Towards this end, the operation of all relevant institutions under a single framework law will eliminate legal gaps and make the management of water resources more systematic. Moreover, the establishment of an institutional infrastructure is imperative to ensure effective coordination and data sharing among all stakeholders. The establishment of such an infrastructure will enhance transparency in the processes of collecting, analyzing, and sharing water quality data, thereby facilitating more expeditious and precise decision-making.

In accordance with the EU Water Framework Directive (WFD), it is evident that an integrated water resources management approach should be incorporated into water quality monitoring and assessment. This approach delineates a comprehensive strategy encompassing all dimensions imperative for the preservation and sustainable management of surface water resources. Adopting a system based on the EU Water Framework Directive for the protection and efficient use of Türkiye's water resources will ensure an effective monitoring and management process at both local and regional levels.

Türkiye's available water resources are becoming increasingly limited due to factors such as increasing demand, drought, and pollution in basins. In this context, the monitoring and control of water quality assumes paramount importance. However, the nation currently faces deficiencies in its monitoring and evaluation systems. The absence of a unified database or information system, constrained stakeholder access to data, and inadequate inter-institutional coordination have collectively resulted in significant challenges in the effective management of water resources. The following recommendations are proposed to address these issues:

1. The enhancement of institutional capacity: Ensuring effective coordination among the institutions/organizations responsible for water quality monitoring and assessment activities is imperative, as is the establishment of a robust infrastructure for data sharing.
2. The legal infrastructure must be strengthened to protect water quality. To that end, the legal framework should be strengthened and a more comprehensive and functional legal structure should be established by combining relevant laws and regulations.
3. The measurement network must be strengthened. The measurement networks and technical infrastructure necessary for the effective implementation of water quality monitoring and assessment processes should be strengthened.
4. The establishment of a common database or information system is imperative. The establishment of a unified database is imperative to enhance the accessibility and dissemination of water quality data. This will ensure that all stakeholders have ready access to current data, facilitating its effective integration into decision-making processes.
5. Ensuring coordination: Inter-institutional coordination and cooperation in water resources

management and monitoring-evaluation studies should be ensured. The enhancement of these processes can be achieved through the initiation of collaborative academic endeavors and joint projects.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	S.A	E.Y
C	50	50
D	60	40
S	20	80
DCP	70	30
DAI	70	30
L	70	30
W	60	40
CR	20	80
SR	60	40

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision.

Conflict of Interest

The authors declare no conflict of interest.

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