JOURNAL OF AGRICULTURAL PRODUCTION

Year: 2023 | Volume: 4 | Issue: 1 | e-ISSN 2757-6620 | agriprojournal.com

JOURNAL OF AGRICULTURAL PRODUCTION

2023 • VOLUME 4 • ISSUE 1

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Journal of Agricultural Production is a peer-reviewed scientific journal published by *Gökhan ARSLAN* twice a year.

(Publisher support: ***Prensip Publishing***)

JOURNAL OF AGRICULTURAL PRODUCTION

2023 • VOLUME 4 • ISSUE 1

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JOURNAL OF AGRICULTURAL PRODUCTION

ISSN: 2757-6620

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RESEARCH ARTICLE

Effects of Boron Forms and Doses on Germination in Sunflower, Soybean, and Opium Poppy Seeds

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ARTICLE INFO

Article History
Received: 12.12.2022
Accepted: 03.02.2023
First Published: 28.04.2023

Keywords
Boron
Germination
Glycine max
Helianthus annuus
Papaver somniferum

ABSTRACT

A laboratory experiment was planned to search the inhibitory effects of boron (B) concentrations constituted by two sources on seed germination and subsequent seedling growth of sunflower, soybean, and opium poppy. Seven levels (0, 2, 4, 8, 16, 30, 60, and 90 mg B L^{-1}) of boric acid (H_3BO_3) and sodium borate ($Na_2B_8O_{13}.4H_2O$) were used. Increasing B levels slightly influenced the germination percentage of sunflower, soybean, and opium poppy. Mean germination time in sunflower and opium poppy was shortened at 60 and 90 mg B L^{-1} by increasing B, while the germination index was promoted in sunflower. However, seedling growth of sunflower, soybean, and opium poppy was considerably prohibited by B doses. The shoot length of sunflower was decreased at 16 mg B L^{-1}, but low B levels enhanced root length, indicating that shoot growth showed more sensitivity to B doses than root length. The shoot and root length of soybean did not exhibit any significant trends against B levels. The seedling length of opium poppy was diminished at 60 mg B L^{-1} and above. There were no significant differences between boric acid and sodium borate except for shoot length in soybean, germination percentage and seedling length in opium poppy. It was concluded that there were no toxic effects of B levels up to 90 mg B L^{-1} on germination of sunflower, soybean, and opium poppy, while seedling growth of sunflower and opium poppy was restricted at 60 and 90 mg B L^{-1}, respectively.

Please cite this paper as follows:

Kaya, M. D., Ergin, N., & Harmancı, P. (2023). Effects of boron forms and doses on germination in sunflower, soybean, and opium poppy seeds. *Journal of Agricultural Production*, *4*(1), 1-6. https://doi.org/10.56430/japro.1217929

1. Introduction

Boron (B) is one of the most important micronutrients for crop plants affecting plant growth and yield. It is needed for many physiological processes such as protein formation, cell division, cell wall construction, cell membrane integrity, and root growth (Marschner, 1995; Gupta, 2016). It regulates the balance between sugar and starch, and pollination and seed development (Gupta et al., 1985; Goldbach et al., 2001). B plays a critical role in seed production because healthy flowers cannot be produced, and no seeds develop in case of deficiency in B (Mozafar, 1993). The arable soils contain between 1 and 467 mg kg^{-1} of B, on average between 9 and 85 mg kg^{-1}, and B availability ranges from 0.5 to 5 mg kg^{-1} (Gupta, 2016). In Türkiye, 46.2% of the agricultural soils suffer from B deficiencies while toxic concentrations of B have been 3.3% of the total area (Kıllıoğlu, 2022). Because irrigation water is another source of boron in the soils, it is very common in arid and semi-arid conditions with low rainfall, where B cannot be sufficiently leached and reaches the toxic levels to the plants (Reid, 2007; Tanaka & Fujiwara, 2008).

The responses of plants to boron levels vary within species, cultivars, and the plant growth stages. In literature, there are a lot of researches in relation to the effects of B on plant growth, morphological and physiological changes, and yield in crop

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plants while limited studies have been focused on germination and early seedling growth. For example, B doses up to 2 kg ha^{-1} had positive effects on seed yield (Jagadala et al., 2020) and foliar application between 28 and 1200 mM increased the total dry biomass of sunflower (Asad et al., 2003). Day (2016) reported depression in plant growth with increasing levels of B up to 128 mg kg^{-1} as H_3BO_3, Beyaz et al. (2018) determined the largest leaf areas in the seedlings irrigated with water containing 1 mg B L^{-1}. In soybean, Silva et al. (2020) found the beneficial effects of 0.95 kg B ha^{-1} on growth and seed yield. However, the toxic effect of B higher than 36 kg ha^{-1} was observed in plant growth and yield of opium poppy cultivars (Günlü & Öztürk, 2008). In this study, we concentrated on determining if there was toxic or promoter levels of boron on seed germination and early seedling growth of sunflower, soybean, and opium poppy.

2. Materials and Methods

Standard germination test was conducted at the Seed Science and Technology Laboratory, Eskişehir Osmangazi University in 2022 to assess the toxicity level of boron on germination and seedling growth of sunflower, soybean, and opium poppy. In the experiment, the seeds of sunflower (LG 59580), soybean (Arısoy), and opium poppy (Çelikoğlu) were germinated at seven boron levels (2, 4, 8, 16, 30, 60, and 90 mg B L^{-1}) prepared from boric acid (17% H_3BO_3) and sodium borate (20.9% $Na_2B_8O_{13}.4H_2O$). Distilled water was attained as a control.

Four replicates of 50 seeds were inserted into three-layer filter papers irrigated with 7 mL for each paper sheet of respective boron solutions. After the filter papers with seeds were rolled, they were placed into a sealed plastic bag to prevent moisture evaporation. The packages were incubated at 20 °C in opium poppy for 10 days and at 25 °C in sunflower for 10 days and in soybean for 8 days under the dark condition. The seed with 2 mm radicle protrusion was considered as the germination criterion and germinated seeds were counted every 24 h to assess the mean germination time (MGT) [ISTA, 2018]. Germination index (GI) was also calculated according to Salehzade et al. (2009) with the following formula.

$$MGT = \frac{\sum Dn}{\sum n} \quad (1)$$

Where, n is the seed number germinated on day D, and D is the number of days from the beginning of the germination test.

$$GI = \frac{Number\ of\ germinated\ seeds}{Days\ of\ first\ count} + \cdots + \frac{Number\ of\ germinated\ seeds}{Days\ of\ the\ final\ count} \quad (2)$$

On the final day, ten seedlings from each treatment were randomly sampled to measure root length, shoot length, and seedling fresh and dry weight. Dry weights were recorded after oven drying at 70 °C for 48 h.

The experiment was arranged at a two-factor factorial in a completely randomized design with four replications. The data were analyzed by ANOVA and the differences were compared by Tukey's test ($p<0.05$) via MSTAT-C computer software program (Michigan State University, v. 2.10).

3. Results

Analysis of variance showed that any significant effects of boron sources on germination and seedling growth of sunflower were determined, while boron levels significantly affected germination percentage, mean germination time, germination index, shoot length, and seedling fresh weight (Table 1). Two-way interaction between boron forms and levels was significant for germination percentage, germination index, shoot length, root length, and seedling dry weight. MGT was shortened and GI was increased under 60-90 mg B L^{-1} compared to control. Also, the shoot length was increased up to 8 mg L^{-1} and then it decreased. Seedling dry weight was influenced by increasing B levels, but heavier SDW was observed at 60 and 90 mg L^{-1}. The interaction showed that a prominent increase or decrease was observed except for shoot length. It was reduced by increasing B levels, but sodium borate affected more adversely it than boric acid. Shoot length was decreased from 9.78 to 7.19 mg L^{-1} in boric acid and from 9.78 to 6.66 mg L^{-1} in sodium borate.

In soybean, a significant variation for boron sources was determined only in shoot length. Also, boron levels did not affect germination percentage, germination index, and seedling dry weight. Two-way interaction was significant for MGT, SL, RL, and SFW; however, a prominent increase or decrease was observed for the investigated characteristics. In each B level, similar germination percentages were observed, and boron sources and levels did not affect it significantly (Table 2). Boric acid gave a longer shoot length than sodium borate, but the other parameters did not change by boron sources.

Table 1. Germination and seedling growth parameters of sunflower under different concentrations of boric acid and sodium borate.

Factors	GP (%)	MGT (day)	GI	SL (cm)	RL (cm)	SFW (mg $plant^{-1}$)	SDW (mg $plant^{-1}$)
Boron forms (A)							
Boric acid (BA)	92.6	2.00	25.7	8.12	5.62	458	43.4
Sodium borate (SB)	93.7	1.97	26.2	8.08	6.32	460	44.5
Dose (B)							
Control	94.0^{a}	2.11^{ab}	24.8^{c}	9.78^{ab}	6.47	502^{ab}†	44.3
2 mg B L^{-1}	90.5^{b}	2.04^{b}	24.5^{c}	9.38^{b}	6.35	477^{bc}	45.1

Table 1. (continued)

Factors	GP (%)	MGT (day)	GI	SL (cm)	RL (cm)	SFW (mg plant^{-1})	SDW (mg plant^{-1})
Dose (B)							
4 mg B L^{-1}	95.0^{a}	1.91^{cd}	27.2^{b}	9.47^{b}	5.31	457^{c}	44.3
8 mg B L^{-1}	95.3^{a}	1.95^{c}	26.3^{b}	10.50^{a}	5.10	462^{c}	42.5
16 mg B L^{-1}	89.0^{b}	2.05^{ab}	24.7^{c}	6.41^{cd}	5.42	405^{d}	44.3
30 mg B L^{-1}	90.0^{b}	2.14^{a}	24.1^{c}	5.96^{d}	5.55	399^{d}	44.8
60 mg B L^{-1}	94.5^{a}	1.88^{cd}	26.8^{b}	6.36^{cd}	6.52	450^{c}	44.1
90 mg B L^{-1}	96.8^{a}	1.82^{d}	29.1^{a}	6.93^{c}	7.00	522^{a}	42.4
A×B							
BA × Control	92.0^{b-e}	2.20	23.3^{gh}	9.78^{ab}	5.56^{bc}	495	44.3^{ab}
BA × 2 mg B L^{-1}	90.5^{c-f}	2.10	23.9^{fgh}	8.96^{b}	5.96^{bc}	485	46.0^{a}
BA × 4 mg B L^{-1}	96.0^{ab}	1.92	27.3^{bc}	8.79^{b}	3.89^{c}	434	44.8^{ab}
BA × 8 mg B L^{-1}	94.5^{abc}	1.94	25.9^{b-f}	10.60^{a}	3.91^{c}	431	39.3^{c}
BA × 16 mg B L^{-1}	89.5^{def}	2.09	25.3^{c-g}	6.04^{de}	5.23^{bc}	410	42.8^{abc}
BA × 30 mg B L^{-1}	86.5^{f}	2.11	22.9^{h}	6.34^{cde}	5.85^{bc}	429	44.0^{ab}
BA × 60 mg B L^{-1}	93.5^{bcd}	1.90	26.1^{b-e}	7.27^{c}	5.86^{bc}	451	45.0^{ab}
BA × 90 mg B L^{-1}	98.0^{a}	1.78	30.7^{a}	7.19^{c}	8.66^{a}	533	41.3^{bc}
SB × Control	96.0^{ab}	2.02	26.2^{b-e}	9.78^{ab}	7.39^{ab}	510	44.3^{ab}
SB × 2 mg B L^{-1}	90.5^{c-f}	1.98	25.1^{d-h}	9.81^{ab}	6.74^{ab}	470	44.3^{ab}
SB × 4 mg B L^{-1}	94.0^{abc}	1.91	27.0^{bcd}	10.20^{a}	6.74^{ab}	481	43.8^{ab}
SB × 8 mg B L^{-1}	96.0^{ab}	1.95	26.8^{bcd}	10.40^{a}	6.30^{b}	494	45.8^{a}
SB × 16 mg B L^{-1}	88.5^{ef}	2.01	24.2^{e-h}	6.78^{cd}	5.62^{bc}	400	45.8^{a}
SB × 30 mg B L^{-1}	93.5^{bcd}	2.17	25.2^{c-g}	5.59^{e}	5.26^{bc}	370	45.5^{a}
SB × 60 mg B L^{-1}	95.5^{ab}	1.86	27.5^{b}	5.45^{e}	7.17^{ab}	449	43.3^{ab}
SB × 90 mg B L^{-1}	95.5^{ab}	1.87	27.5^{b}	6.66^{cd}	5.35^{bc}	511	43.5^{ab}
Analysis of Variance							
A	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
B	**	**	**	**	*ns*	**	*ns*
A×B	*	*ns*	**	**	**	*ns*	*

†: Letter(s) connected with the means show significance levels at p<0.05. *, **: significant at p<0.05 and 0.01; ns: not significant. GP: Germination percentage, MGT: Mean germination time, GI: Germination index, SL: Shoot length, RL: Root length, SFW: Seedling fresh weight, SDW: Seedling dry weight.

Table 2. Germination and seedling growth parameters of soybean under different concentrations of boric acid and sodium borate.

Factors	GP (%)	MGT (day)	GI	SL (cm)	RL (cm)	SFW (mg plant^{-1})	SDW (mg plant^{-1})
Boron (A)							
Boric acid (BA)	93.0	2.05	22.8	10.2^{a}	9.30	759	108
Sodium borate (SB)	92.8	2.07	23.2	9.6^{b}	9.45	768	110
Dose (B)							
Control	91.5	2.05^{bc}	23.3	10.1^{bc}	8.99^{d}	765^{bc}†	106
2 mg B L^{-1}	92.5	2.10^{a}	22.7	11.8^{a}	10.70^{a}	847^{a}	112
4 mg B L^{-1}	94.5	2.10^{a}	20.9	9.8^{bcd}	9.21^{cd}	795^{b}	107
8 mg B L^{-1}	94.0	2.13^{a}	22.9	8.8^{f}	7.93^{e}	712^{d}	112
16 mg B L^{-1}	95.5	2.09^{ab}	23.7	10.4^{b}	9.81^{bc}	781^{bc}	111
30 mg B L^{-1}	91.0	1.98^{d}	23.6	9.1^{ef}	9.29^{cd}	743^{cd}	109
60 mg B L^{-1}	91.8	1.99^{cd}	23.6	9.5^{cde}	10.50^{ab}	750^{cd}	110
90 mg B L^{-1}	92.8	2.03^{cd}	23.2	9.4^{def}	8.97^{d}	715^{d}	105
A×B							
BA × Control	92.0	2.02^{d-g}	23.4	9.2^{fgh}	8.62^{def}	721^{c-f}	105
BA × 2 mg B L^{-1}	95.0	2.07^{b-f}	23.6	13.9^{a}	12.70^{a}	928^{a}	112
BA × 4 mg B L^{-1}	92.5	2.15^{ab}	17.7	10.3^{bcd}	10.20^{c}	812^{b}	107
BA × 8 mg B L^{-1}	93.5	2.09^{bcd}	23.1	8.6^{hıi}	7.96^{f}	694^{f}	113
BA × 16 mg B L^{-1}	96.5	2.05^{def}	24.4	10.6^{bcd}	9.41^{cd}	769^{bcd}	108
BA × 30 mg B L^{-1}	90.8	1.96^{g}	24.2	7.9^{i}	6.59^{g}	710^{ef}	113
BA × 60 mg B L^{-1}	91.3	1.99^{fg}	23.1	10.7^{bc}	11.40^{b}	790^{b}	103
BA × 90 mg B L^{-1}	92.8	2.04^{d-g}	22.9	10.0^{c-f}	8.76^{def}	716^{def}	103
SB × Control	91.0	2.07^{b-e}	23.3	10.9^{b}	9.37^{cd}	809^{b}	108

Table 2. (continued)

Factors	GP (%)	MGT (day)	GI	SL (cm)	RL (cm)	SFW (mg plant^{-1})	SDW (mg plant^{-1})
A×B							
SB × 2 mg B L^{-1}	90.0	2.14^{abc}	21.7	9.7^{d-g}	8.74^{def}	767^{b-e}	112
SB × 4 mg B L^{-1}	96.5	2.06^{c-f}	24.1	9.3^{e-h}	8.27^{ef}	779^{bc}	108
SB × 8 mg B L^{-1}	94.5	2.18^{a}	22.7	9.1^{ghı}	7.90^{f}	729^{c-f}	112
SB × 16 mg B L^{-1}	94.5	2.13^{abc}	23.0	10.3^{bcd}	10.20^{c}	792^{b}	115
SB × 30 mg B L^{-1}	91.3	2.00^{efg}	23.0	10.2^{b-e}	11.90^{ab}	776^{bc}	104
SB × 60 mg B L^{-1}	92.3	1.99^{fg}	24.0	8.3^{ıi}	9.47^{cd}	709^{ef}	116
SB × 90 mg B L^{-1}	92.8	2.01^{efg}	23.6	8.7^{hıi}	9.18^{cde}	714^{def}	107
Analysis of Variance							
A	*ns*	*ns*	*ns*	**	*ns*	*ns*	*ns*
B	*ns*	**	*ns*	**	**	**	*ns*
A×*B*	*ns*	*	*ns*	**	**	**	*ns*

†: Letter(s) connected with the means show significance levels at p<0.05. *, **: significant at p<0.05 and 0.01; ns: not significant. GP: Germination percentage, MGT: Mean germination time, GI: Germination index, SL: Shoot length, RL: Root length, SFW: Seedling fresh weight, SDW: Seedling dry weight.

Table 3. Germination characteristics of poppy seeds under different concentrations of boric acid and sodium borate.

Factors	GP (%)	MGT (day)	GI	SL (cm)
Boron (A)				
Boric acid (BA)	96.1^{a}	2.07	23.5	4.93^{a}†
Sodium borate (SB)	94.6^{b}	2.06	23.3	4.47^{b}
Dose (B)				
Control	97.8^{a}	2.11^{b}	23.5	4.69^{bc}
2 mg B L^{-1}	96.3^{ab}	2.11^{b}	23.2	4.74^{abc}
4 mg B L^{-1}	96.5^{ab}	2.16^{a}	22.9	4.85^{ab}
8 mg B L^{-1}	96.5^{ab}	2.09^{b}	23.4	4.85^{ab}
16 mg B L^{-1}	92.5^{c}	2.00^{d}	23.3	4.80^{ab}
30 mg B L^{-1}	95.3^{abc}	2.04^{c}	23.6	4.95^{a}
60 mg B L^{-1}	94.0^{bc}	1.99^{d}	23.8	4.51^{c}
90 mg B L^{-1}	94.0^{bc}	1.99^{d}	23.8	4.20^{d}
A×B				
BA × Control	98.5	2.13^{bc}	23.5	4.67^{d-g}
BA × 2 mg B L^{-1}	97.5	2.14^{b}	23.3	5.01^{a-d}
BA × 4 mg B L^{-1}	97.0	2.11^{bcd}	23.3	4.57^{efg}
BA × 8 mg B L^{-1}	97.5	2.11^{bcd}	23.5	4.87^{b-e}
BA × 16 mg B L^{-1}	94.0	2.02^{fg}	23.5	5.20^{ab}
BA × 30 mg B L^{-1}	96.0	2.06^{ef}	23.5	5.33^{a}
BA × 60 mg B L^{-1}	94.0	1.99^{g}	23.8	4.90^{b-e}
BA × 90 mg B L^{-1}	94.5	1.99^{g}	23.8	4.89^{b-e}
SB × Control	97.0	2.09^{cde}	23.5	4.72^{d-g}
SB × 2 mg B L^{-1}	95.0	2.08^{de}	23.1	4.47^{fgh}
SB × 4 mg B L^{-1}	96.0	2.21^{a}	22.4	5.13^{abc}
SB × 8 mg B L^{-1}	95.5	2.08^{de}	23.3	4.83^{c-f}
SB × 16 mg B L^{-1}	91.0	1.99^{g}	23.0	4.41^{gh}
SB × 30 mg B L^{-1}	94.5	2.03^{fg}	23.6	4.58^{efg}
SB × 60 mg B L^{-1}	94.0	2.01^{g}	23.9	4.12^{h}
SB × 90 mg B L^{-1}	93.5	1.99^{g}	23.8	3.50^{ı}
Analysis of Variance				
A	*	*ns*	*ns*	**
B	*	**	*ns*	**
A×*B*	*ns*	**	*ns*	**

†: Letter(s) connected with the means show significance levels at p<0.05. *, **: significant at p<0.05 and 0.01; ns: not significant. GP: Germination percentage, MGT: Mean germination time, GI: Germination index, SL: Seedling length.

Seed germination of opium poppy was higher in boric acid than sodium borate, such increases were attained in shoot length (Table 3). In addition, mean germination time and seedling length were reduced at 60 and 90 mg B L^{-1}. Germination index was not changed by B applications. Two-way interaction in MGT and SL exhibited that differences between control and 90 mg B L^{-1} were similar to each other, but the seedling length was reduced by increasing levels of sodium borate.

4. Discussion

This study exhibited the germination and early seedling growth parameters of sunflower, soybean, and opium poppy under various levels of boron sources. Considering germination parameters, soybean was not affected by B levels whose effects were found uneven. Although there were statistical differences among B levels in sunflower and opium poppy, which did not exactly reflect any trends upward or downward. Similar results were also observed in sunflower and opium poppy. It could be easily said that the germination of these species was not influenced by boron doses up to 90 mg B L^{-1} only if a 2 mm of radicle hook from the seeds was considered the germination criterion. Our results showed similarity with the findings of Patil et al. (2012) in soybean, Lima (1998) in pea, and Jadhav and Bhamburdekar (2014) in sorghum, who determined that no significant reduction in germination percentage due to increasing B. In contrast, Ashagre et al. (2014) reported the seeds of safflower did not germinate at 8 and 16 ppm B levels and Bonilla et al. (2004) found a reduction in germination percentage in pea. This may result from that they considered the appearance of radicle and hypocotyl from the seeds as the germination criterion. In sunflower, mean germination time shortened slightly and the germination index increased at 60 and 90 mg B L^{-1}. However, any clear tendency was observed in soybean and opium poppy.

Seedling growth of sunflower, soybean, and opium poppy was more sensitive to B levels than germination. The shoot length of the sunflower declined significantly at 60 and 90 mg B L^{-1}, while a precise B level decreasing root and shoot length in soybean could not be detected. Our results disagree with the findings reported by Ashagre et al. (2014) showed that root and shoot growth of safflower were linearly dropped by increasing B levels and higher levels than 4 ppm B were toxic. Jadhav and Bhamburdekar (2014) found the promoter effect of 5 and 10 ppm B, while higher levels led to decreasing in root and shoot length of sorghum. Culpan et al. (2019) reported a significant reduction in seedling growth of safflower when B doses were increased. In the previous researches, there have been controversial results regarding boron's effects on germination and seedling growth of various plants, which were detected positive neutral, and toxic. Although this may be explained by species and cultivar differences, here we suggest that the seed vigor should be considered for germination performance under boron stress.

5. Conclusion

In conclusion, our results showed that there was no remarkable adverse effect of boron on germination parameters of sunflower, soybean, and opium poppy seeds. Although the shoot length of sunflower and seedling length of opium poppy were diminished by increasing boron levels, the beneficial effects of boron doses between 2 and 8 mg B L^{-1} were found for seedling growth of sunflower, soybean, and opium poppy. It was concluded that the inhibitory effects of boron for sunflower and opium poppy started at 60 mg L^{-1}, while soybean did not show a clear sensitivity to boron levels.

Conflict of Interest

The authors declare that they have no conflict of interest.

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JOURNAL OF AGRICULTURAL PRODUCTION

ISSN: 2757-6620

https://prensip.gen.tr

RESEARCH ARTICLE

Spatial Distribution of Microplastic Contamination in the Invasive Red Sea Mussel *Brachidontes pharaonis* (Fischer P., 1870) Around the İskenderun Bay

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ARTICLE INFO

Article History
Received: 11.01.2023
Accepted: 07.03.2023
First Published: 16.05.2023

Keywords
Microplastic
Mussel
Northeastern Mediterranean Sea
Pollution
Türkiye

ABSTRACT

This study is first study reporting the microplastic abundance in soft tissues of a bivalvae *Brachidontes pharaonis* collected from 4 stations of İskenderun Bay. A total of 245 *B. pharaonis* specimens were examined and results showed that among examined specimens, 95 of them contained microplastic in their soft tissues. When all the data combined, mean MP abundance was found as 0.4±0.5 MPs ind^{-1} and 0.3±0.4 MPs g^{-1} ww. Fibers were predominant type of MPs and accounted for 75% of total extracted MPs, followed by fragments (25%). Majority of MPs were less than 1 mm and black. Fourier transform infrared spectroscopy (FTIR) showed that the extracted MPs were polypropylene (PP), polyethylene (PE), and polyethylene terephthalate (PET). Identified polymer types indicate that aquatic biota impacted by the anthropogenic influences such as agriculture, farming, fishing, household, etc. Results obtained in this study contribute the knowledge related with the microplastic contamination levels in marine biota.

Please cite this paper as follows:

Yücel, N., & Kılıç, E. (2023). Spatial distribution of microplastic contamination in the invasive red sea mussel *Brachidontes pharaonis* (Fischer P., 1870) around the İskenderun Bay. *Journal of Agricultural Production*, *4*(1), 7-15. https://doi.org/10.56430/japro.1232650

1. Introduction

Microplastics (MPs) defined as the plastic particles less than 5 mm (Arthur et al., 2009) are growing global problem. Especially after the establishment of the Marine Strategy Framework Directive which promotes to achieve good ecological status in terms of microplastic density in marine environments (European Commission, 2010), studies dealing with MP distribution, MP density and MP ingestion in aquatic animals are increased considerably.

So far, presence of MPs have been reported from marine environment i.e., sea surface (Suaria et al., 2016; Güven et al., 2017; Gedik et al., 2022a), seabed (Cheang et al., 2018; Erkan et al., 2021), and surface sediments (Wang et al., 2017; Abidli et al., 2018, Aytan et al., 2020). Since MPs are ubiquitous in marine environments, their interaction with marine bioata becomes inevitable. Till date, microplastic ingestion have been reported from many aquatic animals such as zooplankton (Beer et al., 2018), bivalve (Ding et al., 2021; Yozukmaz, 2021),

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crustecean (Yücel, 2022; Yücel & Kılıç, 2022), fish (Kılıç & Yücel, 2022). Among them, bivalves are more vulnerable to MP pollution due to their "extensive filter-feeding activity" (J. Li et al., 2019). Mussels, a member of bivalve class, are exposing to MP contamination by adherence in addition to the ingestion (Kolandhasamy et al., 2018). Laboratory studies showed that MPs intake reduces attachment strength, alters haemolymph proteome (Green et al., 2019), and affects the health and fecundity of mussels (Woods et al., 2018).

Mussels have a key function in benthic-pelagic ecosystem (Strayer et al., 1999). While mussels filter water from the water column, they concentrate the nutrients and particles found in the water column and create biodeposits which are transfer normally unavailable nutrients to the benthic organisms (Harris et al., 2021). In this way, they transfer the MPs found in the sea surface to the sea bottom. Recent study showed that MP presence cause variations in the sinking and resuspension rates (Harris et al., 2021) which lead to variations in the "ecosystem services provided by mussels" (Woods et al., 2018). In addition, MPs transfer to the upper trophic levels is also possible via predation (Santana et al., 2017; Renzi et al., 2018).

Due to their significant ecological role, global distribution, easy collection, high tolerance to environmental pollutants, sessile nature (reviewed from J. Li et al., 2019), mussels are widely used as bioindicator of environmental contaminants. Suitability of mussels as bioindicators of MP pollution have been evaluated by many researchers. some studies showed that mussels are excellent bioindicator of MP contamination (Li et al., 2016; Fossi et al., 2018; J. Li et al., 2019; Ding et al., 2021) and gives information regarding MPs contamination in the both sea surface and littoral zone. Yet, some others report that mussels should be used as global indicators (Ward et al., 2019; Hoellein et al., 2021) which indicates that species based studies needs to be carried out to be obtain a clear result.

The present study investigates the microplastic ingestion in invasive *Brachidontes pharaonis* together with investigating the type, color, size of extracting MPs in the soft tissues. Results obtained in this study contribute the knowledge related with the microplastic contamination levels in marine biota.

2. Materials and Methods

2.1. Study Area and Sampling

İskenderun Bay is located in the northeastern part of the Mediterranean Sea (Figure 1). Highly urbanized coastal area, intense industrial activities lead to discharge of a significant number of pollutants including MPs. Also, dredging activities applied in ports cause resuspension of MPs found in the sediment layer (Preston-Whyte et al., 2021), marine vessels and fishing equipment form an additional source of MPs (Nel et al., 2017). İskenderun Bay collects, accumulates and distributes this incoming pollution load depending on main surface flows and dominant wind directions (Figure 1). Previous studies showed that microplastic pollution is significant in the İskenderun Bay (Güven et al., 2017) which result of microplastic presence in the gastrointestinal tract of marine fish (Kılıç, 2022; Kılıç & Yücel, 2022) and crustecea (Yücel, 2022).

In order to evaluate variations in the MPs accumulation levels along the bay, four monitoring stations (i.e., İskenderun, Çevlik, Dörtyol, and Karataş) were selected (Figure 1). From each station, 70-75 individuals of mussels which were close to the sea surface were randomly collected by hand with a metal knife in September 2022. The selected individuals were washed with distilled water and wrapped with tin foil. Then, they were placed in ice bag and transported to the laboratory.

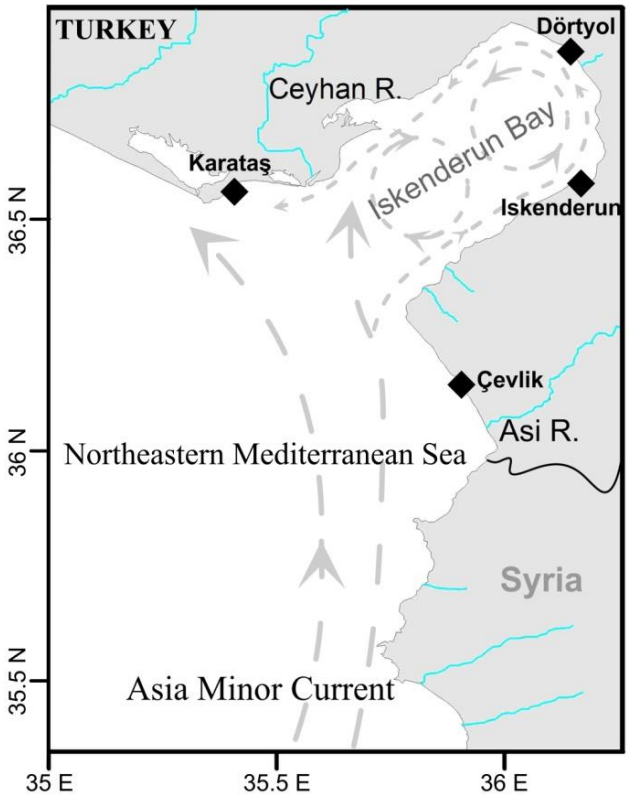

Figure 1. Sampling locations (black rhombus) and sea surface currents [modified from Özsoy and Sözer (2006)] in the İskenderun Bay.

2.2. MP Extraction

At first, the surface of each specimen was cleaned with prefiltered pure water in order to prevent contamination. Then, each specimen was weighed and morphological characteristics were measured (i.e., shell length, width, and height). Next, the soft tissue of the mussel was removed by tweezer forceps carefully and weighed (wet weight). The soft tissue of each specimen was washed with prefiltered pure water and placed in separate glass beakers which were covered with tin foil. When the dissection process completed, 50 mL of H_2O_2 was added to the beakers. Then, the beakers were placed on a hot plate and kept at 65 °C for 12 hours until the solution was homogenized

and soft parts were completely digested (Pazos et al., 2020). Finally, the remaining solution was filtered through 50 µm mesh filters and placed in a sterile glass petri dish.

2.3. Microscopic Examination and Polymer Identification

Filters were examined for the existence of microplastic particles by Olympus SZX7 microscope. Under microscope, each suspected particle was checked with hot needle to estimate the plastic nature. Identified particles were classified according to type (fiber, fragment), color (black, red, blue, white, transparent, green, brown, silver and yellow) and size. Filters which have MPs larger than 1 mm were placed in glass petri dishes and set aside for Fourier transform infrared (FTIR) analysis.

To evaluate the polymer configuration of detected particles, Fourier transform infrared spectroscopy (FTIR) was employed. At this stage, out of 104 MPs, 28 MPs suitable in size were used as subsamples and analyzed for polymer identification. FTIR analysis was carried out on a SHIMADZU QATR10 FTIR spectrophotometer equipped with single reflection attenuated total reflectance (ATR) accessory. The spectrum range was arranged as 4000-400 cm^{-1} and a resolution was set to 4.0 cm^{-1} with 32 scans for each measurement. The polymer type was identified by comparing absorbance spectra to reference libraries of SHIMADZU library

2.3.1. Contamination prevention

The critical step of microplastic examination is contamination prevention. For that reason, comprehensive precautions were employed to prevent airborne contamination. First, each step of the study was performed in closed laboratories with restricted access. Only authorized personnel were allowed to enter the laboratories and they wore nitrile gloves and cotton aprons at all times. Before any analysis, laboratory surfaces, dissection equipment and glass beakers were cleaned twice with prefiltered distilled water. Filters and sterile petri dishes were checked under the microscope for the presence of MPs before use. Three wet blank filters were placed in the laboratory during dissection and microscopic examination steps. Even though we applied all necessary precautions, 1 fiber particle was detected in one blank filter (out of 6 blank filters). Particle detection rate in the blank filters was 0.17±0.41 which indicates that results were scientifically acceptable. The data was corrected by extracting the contamination data.

2.4. Statistical Analysis

Since normality of the dataset could not be verified by the Shapiro-Wilk test, PerMANOVA which is a non-parametric test of significant difference was employed to detect variations in the MPs abundance between stations. Non-parametric spearman correlation analysis was applied to test correlation between morphological features of *Brachidontes pharaonis* (i.e., shell length, height, weight, wet weight) and MPs abundance. Significance level of 0.05 was set for all statistical computations ($p<0.05$).

3. Results

In this study, total of 245 mussels, collected from 4 different stations along to İskenderun Bay, were examined. Mean length and weight of all examined mussels were found as 15.3±13.7 mm and 3.0±0.9 g, respectively. Information regarding other morphological averages depending on stations were given in Table 1.

A total of 104 MPs particles were detected (Figure 2). When all the data combined, mean MP abundance was found as 0.4±0.5 MPs ind^{-1} and 0.3±0.4 MPs g^{-1} ww. Depending on stations mean MP abundance was varied from 0.2±0.5 MPs ind^{-1} to 0.6±0.6 MPs ind^{-1} and from 0.1±0.3 MPs g^{-1} ww to 0.4±0.5 MPs g^{-1} ww (Table 1). The highest MP quantity per individual and per soft tissue (ww) were detected in the Çevlik station. Whereas, the lowest MPs amount per individual and per soft tissue (ww) were found in İskenderun station. The microplastic abundance in the mussels collected from Çevlik stations were found to be significantly different than other stations in terms of both MPs per individual (ind) and MPs per soft tissue ($p<0.05$). In addition, MPs accumulation in terms of MPs per gr, MPs accumulation levels between Karataş and İskenderun, Dörtyol and Çevlik, Dörtyol and İskenderun showed significant variations ($p<0.05$).

Detected MPs were divided into two categories as fiber and fragment. Fiber were the most commonly found microplastic type, accounting for 75% of total extracted MPs. Rest of the extracted MP particles (25%) were fragments (Figure 3). Pellet shape MPs which is major outcome of primary sources were not detected in this study.

In terms of color, approximately half of the extracted MP particle were black (52%) and followed by blue (31%), red (7%), green (7%), brown (3%), and silver (1%), respectively (Figure 3). Size of detected MPs were varied from 80 µm to 3150 µm with a mean of 875±675 µm. Majority of the extracted MPs were smaller than 1000 µm in size.

In this study, the origin of 27% of the total extracted MPs was used in the FTIR analysis. Among 28 examined MPs, 4 suspected particles were found to be organic in nature and polymer type of 5 suspected particles could not be determined. Remaining particles were found to be polyethylene (PE) [32%], polypropylene (PP) [21%], polyethylene terephthalate (PET) [14%].

Table 1. Microplastic abundance in the soft tissue of *Brachidontes pharaonis* along the İskenderun Bay, Türkiye.

Station	n	Length (mm)	Width (mm)	Height (mm)	Total weight (g)	Soft tissue weight (g)	Total number of MPs	MPs abundance (MPs ind^{-1})	MPs abundance (MPs gr^{-1} ww)	Occurrence rate (%)
Karataş	70	25.8±10.1	11.4±4.4	10.6±4.4	2.7±1.0	1.4±0.5	24	0.3±0.5^{a}	0.3±0.5^{ab}	33
Dörtyol	70	14.9±7.6	5.1±3.1	8.1±3.1	2.9±0.8	1.5±0.4	20	0.3±0.5^{a}	0.2±0.3^{ac}	27
İskenderun	70	15.0±8.6	6.1±2.9	7.2±3.4	3.2±0.9	1.6±0.4	16	0.2±0.5^{a}	0.1±0.3^{c}	21
Çevlik	75	24.7±11.2	10.7±4.8	9.8±4.7	3.0±0.8	1.5±0.42	44	0.6±0.6^{b}	0.4±0.5^{bc}	50

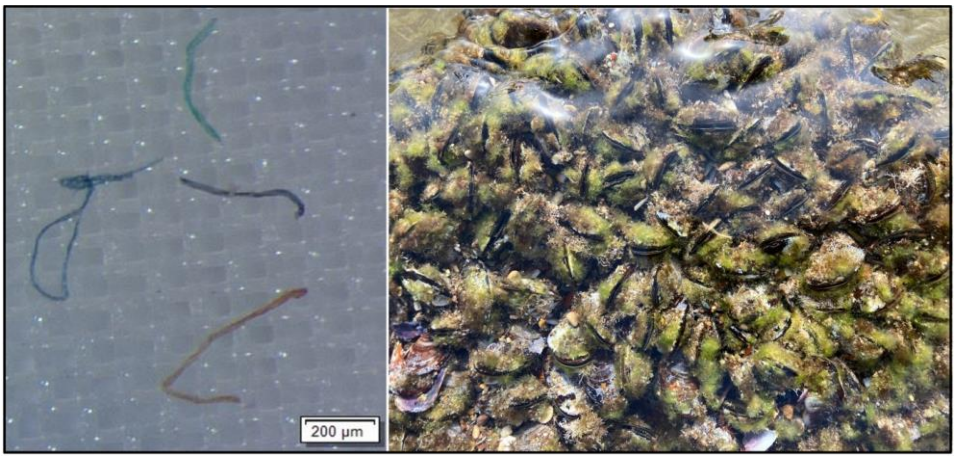

Figure 2. Representative image of fibers extracted from the soft tissues of *Brachidontes pharaonis* (left), photograph of *Brachidontes pharaonis* colony (right).

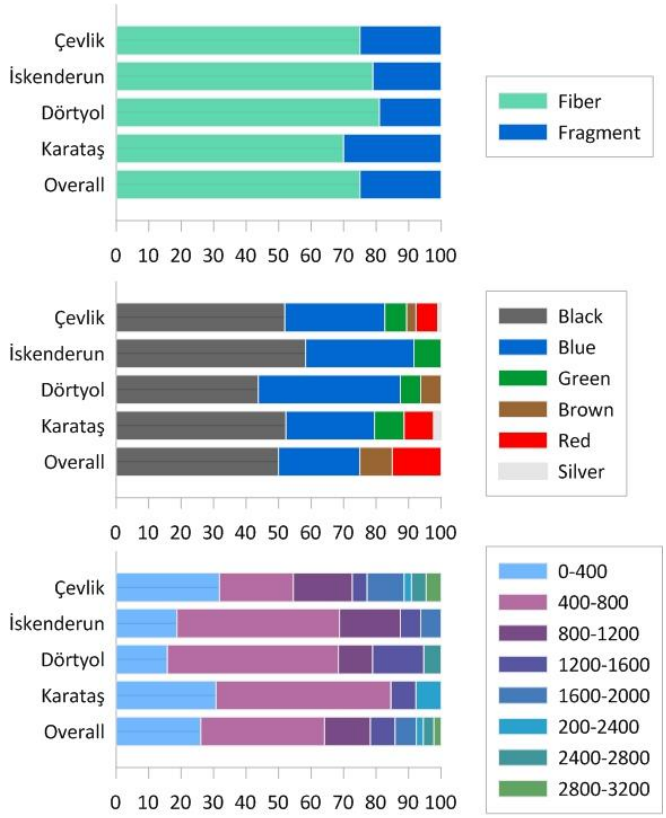

Figure 3. Percentage distribution of MPs characteristics in terms of shape (above), color (middle), and size in µm (below).

4. Discussion

İskenderun Bay is located inside the biggest plastic hotspot of the Mediterranean Sea (Papadimitriu & Allinson, 2022). As result of urbanized coastal line and intense industrial activities, severe microplastic pollution has been reported in the study area (Güven et al., 2017). Studies evaluating microplastic abundance in marine biota in the İskenderun Bay have been limited with fish (Kılıç, 2022; Kılıç & Yücel, 2022) and crustecea (Yücel, 2022) and missing in terms of bivalves. This study is first study reporting the microplastic abundance in soft tissues of a red sea mussel *Brachidontes pharaonis* from İskenderun Bay.

Mussels are sessile species and they can uptake microplastic particles from marine environments while filtering the water. In addition laboratory studies have demonstrated that MP uptake could be driven by adherence (Kolandhasamy et al., 2018) and fusion by mussel byssus (Q. Li et al., 2019). In this study, almost half of examined *Brachidontes pharaonis* specimens contained MPs in their soft tissues (45% of 245 specimens). Considering their biological mechanisms of mussels, results demonstrate that MP contamination is inevitable when MPs are present in the littoral zone. Similar to this study, previous studies also showed that mussels can uptake significant amount of microplastics in their soft tissues (Pedersen et al., 2020; Wakkaf et al., 2020; Joyce & Falkenbercg, 2023).

Since this study is the first attempt to report microplastic abundance in the *Brachidontes pharaonis*, results could only be compared with studies employing other mussel species. Previous studies reported higher MPs occurrence rates than the results of this study. The MPs occurrence frequency in the *Mytilus galloprovincialis* as 46.3% in northern Ionian Sea (Digka et al., 2018), 64% in Turkish coastline (Gedik & Eryaşar, 2020), 97% in Bizerte lagoon, northern Tunisia (Wakkaf et al., 2020). Joyce and Falkenbercg (2023) reported the microplastic occurrence in the soft tissues of *Brachidontes variabilis, Perna viridis, Xenostrobus secures* as 67% from Hong Kong. Lastly, microplastic occurrence rate in the *Limnoperna fortune from* Río de la Plata estuary, Argentina was reported as 96% (Pazos et al., 2020).

Microplastic abundance in terms of both MPs per ind and MPs per gr (ww) were consisted with the previous reports (Table 2). Only exception is Digka et al. (2018) who reported higher MPs accumulation rates than this study. Different from this study, they used gills and digestive glands of the mussels rather than whole soft tissue of mussel which might cause variations in the MPs accumulation.

Table 2. Some studies reporting the microplastic abundance in mussel species.

Location	Species	MPs abundance (MPs ind^{-1})	MPs abundance (MPs gr^{-1} ww)	Dominant type	Dominant polymer	References
İskenderun Bay, Türkiye	*Brachidontes pharaonis*	0.4±0.5	0.3±0.4	Fiber	PE, PP	This study
Aegean Sea, Türkiye	*Pinctada imbricata radiata*	2.16	-	Fiber	PE	Aksakal et al. (2021)
Bizerte lagoon, Northern Tunisia	*Mytilus galloprovincialis*	0.4±0.2	2.1±1.0	Fiber	PE, PP	Wakkaf et al. (2020)
Tolo Harbour, Hong Kong	*Brachidonte variabilis*	0.2-0.7	0.9-3.1	Fiber	-	Joyce and Falkenbercg (2023)
Tolo Harbour, Hong Kong	*Perna viridis*	0.6-3.1	2.4-15.1	Fiber	-	Joyce and Falkenbercg (2023)
Tolo Harbour, Hong Kong	*Xenostrobus securis*	2.88-14.67	1.5-8.4	Fiber	-	Joyce and Falkenbercg (2023)
Marmara Sea, Türkiye	*Mytilus galloprovincialis*	0.30 -7.53	0.11 to 4.58	Fiber	PET	Gedik et al. (2022b)
Black Sea	*Chamelea gallina*	0.22–2.17	-	Fiber	PET, PE, PP	Gedik and Gozler (2022)
Pays de la Loire region, France	*Mytilus edulis*	0.60±0.56	0.23±0.20	Fragment	PE, PP	Phuong et al. (2018)
Turkish coast	*Mytilus galloprovincialis*	0.06-2.47	0.02-1.12	Fragment	PET, PE, PP	Gedik and Eryaşar (2020)
Río de la Plata estuary, Argentina	*Limnoperna fortune*	0.43±0.35	2.08±1.33	Fiber	-	Pazos et al. (2020)
Northern Ionian Sea	*Mytilus galloprovincialis*	1.9±0.2	5.3±0.5	Fragment	PE	Digka et al. (2018)

Even though MP uptake is common report in all mentioned studies, variations observed in MPs accumulation and occurrence rate are arise from many environmental and methodological differences. First of all, since these species sessile, their MPs ingestion is primary affected by the MPs concentration in the surrounding environment. In addition, environmental factors such as salinity (Khoironi et al., 2018) and season (Ding et al., 2021) cause variations in the microplastic uptake. Lastly, lack of common applied methodology makes comparison harder since different methodologies may lead to under or overestimation.

In this study, MP accumulation in terms of MPs per ind and MPs per gr were significantly higher in Çevlik station ($p<0.05$). Significant amount of plastic were transported to coastal part of Çevlik station due to main current system and wind directions (Yılmaz et al., 2022). In addition, many waste deposition peaks were formed around the discharge zone of Orontes River (Yılmaz et al., 2022) which seems to be increase the microplastic amount in the coastal shore. In a basic sense, the higher microplastic contamination in the habitat of *Brachidontes pharaonis*, the higher the microplastic accumulation in the mussel. This tendency has been also demonstrated by previous studies (Li et al., 2016; Phuong et al., 2018).

Statistical analysis did not reveal a correlation between mussel length and MP abundance as well as mussel weight and MP abundance. Similar outcome was also reported in previous reports in mussels (Phuong et al., 2018; Scott et al., 2019; Gedik et al., 2022b). This outcome clarifies that MPs accumulation is mostly depended on the contamination level in the animals' habitat rather than its size.

Shape of MPs is directly related with the bioavailability of MP particle since it affects their accumulation potential and adverse effects (Qu et al., 2018; Fernández & Albentosa, 2019). Only fiber and fragment MPs were detected in this study, which is reported to be most commonly extracted MPs shapes (J. Li et al., 2019). We did not find any pellet shape MPs which is probably related with the selective ingestion of *Brachidontes pharaonis*. Because, Alnajar et al. (2021) detected that filter feeding mussels do not ingest regular shape particles (i.e. pellet, microbead) under controlled conditions. On the other hand, fiber shape MPs more easily ingested by mussels and get trapped in the gills and hepatopancreas (Renzi et al., 2018). In parallel with this, majority of the extracted MPs (75%) were fiber in shape which coincide with previous studies in mussels (Li et al., 2016; Qu et al., 2018; Renzi et al., 2018; Pazos et al. 2020; Wakkaf et al., 2020; Ding et al., 2021; Yozukmaz, 2021; Gedik & Gozler, 2022; Gedik et al., 2022b; Joyce & Falkenbercg, 2023). A recent study showed that microfibers act like a vectors leading to an indirect toxicity of chemicals, metals, monomers, dyes (Alnajar et al., 2021).

On the other hand, dominance of fragment shape MPs was also reported in many studies (Digka et al. 2018; Phuong et al. 2018; Gedik & Eryaşar, 2020). According to the Digka et al. (2018), variations in the predominant shape is related with the anthropogenic activities around the study area such as poor waste management strategies, recreational fishing and tourism. These activities are reported to be increase the fragment type MPs in the marine environment. Similarly, poor sewage system could cause increase in the fiber shape MPs since millions of fibers could be released from a single laundry (Galafassi et al., 2019).

Size of extracted MP was is usually limited with the size of the animal (Jâms et al., 2020). In line with this, small size MP (<1 mm) particles were dominant in this study. Similar observation was also reported in the previous studies (Li et al., 2016; Bråte et al., 2018; Digka et al., 2018; Pazos et al., 2020; Gedik et al., 2022b). While considering the lab scale studies which reporting the translocation of small size MPs in the circularity systems and other organs (Browne et al., 2008; von Moos et al., 2012), dominance of small size MPs might also be an outcome of translocation and accumulation besides ingestion organs.

In terms of color, black colored MPs were dominant in all examined stations which overlap with the previous results reporting the MPs abundance in the fish (Kılıç, 2022; Kılıç & Yücel, 2022) and crustecea (Yücel, 2022) samples from the İskenderun Bay.

Polymer type is an important parameter which influences the uptake of MPs by mussels. In this study, PE, PP, and PET were the dominant type of polymers which is similar with the previous findings (see Table 2). These polymers were globally most demanded and produced polymers (Plastic Europe, 2022) which increase the exposure risk of marine biota. They are widely used in many areas including but not limited to agriculture, farming, fishing ropes, household, water bottles, packaging (Plastic Europe, 2022).

5. Conclusion

Till date, most of the research was focused on commercially important mussel species, while neglecting other "ecosystem engineering species" (Joyce & Falkenbercg, 2023). This study is first study reporting the microplastic abundance in soft tissues of a bivalve *Brachidontes pharaonis* from İskenderun Bay. Results showed the severity of MPs contamination in the region. Fibers were the major dominant type of polymers which demonstrates the pressure of highly urbanized and industrialized coastal area. Polypropylene and polyethylene are the main polymer types which also indicate the impact of anthropogenic activities in the marine biota. More studies needs to be carried out to understand the MPs transport in the study area.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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JOURNAL OF AGRICULTURAL PRODUCTION

ISSN: 2757-6620

https://prensip.gen.tr

RESEARCH ARTICLE

Modeling and Forecasting Uganda's Beef and Cattle Milk Production using the Box-Jenkins Methodology

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ARTICLE INFO

Article History
Received: 31.10.2022
Accepted: 16.03.2023
First Published: 16.05.2023

Keywords
ARIMA
Beef production
Box-Jenkins
Dairy production
Forecasting
Uganda

ABSTRACT

Beef and cattle milk production play a significant role in reducing hunger, malnutrition, and rural poverty, improving rural livelihoods, creating employment opportunities, and supporting the overall development of Uganda's economy. This study was conducted to find a suitable ARIMA model for forecasting Uganda's beef and cattle milk production using annual time series data from 1961 to 2020, extracted from the Food and Agriculture Organization Corporate Statistical Database (FAOSTAT). Following patterns of the Autocorrelation Function and Partial Autocorrelation Function plots of the differenced series, 4 tentative ARIMA models were identified for milk production, i.e., ARIMA (0,1,0), ARIMA (1,1,0), ARIMA (0,1,1), and ARIMA (1,1,1). While 3 tentative ARIMA models were identified for beef production, i.e., ARIMA (1,1,1), ARIMA (1,1,0), and ARIMA (0,1,1). ARIMA (0,1,0) model was selected to be the most suitable for forecasting cattle milk production because it had the smallest MAPE and Normalized BIC values. On the other hand, ARIMA (1,1,0) was selected to be the best model for forecasting beef production because it had the smallest normalized BIC value and a significant coefficient of the autoregressive component. Forecasts show that milk production will increase at an annual average rate of 1.63%, while beef production will increase at an annual average rate of 0.39% in the five-year forecast period (2021-2025). These findings are important in designing strategies to improve the beef and dairy livestock sub-sectors in Uganda.

Please cite this paper as follows:

Waiswa, D. (2023). Modeling and forecasting Uganda's beef and cattle milk production using the Box-Jenkins methodology. *Journal of Agricultural Production*, *4*(1), 16-29. https://doi.org/10.56430/japro.1197299

1. Introduction

The livestock subsector contributes about 3.5% of Uganda's national Gross Domestic Product (GDP) and 15.01% of the agricultural GDP (UBOS, 2022). Uganda's livestock sector consists of cattle, goats, pigs, sheep, poultry, rabbits, beekeeping, and other animals. Among these, cattle are the most important livestock species, with products that contribute approximately 73% of the gross value of all livestock output, majorly beef and milk (Behnke & Nakirya, 2012; Waiswa et al., 2021). The total national herd consists of 15.5 million heads of cattle as of 2020, representing a 2.7% increase from 15.09 million heads in 2019 (FAO, 2022). According to statistics provided by the United Nations Food and Agriculture Organization (FAO), there has been a 3.63% annual average reduction in beef production in Uganda from 2015 to 2020. This has been attributed to the decrease in the number of animals slaughtered, which has decreased at an annual average rate of 3.09% (FAO, 2022).

Beef production in Uganda stood at 163,889 tons in 2020 (FAO, 2022), produced predominantly by indigenous breeds that make up 93.5% of the total national herd (Waiswa et al., 2021). Among these breeds are the East African short-horned zebu and the long-horned Ankole cattle, which are mainly kept under an extensive management system. The exotic tropical

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beef breeds constitute 0.9% of the total cattle population, most notably the Boran (UIA, 2016; Waiswa et al., 2021). The greatest percentage of Uganda's beef is obtained from culled animals (UIA, 2016). Compared to other meat types, beef is the most widely consumed since it is not affected by cultural or religious restrictions. For example, annual per capita consumption of beef stands at 6 kg compared to 3.2 kg, 0.9 kg, and 0.3 kg for pork, goat meat, and mutton, respectively (Agriterra, 2012; Waiswa et al., 2021).

Milk production on the other hand stood at 2.04 million tons in 2018 (Waiswa et al., 2021; FAO, 2022; Waiswa & Günlü, 2022), 51% of this was produced from exotic dairy breeds and their crosses (UBOS, 2022) which make up 5.6% of the total cattle population (Waiswa et al., 2021). The dairy sub-sector grows at an annual average rate of 7-10% (Waiswa et al., 2021). The industry has significant potential to reduce hunger, malnutrition, and rural poverty, improve rural livelihoods, promote food security and nutrition, create employment opportunities, promote gender equality, and support the overall development of Uganda's economy (Waiswa & Akullo, 2021; Waiswa et al., 2021; Waiswa & Günlü, 2022). Dairy exports contribute to 89% of the total livestock products' exports, and only 7% of the total agricultural exports as of 2021 (DDA, 2021). Additionally, the dairy industry is among Uganda's largest foreign exchange-earners. Dairy exports stood at US$ 139.5 million in 2019, while imports stood at US$ 5.19 million in the same year (DDA, 2020). The higher level of exports compared to imports is an indication of the industry's significant growth levels. The increase in Uganda's dairy export value is attributed to improved compliance of Uganda's dairy products to regional and international market standards, increased adoption of dairy cattle farming as a business by the private sector, and the annual increase in dairy processing capacities (DDA, 2020).

Considering the significant roles played by both sectors in Uganda, this study employs the Box-Jenkins methodology to determine the most appropriate Autoregressive Integrated Moving Average (ARIMA) models for the 1961 to 2020 time series of Uganda's beef and cattle milk production, and make five-year (2021-2025) forecasts with appropriate prediction intervals. Forecasting is important in different fields to enhance short and long-term planning. The Box-Jenkins methodology is one of the widely used forecasting methodologies and has been reported to provide good forecasts (Sánchez-López et al., 2015). This approach has been widely used in several studies to forecast several parameters within the agricultural sector such as production, yield, demand and consumption, and trade (prices, imports, and exports) of agricultural products. Among the available studies, this approach has been used to forecast the production and yield of animal products such as milk (Kaygisiz & Sezgin, 2017; Hassan et al., 2018; Akin et al., 2020; Ganesan et al., 2020; Eştürk, 2021; Taye et al., 2021), beef (Eroğul et al., 2019), and poultry (Sankar, 2014; Hussain et al., 2021).

While comparing with other forecasting methods, the ARIMA model gave the best forecasts for domestic and international beef prices in Indonesia (Putri et al., 2019), and wheat production in Pakistan (Masood et al., 2018), compared to the Linear, Quadratic, Exponential, S-Curve, Double Exponential Smoothing, and Single exponential smoothing models. The ARIMA method was also the most appropriate for forecasting the amount of beef and goat meat in Türkiye (Muhammed & Zengin, 2020). In all these studies, models with the lowest Root Mean Square Error (RMSE), Mean Absolute Error (MAE), Mean Percentage Error (MPE), and Mean Absolute Percentage Error (MAPE) values were considered to be the best fit in forecasting the given variables.

This paper is organized as follows: Section 1 was an introduction to the subject and provided an overview of the available literature on the subject. Section 2 describes the data and econometric methodology; section 3 presents the results and discussion while section 4 provides the conclusion of the study.

2. Materials and Methods

2.1. Econometric Methodology

Time series data of beef and cattle milk production for 60 years, covering the period from 1961 to 2020 was used in this study. The data was extracted from the FAOSTAT website (FAO, 2022). Stationarity of the data set was tested using the Augmented Dickey-Fuller (ADF) unit root test in EViews version 10 while the rest of the analyses and forecasts were conducted in SPSS statistical program version 26.0. Beef and cattle milk production for the period from 2021 to 2025 was forecast using the Box-Jenkins methodology (ARIMA models).

The Box-Jenkins methodology was first introduced by Box and Jenkins in 1976 (Asteriou & Hall, 2007; Suleman & Sarpong, 2012; Rahman et al., 2016). The general ARIMA model is denoted as ARIMA (p, d, q), where p is the number of lags of the dependent variable (the AR terms), d is the number of differences to be taken to make the series stationary, and q is the number of lagged terms of the error term (the MA terms) (Asteriou & Hall, 2007). The AR(p) model is represented as;

$$Y_t = \sum_{i=1}^{p} \phi_i Y_{t-i} + u_t \quad (1)$$

The MA(q) model is represented as;

$$Y_t = u_t + \sum_{j=1}^{q} \theta_j u_{t-j} \quad (2)$$

A combination of the AR(p) and the MA(q) models form the Autoregressive Moving Average ARMA (p, q) models which are represented as;

$$Y_t = \sum_{i=1}^{p} \phi_i Y_{t-i} + u_t + \sum_{j=1}^{q} \theta_j u_{t-j} \quad (3)$$

Where Y_t is the dependent variable at time t, Y_{t-i} is the response variable at time lags t-i, ϕ, and θ are the coefficients

to be estimated, u_{t-j} is the error in previous periods that are incorporated in the response Y_t, u_t is the error term at time t, and $|\phi|$ and $|\theta| < 1$.

ARMA models can only be made on stationary time series i.e., the series exhibit constant mean and variance over time, and the covariance between two values from the series depends only on the length of time separating the two values, and not on the actual times at which the variables are observed (Asteriou & Hall, 2007; Gujarati & Dawn, 2009; Kunst, 2012). Non-stationary series can be transformed to stationary by differencing (Asteriou & Hall, 2007; Gujarati & Dawn, 2009; Kunst, 2012). In this study, the series became stationary after the first difference, which was taken using the formula; $\Delta Y_t = Y_t - Y_{t-1}$, where Y_t is the dependent variable at time t, Δ is the change in Y_t, and Y_{t-1} is the dependent variable at time t-1. Differencing non-stationary series forms ARIMA (p, d, q) models, where "d" is the number of differences it takes to make the series stationary. The Box-Jenkins methodology consists of three stages aimed at selecting an appropriate ARIMA model for forecasting purposes. These are; identification, estimation, and diagnostic checking (Asteriou & Hall, 2007).

Identification: At this stage, the time plots of the series autocorrelation function, and partial autocorrelation function are examined to check for stationarity of the data, and find out the appropriate values of p, d, and q (Asteriou & Hall, 2007; Gujarati & Dawn, 2009). As shown in Figure 1 and 3, and the correlograms presented in Figure 5, 6, 9, and 10, the original series were non-stationary. Examination of the correlograms was supplemented by the ADF unit root test to test for stationarity of the series as shown in Table 1. The data was then differenced once to make it stationary. The autocorrelation function (ACF), and partial autocorrelation function (PACF) of the differenced series were plotted again to examine stationarity. As shown in Figure 2, 4, 7, 8, 11, and 12, the series were stationary after the first differencing. Therefore, the "d" value was determined as 1. After achieving stationarity, the p and q orders of the ARIMA models were identified using the autocorrelation function, and partial autocorrelation plots. Following Gujarati and Dawn (2009)'s recommendation on the choice of lag length, a third of the time series (20 lags) was used as the lag length while calculating ACFs and PACFs.

Estimation: Each of the identified ARIMA models was estimated and the various coefficients were examined. The estimated ARIMA models were compared in terms of significant coefficients of the ARMA parameters, the Stationary R-squared, Normalized Bayesian Information Criterion (BIC), Root Mean Square Error (RMSE), Mean Absolute Error (MAE), Mean Percentage Error (MPE), and Mean Absolute Percentage Error (MAPE) values. Models with the lowest BIC, RMSE, MAE, MPE, and MAPE values, while those with the highest Stationary R^2 value were considered to be the most appropriate, and were therefore used for forecasting beef and cattle milk production for the 2021-2025 period. These parameters are expressed as shown below (Oni & Akanle, 2018; Celik, 2019):

$$\text{RMSE} = \sqrt{\frac{\sum e_t^2}{n}} \tag{4}$$

$$\text{MAE} = \frac{1}{n}\sum_{t=1}^{n} |e_t| \tag{5}$$

$$\text{MPE} = \frac{1}{n}\sum_{t=1}^{n} \frac{e_i}{X_i} \times 100 \tag{6}$$

$$\text{MAPE} = \frac{1}{n}\sum_{t=1}^{n} \left|\frac{e_t}{X_t}\right| \times 100 \tag{7}$$

Where e_t is the estimation error at time t (the actual value at time t minus the estimated value at time t), and n is the number of the estimated periods. When MAPE < 10, the forecasting model has a high accuracy, $10 \leq \text{MAPE} \leq 20$, the forecasting model has a good accuracy, $20 \leq \text{MAPE} \leq 50$, the forecasting model has a reasonable accuracy, and MAPE > 50, the forecasting model is unreliable (Celik, 2019).

$$\text{Stationary R-Squared} = 1 - \frac{\sum_t (Y_t - \hat{Y}_t)^2}{\sum_t (\Delta Y_t - \overline{\Delta Y})^2} \tag{8}$$

Where, ΔY_t is the differenced series.

$$\text{BIC} = \ln\left(\hat{\sigma}_e^2\right) + k\ln(n)/n \tag{9}$$

Where, $\hat{\sigma}_e^2$ is the error variance.

Diagnostic checking: At this stage, the goodness of fit of the model is examined by plotting the residuals and looking out for outliers and evidence of periods in which the model does not fit the data well (Asteriou & Hall, 2007). The Ljung-Box (LB) Q-statistic, which tests for autocorrelations of the residuals was also used.

3. Results and Discussion

According to Figure 1 and 3, there is a generally increasing trend in the original series of Uganda's beef and cattle milk production over the years. This is an indication of the non-stationarity of the series. In addition to Figure 1 and 3, the ADF unit root test was performed to test for the stationarity of the data, and the results are presented in Table 1. The null hypothesis (H_0) was that the series had a unit root, i.e., they were non-stationary, while the alternative hypothesis (H_1) was that the series did not have a unit root, i.e., they were stationary. The ADF test was conducted following Hill et al. (2018). Because the original series appear to be fluctuating around a linear trend as can be observed in Figure 1 and 3, the test equation with the intercept term and trend was used to test for stationarity. In addition, because the differenced series fluctuate around a non-zero sample average and show no trend as can be observed in Figure 2 and 4, the test equation with only the intercept term and no trend was used to test for stationarity.

According to the test results, before differencing, the absolute values of the ADF test statistic for both beef and milk production are smaller than the test critical values at the 5% level, with probabilities greater than 0.05. Therefore, H_0 is accepted, which means that the series (beef and cattle milk production series) have a unit root. In contrast, after the first difference, the absolute values of the ADF test statistic are larger than the test critical values at the 5% level, with probabilities less than 0.05. Therefore, H_0 is rejected, which means that the series did not have a unit root after taking their first difference. Differenced values are also shown in Figure 2 and 4. An important feature to note in these figures is the absence of any sustained increase or decline in the level of the series over the observation period; in other words, they fluctuate around a constant mean level and have no trend-like behavior, a characteristic of stationary series.

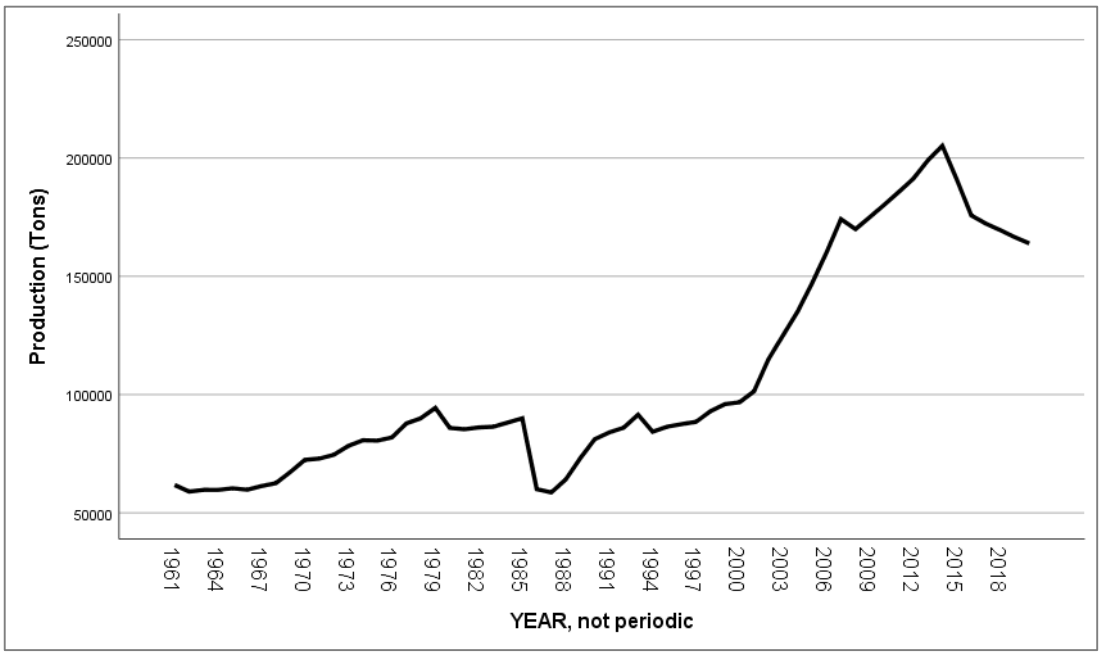

Figure 1. The trend of original beef production series from 1961 to 2020.

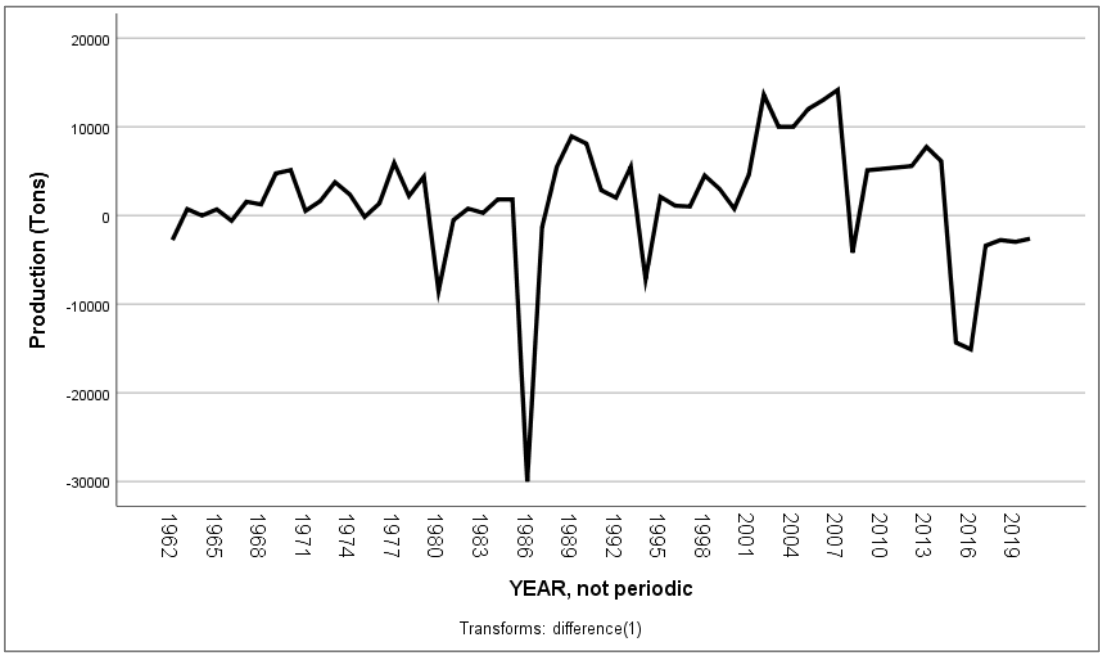

Figure 2. Differenced beef production series from 1961 to 2020.

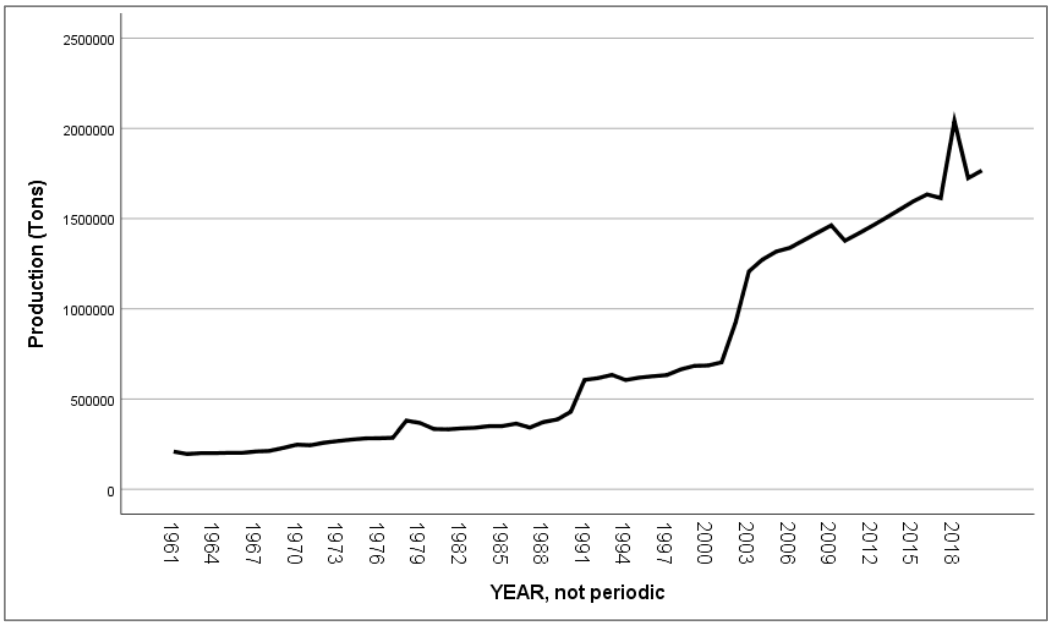

Figure 3. The trend of original milk production series from 1961 to 2020.

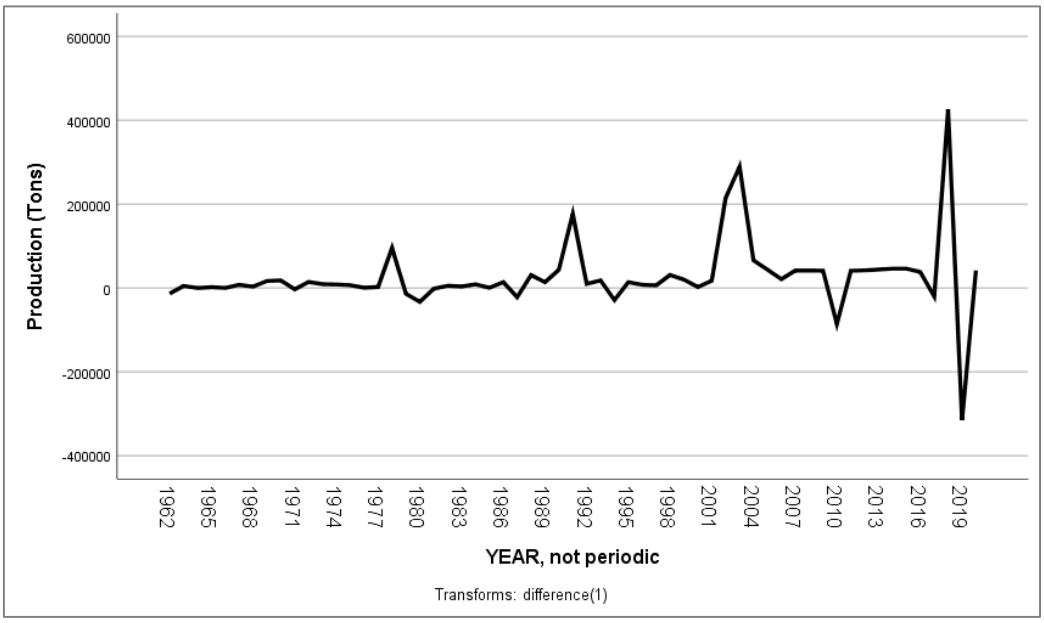

Figure 4. Differenced milk production series from 1961 to 2020.

Table 1. ADF unit root test results for beef and cattle milk production.

			Before differencing		**After first difference**	
Exogenous variable in test equation			Trend and Intercept		Intercept	
			t-Statistic	**Prob.**	**t-Statistic**	**Prob.**
Beef Production (Tons)	ADF test statistic		-1.7909	0.6964	-5.1270***	0.0001
	Test critical values:	1% level	-4.1243		-3.5482	
		5% level	-3.4892		-2.9126	
		10% level	-3.1731		-2.5940	
Cattle milk Production (Tons)	ADF test statistic		-1.988	0.5960	-9.225***	0.0000
	Test critical values:	1% level	-4.121		-3.548	
		5% level	-3.488		-2.913	
		10% level	-3.172		-2.594	

*** denotes rejection of the null hypothesis of the presence of unit root at the 1% level of significance.

Correlograms of the original series as shown in Figure 5, 6, 9, and 10 also show non-stationarity in the data. The Autocorrelation Function (ACF) plots show significant autocorrelations that are outside the 95% confidence interval. From lag 1 up to lag 11 for beef production and lag 14 for milk production, the autocorrelations are significant and their decline is very gradual to zero. While for Partial Autocorrelation Function (PACF) plots, only the first lag is significant for both beef and milk production series while the rest are within the standard error bound. ACF and PACF plots of the differenced series of beef production (Figure 7 and 8) show that only autocorrelations of the first lag are outside the standard error bound, and therefore significant, while the rest of the lags are within the standard error bounds. On the other hand, the ACF and PACF plots of the differenced series of milk production in Figure 11 and 12 show that autocorrelations of all lags lie within the 95% confidence interval.

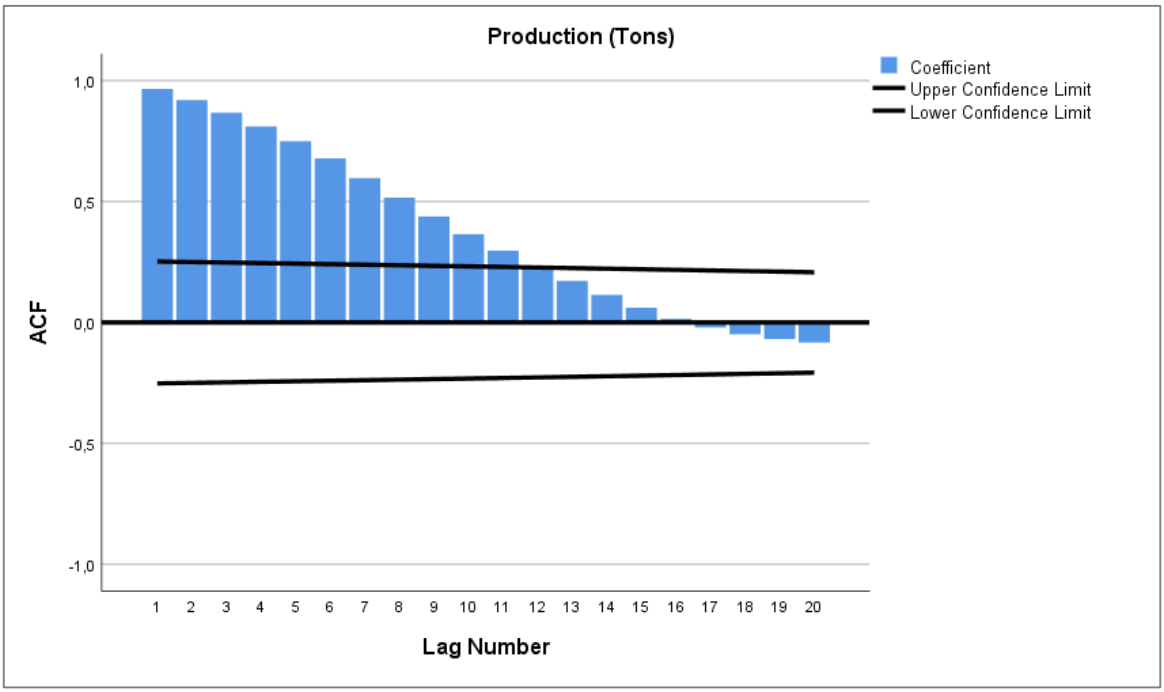

Figure 5. A plot of ACFs of the original beef production series.

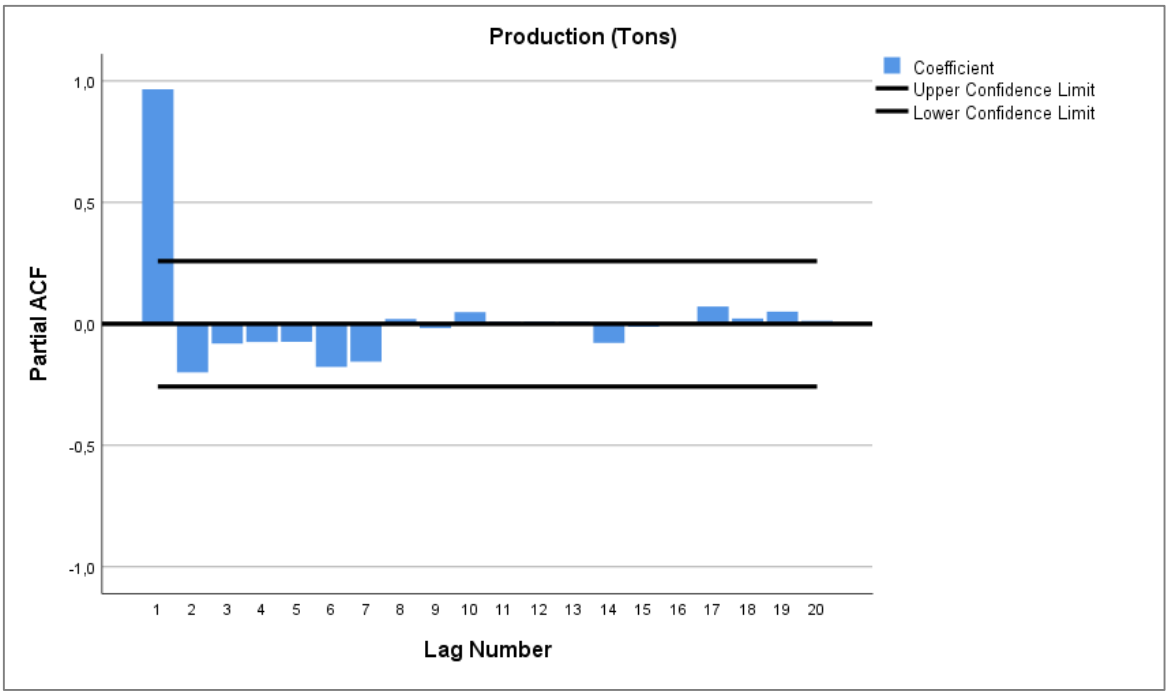

Figure 6. A plot of PACFs of the original beef production series.

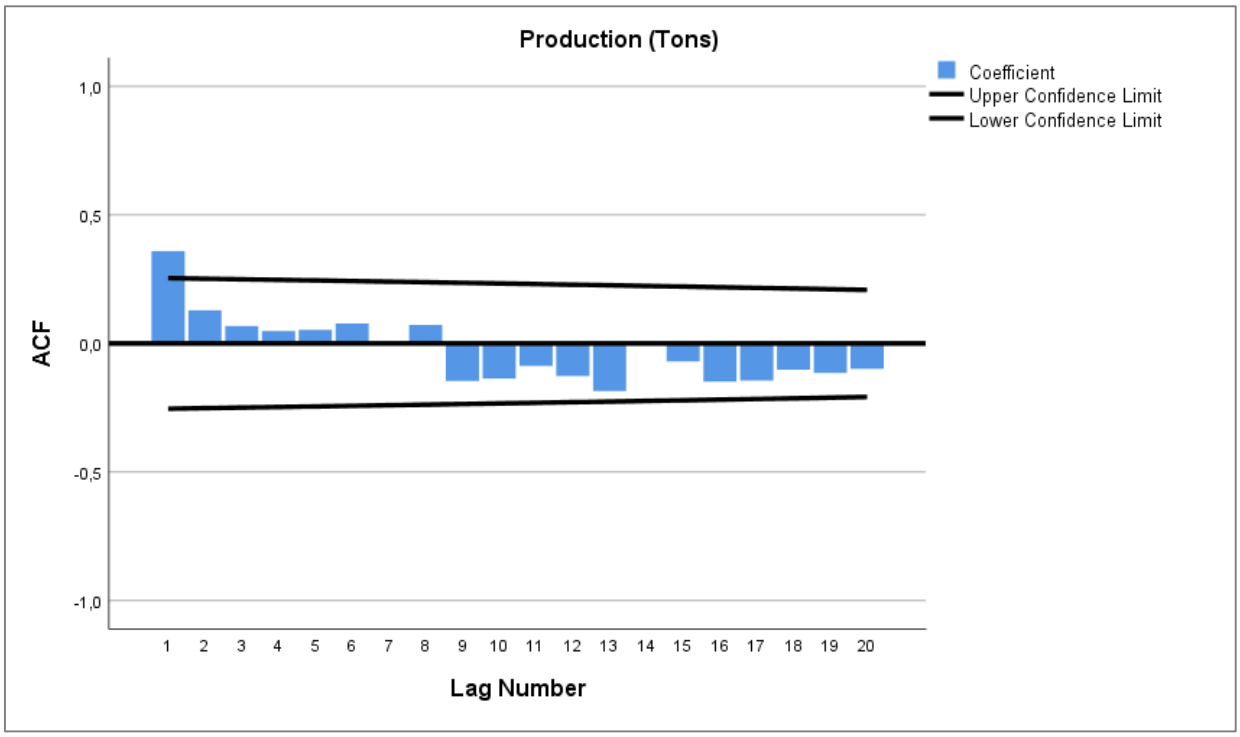

Figure 7. A plot of ACFs of the differenced beef production series.

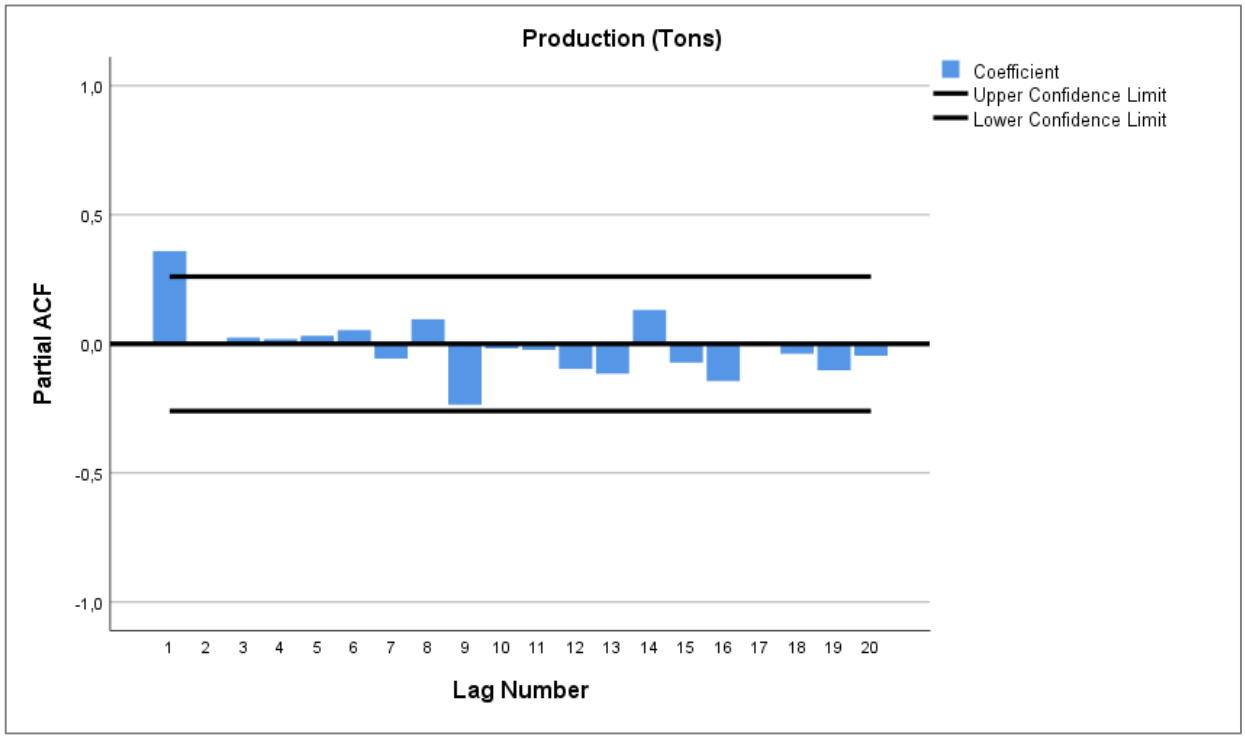

Figure 8. A plot of PACFs of the differenced beef production series.

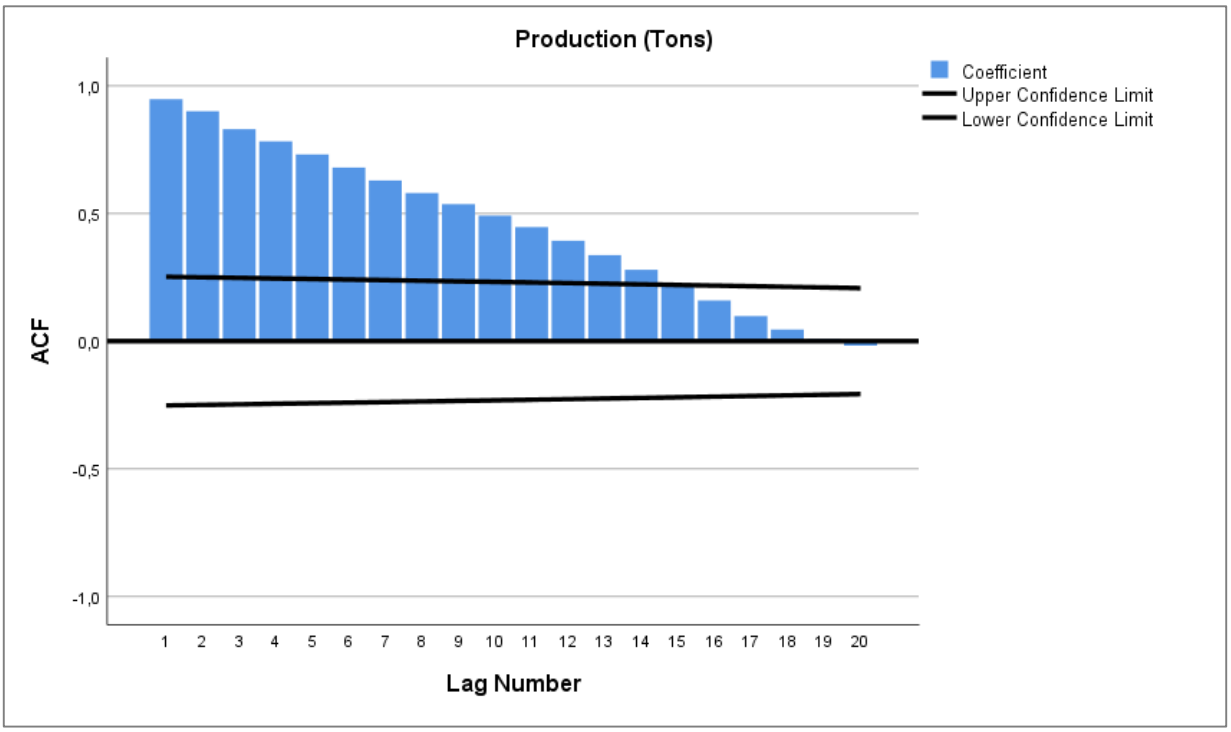

Figure 9. A plot of ACFs of the original milk production series.

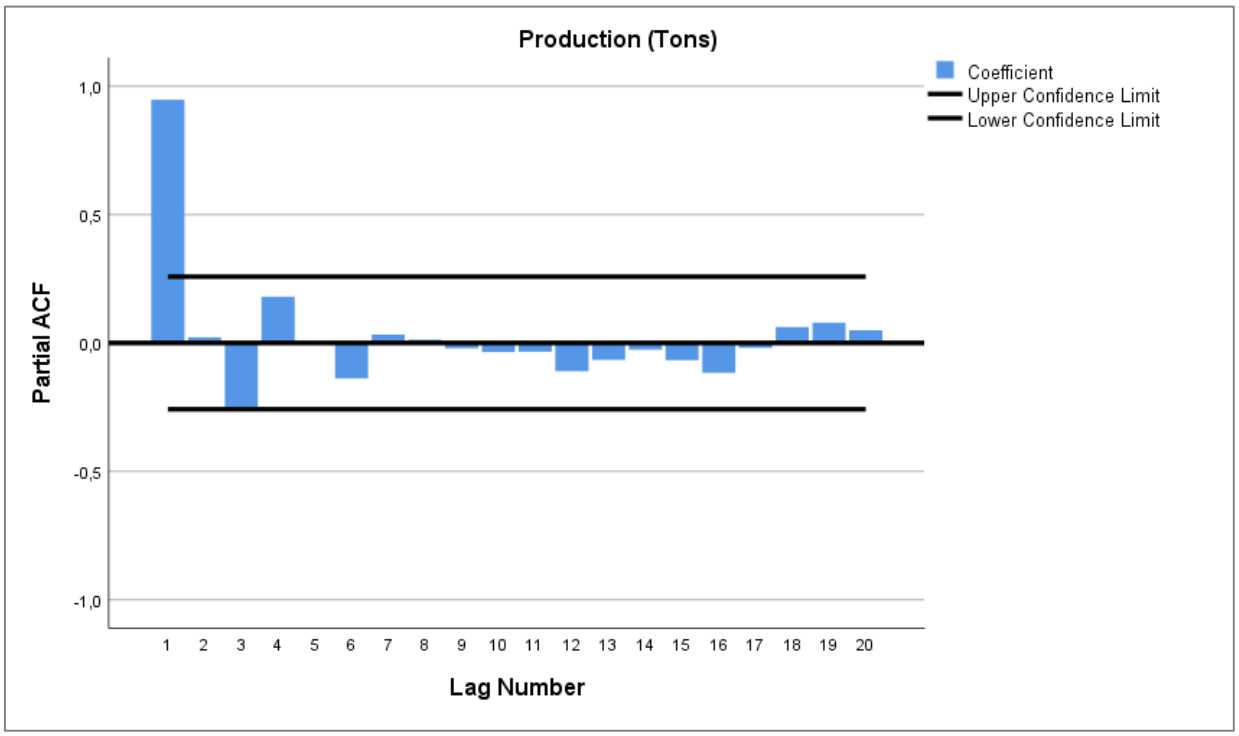

Figure 10. A plot of PACFs of the original milk production series.

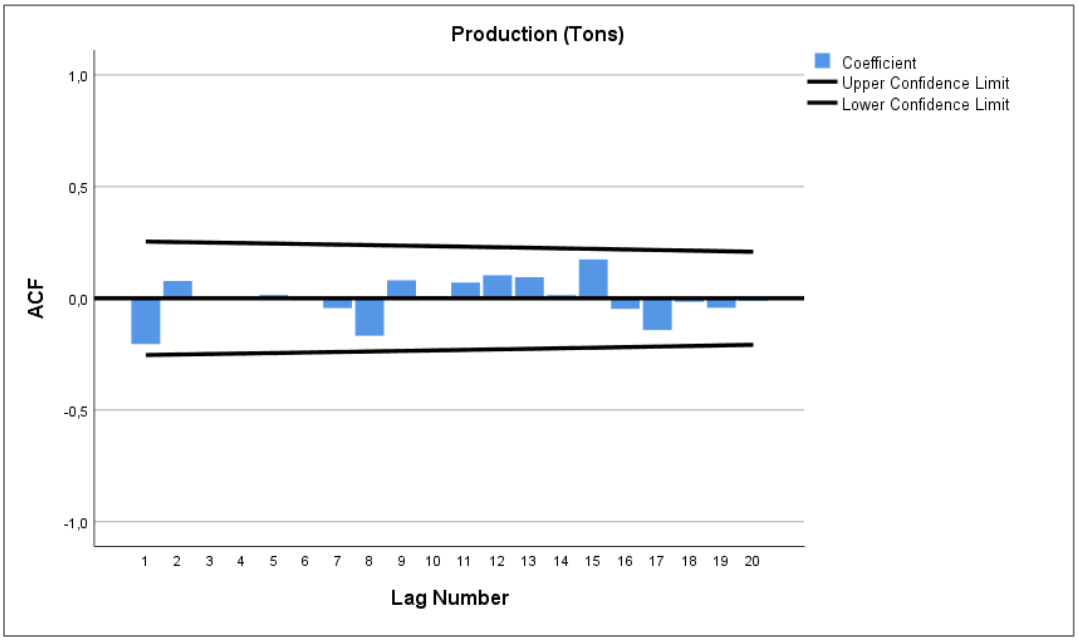

Figure 11. A plot of ACFs of the differenced milk production series.

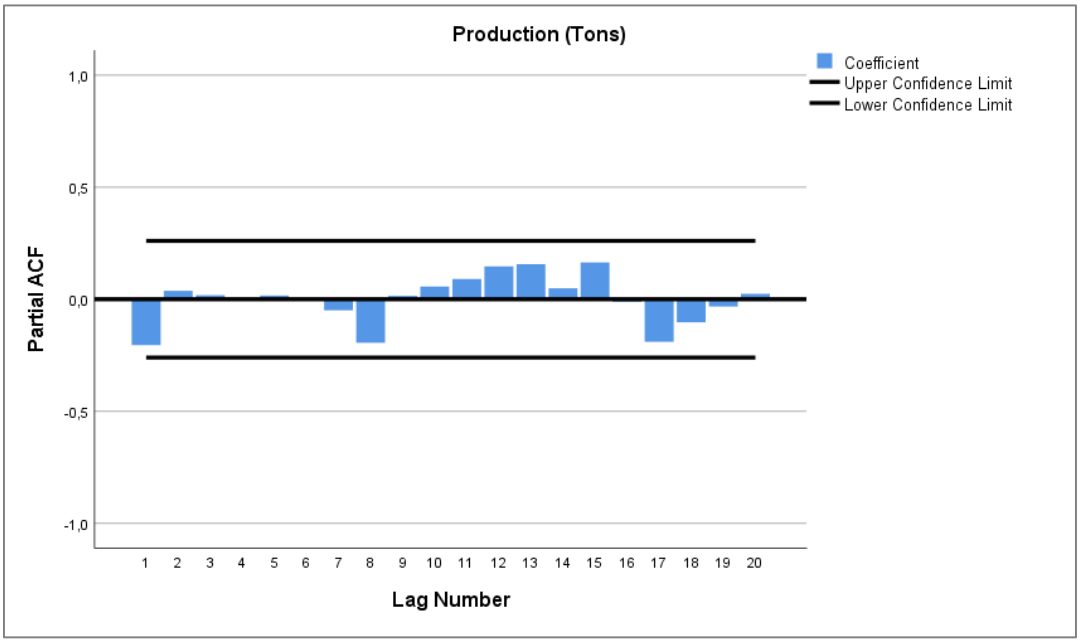

Figure 12. A plot of PACFs of the differenced milk production series.

Following the patterns of the ACF and PACF plots of the differenced series, 3 tentative ARIMA models were identified for beef production, i.e., ARIMA (1,1,1), ARIMA (1,1,0), and ARIMA (0,1,1). On the other hand, 4 tentative ARIMA models were identified for milk production, i.e., ARIMA (0,1,0), ARIMA (1,1,0), ARIMA (0,1,1), and ARIMA (1,1,1). These models were estimated and fit statistic results are presented in Table 2.

According to the results presented in Table 2., among the ARIMA models for milk production, ARIMA (1,1,1) had the largest stationary R^2, ARIMA (1,1,0) had the smallest RMSE and MaxAE values, while ARIMA (0,1,0) had the smallest MAPE, MaxAPE, MAE, and Normalized BIC values. The ARIMA (0,1,0) model having the smallest MAPE, and Normalized BIC value, was selected to be the most appropriate model for forecasting Uganda's milk production. In comparison with other studies that have been conducted using the same methodology to forecast milk production, this study's finding is different from the results of the available studies in the literature. Among the available studies, Eştürk (2021) estimated milk production in Türkiye's Ardahan province and concluded that ARIMA (0,0,1) was the most suitable model. While comparing Artificial Neural Networks and Box-Jenkins models to forecast goat milk production in Türkiye, the results

of Kaygisiz and Sezgin (2017)'s study revealed that ARMA (2,1) model was the most appropriate model for forecasting goat's milk production among the identified ARIMA models. Hassan et al. (2018) also used the same methodology to forecast milk production in Sudan's Khartoum state and concluded that ARIMA (1,0,0) model was the most appropriate. While Ganesan et al. (2020) revealed that ARIMA (1,1,0) was the appropriate model for estimating milk production in India. It was also concluded that ARIMA (1,2,1) was the model suitable for forecasting cow milk production at Andassa dairy farm, West Gojam Zone, Amhara Region in Ethiopia (Taye et al., 2021).

Table 2. Tentative ARIMA models for beef and cattle milk production.

Fit Statistic		Cattle Milk Production (Tons)				Beef Production (Tons)		
		ARIMA (0,1,0)	ARIMA (1,1,0)	ARIMA (0,1,1)	ARIMA (1,1,1)	ARIMA (1,1,1)	ARIMA (1,1,0)	ARIMA (0,1,1)
Stationary R-squared		-2.22E-16	0.042	0.038	0.044	0.130	0.130	0.117
R-squared		0.974	0.975	0.975	0.975	0.979	0.979	0.979
RMSE		88308.81	87194.017	87386.524	87881.331	6681.213	6622.366	6670.689
MAPE		7.11	7.888	7.95	7.667	4.195	4.196	4.198
MaxAPE		24.771	25.323	25.158	25.48	52.838	52.838	52.800
MAE		44509.952	47374.031	47300.937	46732.296	4059.830	4061.253	4146.279
MaxAE		399602.78	390143.81	391364	390548.91	31702.580	31702.859	31680.018
Normalized BIC		22.846	22.89	22.894	22.975	17.821	17.735	17.749
Ljung-Box Q(18)	Statistics	11.909	12.856	12.606	12.702	10.169	10.191	12.175
	DF	18	17	17	16	16	17	17
	Sig.	0.852	0.746	0.762	0.694	0.858	0.895	0.789

Among ARIMA models for beef production, all the identified models had very slight differences in the parameters used for estimation. ARIMA (1,1,1) and ARIMA (1,1,0) had the same stationary R^2, R^2, and MaxAPE values. ARIMA (1,1,1) had the smallest MAPE and MAE values, ARIMA (1,1,0) had the smallest RMSE and normalized BIC values, while ARIMA (0,1,1) had the smallest MaxAPE and MaxAE values. Based on the normalized BIC values, ARIMA (1,1,0) is the best model for forecasting Uganda's beef production because it has the lowest normalized BIC value among the identified ARIMA models. In addition to having the smallest normalized BIC value, this model also had a statistically significant coefficient of the autoregressive component moreover at the 1% level of significance (see Table 3) as recommended in Mahapatra and Satapathy (2019)'s study. All components of the ARIMA (1,1,1) model were not statistically significant as can be observed in Table 3.

It can also be noted that all the identified ARIMA models for both beef and milk production analyzed in this study have a high accuracy in forecasting Uganda's beef and milk production because they have MAPE values of less than 10 as put forward in Celik (2019) and Hassan et al. (2018)'s studies.

Table 3. Parameters of the tentative models for beef and cattle milk production.

	Models	Variable	Coefficient	Std. Error	t-Statistic	Prob.
Beef Production (Tons)	ARIMA (1,1,0)	C	1648.685	1329.957	1.240	0.220
		AR(1)	0.358***	0.124	2.891	0.005
	ARIMA (1,1,1)	C	1647.927	1345.167	1.225	0.226
		AR(1)	0.364	0.349	1.041	0.302
		MA(1)	0.007	0.375	0.018	0.986
	ARIMA (0,1,1)	C	1689.093	1147.509	1.472	0.147
		MA(1)	-0.327**	0.126	-2.605	0.012
Cattle Milk Production (Tons)	ARIMA (0,1,0)	C	26397.220**	11496.828	2.296	0.025
	ARIMA (1,1,0)	C	26468.834***	9470.224	2.795	0.007
		AR(1)	-0.202	0.130	-1.558	0.125
	ARIMA (0,1,1)	C	26644.232***	9336.521	2.854	0.006
		MA(1)	0.183	0.131	1.392	0.169
	ARIMA (1,1,1)	C	26193.357**	9968.750	2.628	0.011
		AR(1)	-0.440	0.840	-0.524	0.603
		MA(1)	-0.248	0.891	-0.278	0.782

** and *** denote significance at the 5% and 1% levels of significance, respectively.

Among the diagnostic tests, plots of residuals of ACF and PACF of ARIMA (0,1,0) and ARIMA (1,1,0) for milk and beef production, respectively as shown in Figure 13 and 14 show that all autocorrelations lie within the 95% confidence interval, all lags are not significant. This implies that all information has been captured by both models. Additionally, the null hypothesis of no serial correlation is accepted in the Ljung-Box Q test for both models as shown in Table 2 ($p>0.05$). This implies that the data are independently distributed.

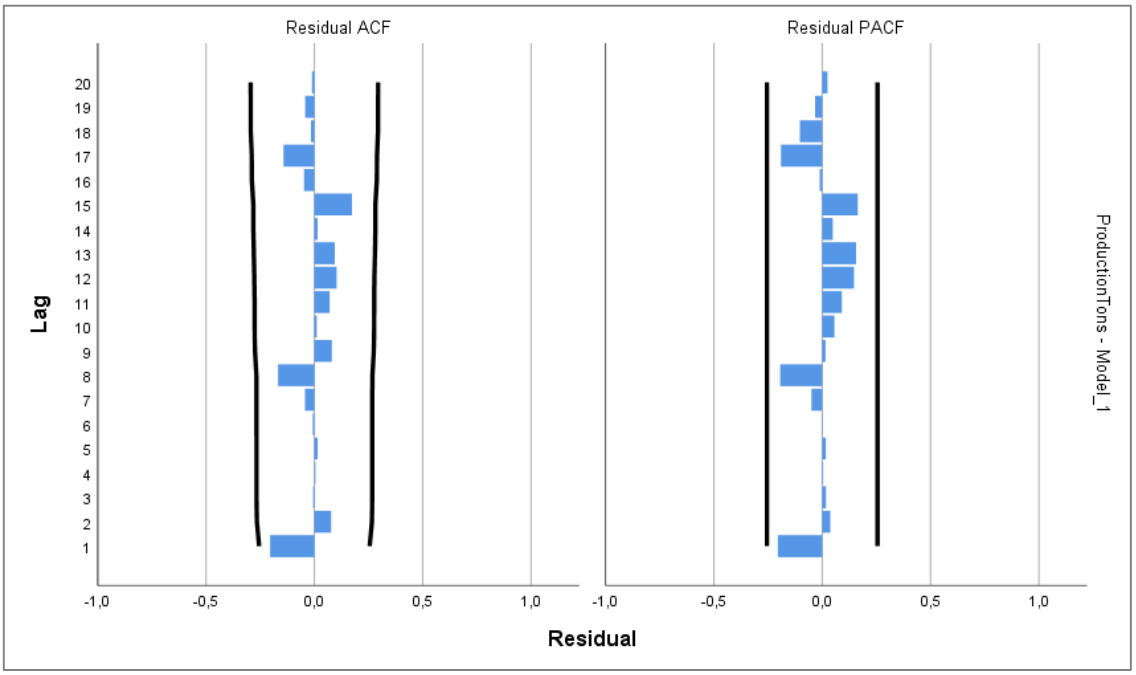

Figure 13. ACF and PACF plots of residuals of ARIMA (0,1,0) for milk production.

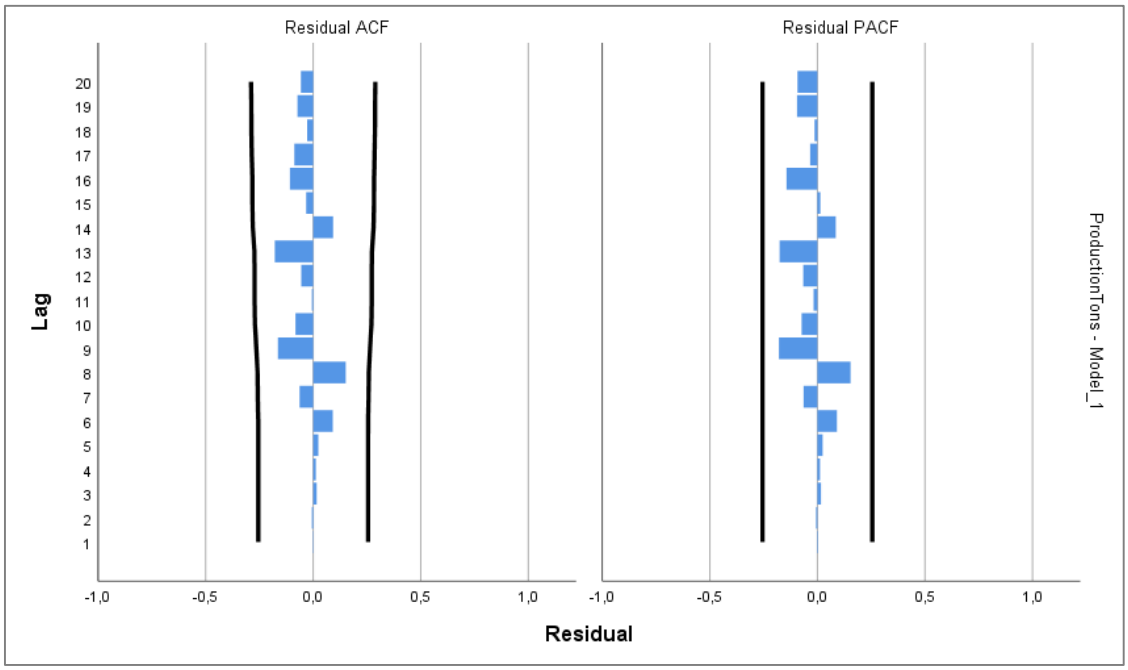

Figure 14. ACF and PACF plots of residuals of ARIMA (1,1,0) for beef production.

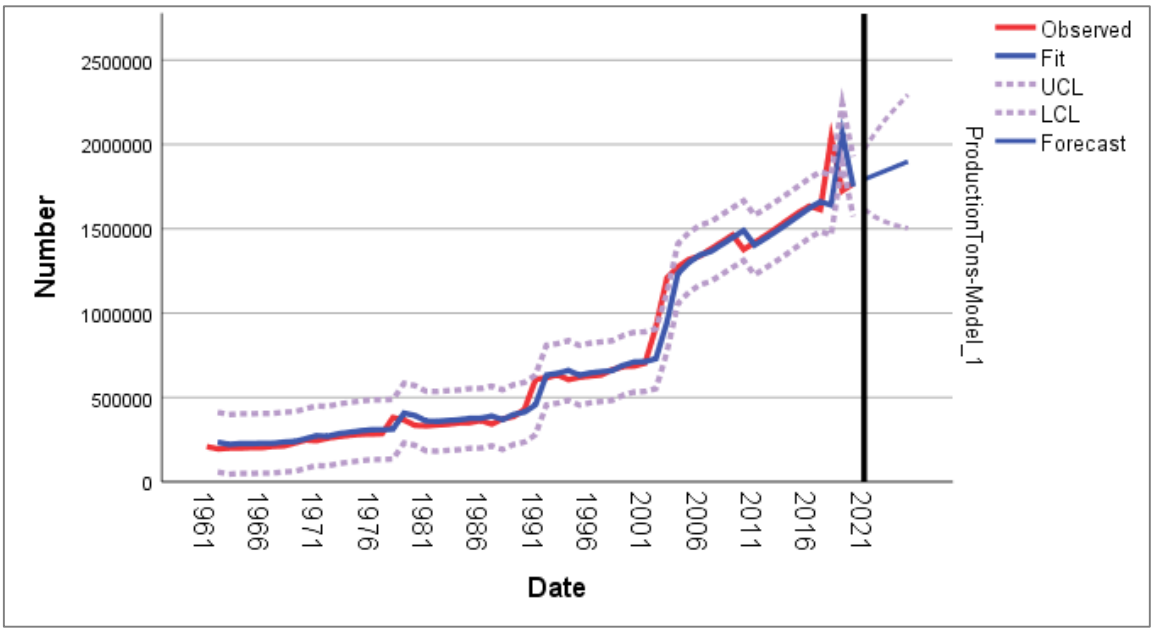

Figure 15. A plot of the observed, fit, and forecast values of ARIMA (0,1,0) for milk production.

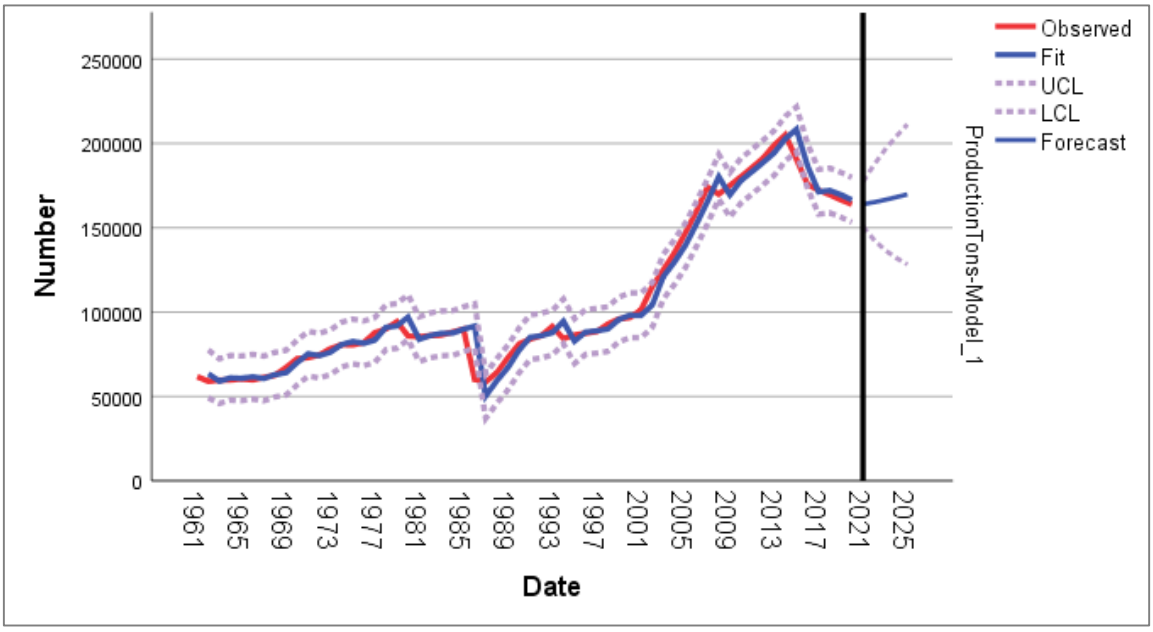

Figure 16. A plot of the observed, fit, and forecast values of ARIMA (1,1,0) for beef production.

Forecasts of both ARIMA (0,1,0) and ARIMA (1,1,0) for milk and beef production are presented in Figure 15 and 16. Additionally, Table 4. presents actual values, estimated values, and forecasts of both models from 2021 to 2025. According to these results, ARIMA (0,1,0) forecasts show that milk production will increase at an annual average rate of 1.629% between 2021 and 2025 making 1.898 million tons in 2025. On the other hand, ARIMA (1,1,0) forecasts show that beef production will increase at an annual average rate of 0.393% between 2021 and 2025 making 169,763 tons in 2025. These rates of growth are far below the actual annual average growth rates registered in the sample period i.e., according to the data presented by FAO, beef and cattle milk production in Uganda increased at an annual average rate of 1.92% and 4.09%, respectively in the period between 1961 and 2020. This suggests the need to increase production in the two livestock sub-sectors through increased investment to exploit the benefits of the increasing demand for beef and cattle milk both domestically and regionally. The current beef production levels can only meet half the domestic and regional beef demand (UIA, 2016). This together with the fact that Uganda's beef is highly preferable owing to its yellow fat that does not contain cholesterol mainly because the cows are grazed on natural pastures (UIA, 2016), present a high potential of the beef sector for the domestic and export market. One of the ways to take advantage of such potential is by increasing production through large-scale commercial farming.

Table 4. Actual and predicted values of ARIMA (0,1,0) for milk production and ARIMA (1,1,0) for beef production.

Year	Milk Production (Tons) (ARIMA (0,1,0))				Beef Production (Tons) (ARIMA (1,1,0))			
	Actual	Predicted	LCL	UCL	Actual	Predicted	LCL	UCL
2015	1596000	1576397	1399628	1753167	190785	208372	195118	221626
2016	1634000	1622397	1445628	1799167	175684	186710	173455	199964
2017	1614000	1660397	1483628	1837167	172275	171336	158082	184590
2018	2040000	1640397	1463628	1817167	169496	172113	158859	185367
2019	1724655	2066397	1889628	2243167	166515	169559	156305	182814
2020	1766386	1751052	1574283	1927822	163889	166506	153252	179761
2021		1792783	1616014	1969553		164007	150753	177262
2022		1819180	1569191	2069170		165108	142755	187461
2023		1845578	1539404	2151751		166561	136766	196355
2024		1871975	1518436	2225513		168139	132082	204196
2025		1898372	1503104	2293640		169763	128273	211252

4. Conclusion

Considering the importance of forecasting in short and long-term planning, this study employs the Box-Jenkins methodology to determine the most appropriate ARIMA models for the 1961 to 2020 time series of Uganda's beef and cattle milk production. The study further makes five-year (2021-2025) forecasts of Uganda's beef and cattle milk production with appropriate prediction intervals. Following patterns of the Autocorrelation Function and Partial Autocorrelation Function plots of the differenced data, 4 tentative ARIMA models were identified for milk production, i.e., ARIMA (0,1,0), ARIMA (1,1,0), ARIMA (0,1,1), and ARIMA (1,1,1) for milk production. While 3 tentative ARIMA models were identified for beef production, i.e., ARIMA (1,1,1), ARIMA (1,1,0), and ARIMA (0,1,1). ARIMA (0,1,0) model was selected to be the most suitable for forecasting cattle milk production because it had the smallest MAPE and Normalized BIC values. Although results revealed very slight differences in the parameters used for estimation in all identified models, ARIMA (1,1,0) was selected to be the best model for forecasting Uganda's beef production because it had the smallest normalized BIC value and a significant coefficient of the autoregressive component at the 1% level of significance. Forecasts using the selected ARIMA models show that milk production will increase at an annual average rate of 1.63%, while beef production will increase at an annual average rate of 0.39% in the 2021-2025 forecast period.

Conflict of Interest

The author declares that he has no conflict of interest.

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http://agriprojournal.com

JOURNAL OF AGRICULTURAL PRODUCTION

ISSN: 2757-6620

https://prensip.gen.tr

RESEARCH ARTICLE

Impact of Different Shed Houses and Growing Media on Growth, Yield and Quality of Strawberry

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ARTICLE INFO

Article History
Received: 15.02.2023
Accepted: 03.04.2023
First Published: 25.05.2023

Keywords
Growing media
Net house
Quality
Strawberry
Yield

ABSTRACT

Partial controls of the microclimatic conditions have a major influence on plant growth and productivity. Moreover, plant growth is largely dependent on the physicochemical properties of the growing media. The purpose of this study was to see how different growing media and shade houses affected strawberry plant growth, yield, and quality attributes. The experimental treatments include net house, UV poly shade house, and open field (control) conditions; and three different growing media i.e., 50% soil + 50% cowdung, 50% soil + 50% vermicompost, and 50% soil + 50% cocopeat were studied. According to the findings, strawberries grown in a net house with cocopeat substrate had the highest chlorophyll content (SPAD value) (46.1), fruit yield (289.16 g plant^{-1}), total soluble solid (8.0%), reducing sugar (8.75 mg g^{-1}) and total anthocyanin (30.80 mg 100 g^{-1}). In contrast, fruits grown under UV poly shed with cocopeat substrate exhibited increased plant height (20.33 cm) and ascorbic acid (46.94 mg 100 g^{-1}). Vermicompost based growing media showed no satisfactory improvement in the reproductive growth characteristics of strawberry plants. Therefore, cocopeat based growing media and net house shade may be recommended to obtain better strawberry yield and quality.

Please cite this paper as follows:

Islam, N., Hossain, I., & Choudhury, S. (2023). Impact of different shed houses and growing media on growth, yield and quality of strawberry. *Journal of Agricultural Production*, *4*(1), 30-38. https://doi.org/10.56430/japro.1250832

1. Introduction

The strawberry (*Fragaria x ananassa*) is a member of the Rosaceae family's genus Fragaria (Hancock, 1999). Strawberry is a short-day herbaceous perennial plant that grows well at temperatures ranging from 22 to 25 °C during the day and 7 to 13 °C at night (De & Bhattacharjee, 2012). Strawberries are valued for their taste and essential nutrition, and are rich in anthocyanins, and flavanols that are bioactive compounds. As well as preventing cardiovascular disease, inflammation, and some forms of cancers, with the advantages of slowing down aging (Miao et al., 2017). The intake of fresh strawberries per capita has risen from 0.90 kg in 1980 to 3.26 kg in 2018 (AgMRC, 2019). With ever-limiting resources of cultivated land, it is desirable to increase the yield of horticultural crops not only quantitatively, but also qualitatively.

The climate, which has a high degree of variability, is the most crucial factor in agricultural production because it can alter the atmosphere in which crops are grown (Iizumi & Ramankutty, 2015). It is necessary to measure the circumstances under which plants are grown and to take into account how environmental factors affect the fruit's quality (Martínez et al., 2017). Temperature and relative humidity are two environmental factors that have a significant impact (Rivera et al., 2017). The most significant environmental factor that affects the growth of cymes, flowers, and fruit is

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temperature. The microclimate in the shed house was more conducive to the fruit crop's growth and yield attributes. The enhanced rate of photosynthate transfer from the vegetative component (source) to the reproductive organs (sink) may have boosted fruit size and weight, resulting in greater tomato fruit yield (Kuscu et al., 2014). Sugar-acid ratio, titratable acids, and pH varied between cultivars, suggesting that these traits may be heritable as they were less affected by environmental factors. Kumakura and Shishido (1995) observed that the fruit weight in the glasshouse dropped as the mean temperature increased.

The growing medium used has an effect on plant growth. Strawberries grow best in peat moss, rock wool, coir, perlite, or other mixtures. Growing media serves as both a growing medium and a source of plant growth nutrients. Soil is commonly used as a basic medium because it is the cheapest and easiest to obtain (Bhardwaj, 2013). Vermicompost provides adequate levels of oxygen to the roots, as well as adequate water storage and nutrient for the plants. Farmyard manure (FYM) has a high water retention capacity as well as adequate porosity.

These growing substrates are either single components or mixtures that provide water, air, and nutrients to plants (Olaria et al., 2016). In spite of that, the composition, particle size, pH, aeration, and ability to hold water and nutrients vary greatly between growing media (Oagile et al., 2016). The quality and performance of strawberries greatly depend on the growing media used. The requirement for special media is a critical step in ensuring the plant's efficient growth in the container, as their growth is heavily reliant on the physicochemical properties of the media used (Riaz et al., 2008).

Protected agriculture has expanded nowadays to help improve agricultural productivity. To date, there is not much research available on strawberry cultivation in the shade house. Limited work has been reported on strawberry cultivation regarding the application of growing media under different shades. Therefore, the study aimed to assess the response of different growing media and shade houses on plant growth, yield, and quality attributes of strawberries.

2. Materials and Methods

2.1. Plant Materials and Growing Conditions

During 2019-2020, the experiment was carried out at the Horticulture farm of Sher-e-Bangla Agricultural University in Dhaka, Bangladesh, under natural lighting conditions. The present investigation was carried out in three different shades i.e., net house, plastic shade house, and open field condition (control), and different growing media were used in different ratio i.e., Soil:Cowdung (1:1), Soil:Vermicompost (1:1), and Soil:Cocopeat (1:1). The total nitrogen (N) content was determined using the method of Bremner (1960), while the phosphorus (P) and potassium (K) concentrations (Table 1) were determined using the Motsara and Roy (2008) method. The recommended dose of fertilizers was applied insoluble form based on the nutrient condition of the growing media. The experiment was set up with six replications in plastic pots in a completely randomized design. Dhaka is located at 23°42′37″ N (Latitude), 90°24′26″ E (Longitude) and has an average elevation of 4 meters, according to the National Mapping Organization of Bangladesh. All fundamental cultural practices and plant protection techniques were applied uniformly across all plots throughout the experiment. Five plants were chosen at random from each replication for observations on growth, yield, and physicochemical parameters. Between November 2019 and March 2020, temperatures and relative humidity were measured in all environments to keep track of the actual environmental conditions that the plants were grown in (Table 2).

Table 1. Initial nutrient composition of the following substrates.

Growing media	Nutrients		
	N (%)	P (%)	K (%)
Cowdung	0.85	0.12	1.49
Cocopeat	0.41	0.81	1.32
Vermicompost	1.25	1.14	1.19

Table 2. Average monthly temperature and relative humidity (%) at 12 hours in different shade house and open field.

Month	12 hours					
	Open field		UV poly shed		Net house	
	Temperature (°C)	Humidity (%)	Temperature (°C)	Humidity (%)	Temperature (°C)	Humidity (%)
November, 2019	29.33	66.98	30.03	60.07	26.87	69.19
December, 2019	24.03	68.75	24.93	62.95	20.45	70.01
January, 2020	23.58	70.6	24.12	66.53	21.03	72.21
February, 2020	26.48	66.72	27.21	65.93	23.09	69.01
March, 2020	31.41	62.51	32.05	61.9	27.55	66.19

2.2. Measurement of Growth Parameters

Five plants in each treatment and replication were used to measure the height of the plant and the number of leaves at harvest. From the bottom to the top of the main plant, the height of the plant was measured.

2.3. SPAD Chlorophyll Meter Reading

An SPAD-502 chlorophyll meter was used to measure the chlorophyll content of the first fully expanded leaves (Minolta, Tokyo, Japan.). Measurements were taken from the middle of the leaf lamina of each treated and control plant.

2.4. Measurement of Yield and Yield Traits

The yield/plant (g) was calculated by adding the harvests from five plants in each treatment and replication. Strawberries were picked every two days for a total of five to seven pickings. The weight of fruits (g) from each selected plant was measured using an electronic top pan balance on each date of harvest. The number of fruits/plants was determined by counting the fruits that were ripe enough to be harvested.

2.5. Measurements of Quality Parameters

2.5.1. Total soluble solids content

A digital refractometer was used to determine the TSS content of strawberries (MA871, Romania). Using a dropper, a drop of strawberry juice was applied to the prism of the refractometer Total soluble solids were measured using a refractometer.

2.5.2. pH determination

Separate strawberry fruit juices from each treatment were filtered, and a digital pH meter was used to measure the pH (HI 2211, Romania).

2.5.3. Titratable acidity (TA %)

A 5 g sample was macerated with a mortar and pestle to determine titratable acidity. Then filtered and distilled water rendering 100 ml of total volume was added. In a conical flask, 10 ml of the stock solution was then added along with 2 drops of phenolphthalein. Titration of the solution was done using 0.1 N NaOH. Total acid content was determined in maleic acid equivalents and is reported as the mean value of triplicate analyses.

2.5.4. Vitamin C determination

The Vitamin C content of strawberries was determined using the Oxidation-Reduction Titration Method (Tee et al., 1988). 100 ml of a 5% oxalic acid solution were used to create the volume. For the titration, the dye solution 2, 6-dichlorophenol indophenol was used. The mean observations demonstrated how much dye was required to oxidize a particular quantity of an unknown concentration of a L-ascorbic acid solution using L-ascorbic acid as the known sample. The final point of titration, which lasted 10 seconds and required a 5 ml solution each time, was determined by the pink color. The reading from the burette was recorded.

2.5.5. Reducing sugars content

Determination of reducing sugars was based on the phenol-sulphuric acid method (DuBois et al., 1956). With deionized water, a total of 0.2 g fresh fruit was homogenized and the extract was filtered out. 2 ml of the solution was combined with 0.4 ml of 5% of phenol. Subsequently, the mixture was rapidly added to 2 ml of 98% sulphuric acid. The test tubes were allowed to keep at room temperature for 10 min and positioned for color development in a water bath at 30 ℃ for 20 min. Light absorption with the spectrophotometer was then measured at 540 nm. A blank solution was prepared in the same manner as described above, except that the fruit extract was replaced with distilled water. Reducing sugar content was expressed as mg g^{-1} fresh weight (FW).

2.5.6. Anthocyanin content

1.0 g fruit pulp mixed with 1 ml 85% ethanol + 15% HCl 1.5 N. After the extraction, 1 ml sample solution was taken and then diluted to 10 ml. The absorbance reading was taken at 535 nm. Then calculated the anthocyanin concentration as follows: Anthocyanin (mg per 100 g fresh weight) = (absorbance at 535 nm x volume of extraction solution x 100)/ weight of the sample in g x 98.2. The same procedure is used to prepare reference solutions as described above, except that the fruit extract is replaced with distilled water (Lapornik et al., 2005).

2.6. Statistical Analyses

A randomized complete block design (RCBD) was used in the experiments, with four replications for each treatment and five plants in each replicate. IBM SPSS Statistics 21 was used to conduct the statistical analyses (IBM Corp, Armonk, NY, USA). When $p<0.05$, the mean value across the treatments was regarded as statistically significant. The mean value among the treatments was considered to be statistically significant when. The mean±SE from the replicates was used to present all results. The graphs were created in Microsoft Excel. ANOVA was used to test the effect of the shade house, growing media, and their interaction on yield and biochemical parameters.

3. Results and Discussion

3.1. Environmental Conditions

The main environmental factor influencing the growth and development of short-day strawberry plants is temperature (Palencia et al., 2013). Our experiment revealed that the air temperature in UV poly shade was consistently higher than the conditions in the open field and net houses (Table 2). For photosynthesis in strawberries, ideal temperatures range from 15 to 23 °C (Hancock, 1991). Higher temperatures affect net photosynthesis more adversely than lower temperatures, leading to lower photosynthesis output above a certain temperature (Reddy et al., 1999). Relative humidity increases the net energy availability for crop growth and enhances crop

survival under conditions of moisture stress. A relative humidity level of 65 to 75% during the day was considered to be optimal for good growth and yield of strawberries in the greenhouse (Lieten, 2000). In our experiment, the relative humidity was therefore at an optimum level in all the growing conditions.

3.2. Effect of Different Shade Houses and Growing Media on Plant Growth

Figure 1 shows that different treatments were found to have an impact on various plant growth parameters like plant height, number of leaves, and SPAD reading. The highest plant height (20.33 cm) was found in UV poly shade with cocopeat treatment and the lowest (14.66 cm) was observed in vermicompost treatment in both net house and open field conditions (Figure 1A). However, the net house with cocopeat treatment had the most leaves (13) while the plants treated with vermicompost and grown in UV poly sheds had the fewest leaves (9) (Figure 1B).

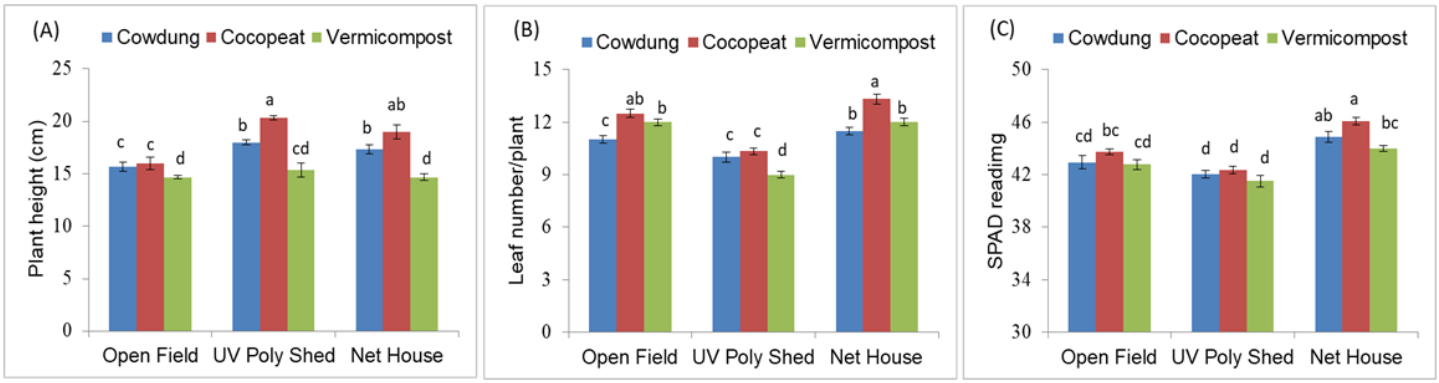

Figure 1. Average plant height (cm) (A), leaf number/plant (B), and leaf content of chlorophyll (SPAD reading) (C) of strawberry grown with different growing media under different shade houses. Mean±S.E (n=15). Different letters mean significant differences between the treatments according to Duncan's multiple range test ($p<0.05$).

The plants grown in the net house with cocopeat-treated soil displayed the highest chlorophyll content (46.10), and the plants grown in the UV poly shade with vermicompost-treated soil displayed the lowest chlorophyll content (41.50) (Figure 1C). Lettuce plants grew taller and had more leaves when grown on cocopeat-based media, which provided adequate microclimate conditions in the root zone (Sarkar et al., 2021). Under cocopeat substrate, plant growth and development were accelerated, resulting in greater chlorophyll content in leaves. This could be owing to the availability of nutrients in cocopeat-based media. Sweet pepper growth parameters were much greater in peat-treated pots than in control pots (100% soil) (Rekani et al., 2016). Due to different temperatures, aeration, and soil moisture ability, different growing media can impact water and mineral uptake in the plant; thus, affect plant growth. An increase in enzymatic activity, microbial population, and plant growth hormones due to the use of sufficient increasing media may be responsible for an improvement in the physiochemical properties of the soil (Singh et al., 2011). It was also reported that the water holding capacity is better cocopeat compared to other growing media (Ozgumus, 1985). In our experiment, open field conditions and UV poly shed showed a significant decrease in leaf chlorophyll content. Due to decreased photosynthetic activity and increased rate of respiration, the higher temperature is likely to have a significant impact on limiting plant growth (Darnell & Hancock, 1996).

3.3. Effect of Different Shade Houses and Growing Media on Yield and Yield Traits

From the data presented in Figure 2, it is apparent that the differences among various treatments were found to be significant in respect of the number of flowers, fruits, individual fruit weight, and fruit yield per plant. The data reveals that the maximum number of flowers (19) and fruits (17 g) per plant was observed under in net house with cocopeat treatment. However, the lowest number of flowers (11) and fruits per plant (8 g) was recorded in a vermicompost treated plant grown under UV poly shed (Figures 2A & 2B). The highest fruit weight (18.03 g) was observed in plants treated with cocopeat grown in net houses, and the lowest fruit weight was observed in plants treated with vermicompost grown in all growing conditions i.e. open field, net house and UV poly shed (approximately 12 g) (Figure 2C).

There was a significant variation among shade houses and different growing media in response to fruit yield/plant. The highest fruit yield (307.11 g) was found in cocopeat treated plants grown in net house and the lowest yield (108 g) was found in vermicompost treated plants under UV poly shade condition (Figure 2D). Temperature above 25 ℃ can reduce fruit set of strawberry (Abdelrahman, 1984). In our experiment, air temperature in open field and UV poly shade was higher in January and February (flowering and fruiting stage) than the net

houses condition, thus, affects the yield of strawberry. Strawberry fruit yield and quality can be affected by higher temperatures after bloom (Wang & Camp, 2000). High temperatures harm fruit set in strawberries, decreasing pollen viability and inhibiting pollen tube growth and pollen tube elongation (Ledesma & Sugiyama, 2005). In order to boost aeration resulting in the development of a better root system, various combinations of increasing media have been recorded (Verdonck & Demeyer, 2004) and result in higher yields (Albaho et al., 2009). Rostami et al. (2014), who also reported that when substrates composed of different growing media were used, the yield of strawberry significantly differed. The positive impact of cocopeat and its mixtures on improved root growth may lead to improved aeration, thereby creating a higher root system that may have facilitated shoot nutrient uptake leading to increased berry yield.

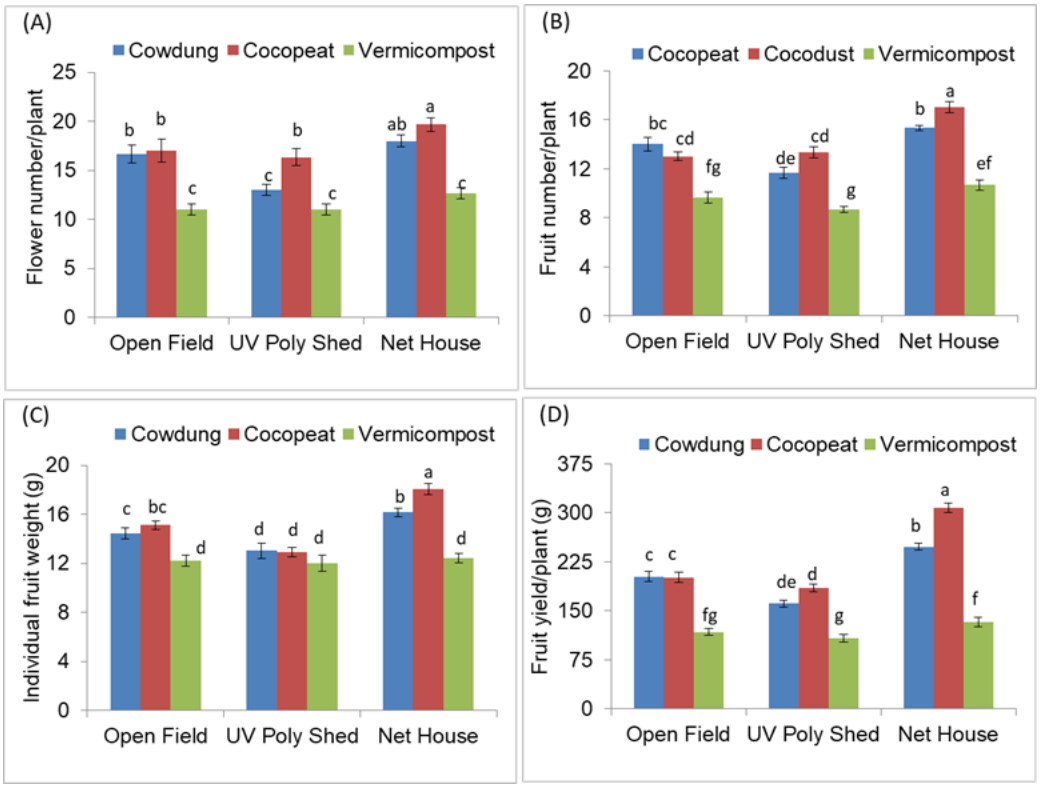

Figure 2. Average flower number/plant (A), fruit number/plant (B), individual fruit weight (g) (C), and fruit yield/plant (g) (D) of strawberry grown with different growing media under different shade houses. Mean±S.E (n=15). Different letters mean significant differences between the treatments according to Duncan's multiple range test ($p<0.05$).

3.4. Effect of Different Shade Houses and Growing Media on Fruit Quality

The fruits of vermicompost-treated plants that are grown in net houses have the lowest ascorbic acid levels (35.83 mg 100 g^{-1}), while the fruits of cocopeat-treated plants that are grown in UV poly sheds and open fields have the highest levels (approximately 46 mg 100 g^{-1}) (Figure 3A). The quality of crops has also been improved by increasing the content of ascorbic acid in the fruit and, in particular, by increasing the sugar content (Wuzhong, 2002).

The higher TSS content was found in fruits grown with vermicompost treated soil and the lowest TSS content was found in fruits grown in cowdung treated soil in all growing environments (Figure 3B). The increased TSS and ascorbic acid content of the fruit could be attributed to better growing media, which aided in the uptake of NPK nutrients, including micronutrients that influence fruit quality traits (Lata et al., 2018). The fruits grown in UV poly shade with cowdung-treated soil had the lowest titrable acidity (0.37%), while those grown in the net house with cocopeat-treated fruit plants had the highest (0.62%) (Figure 3C). For strawberries to taste good, the acid content must be relatively high (Kader, 1991). However, the higher pH was found in UV poly shed with cowdung treated plant (3.69) and the lower pH was found in the net house with cocopeat and vermicompost treated plants (approximately 3.45) (Figure 3D). High fruit quality is associated with low pH (Davies et al., 1981). Loss of organic acids from ripe fruit tends to be largely due to respiration (Halinska & Frenkel, 1991).

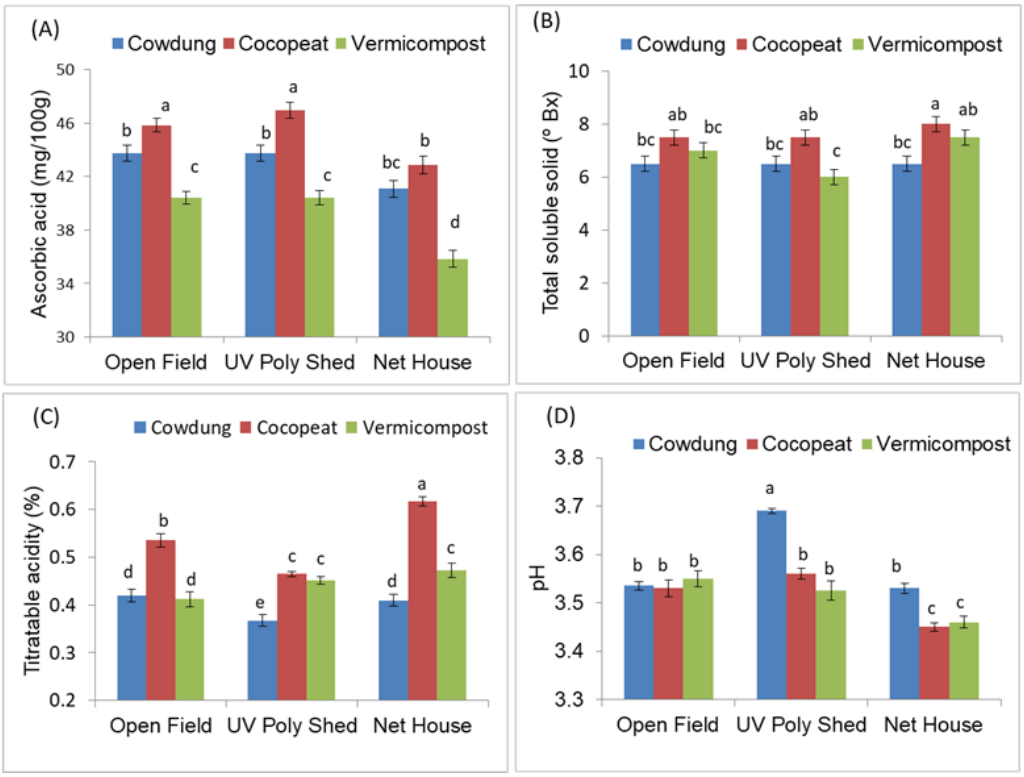

Figure 3. Average content of ascorbic acid (mg 100 g FW^{-1}) (A), total soluble solid (°Brix) (B), titratable acidity (%) (C), and pH (D) of strawberry grown with different growing media under different shade houses. Mean±S.E (n=15). Different letters mean significant differences between the treatments according to Duncan's multiple range test ($p<0.05$).

There was a significant difference in reducing sugar content in fruits grown with different growing media under different shade houses. The higher reducing sugar was found in cocopeat treated plants grown under the net house (8.75 mg g FW^{-1}), and the lower reducing sugar was found in the fruits grown in cowdung treated plants under open field conditions (8.1 mg g FW^{-1}) (Figure 4A). Likewise, Voca et al. (2009) showed that fruits grown under a tunnel were usually more reducing sugar than in open field cultivated fruits. The acid and sugar content of the fruit is related to its ripeness (Pérez et al., 1997), and higher sugar content is required for good strawberry flavor (Kader, 1991). The red-colored strawberry attracts buyers and hence serves as an effective fruit marketing criterion. The content of anthocyanin is influenced by many factors, such as temperature, light, food, hormones, etc (Karanjalker et al., 2018). In our result, the growing media and shade houses have significantly affected the total content of anthocyanin. As observed in Figure 4B, anthocyanin content decreased in strawberry fruit grown with cowdung under open field conditions (27.50 mg 100 g^{-1}). Meanwhile, anthocyanin content significantly increased in fruits grown in the net house with cocopeat treated soil (30.80 mg 100 g^{-1}). Increased content of anthocyanins was observed in fruits from plants grown on the peat-coconut substrate (Wysocki et al., 2017). This study showed the positive influence of shade on the total anthocyanin content of the strawberry. It was speculated that the content of anthocyanin could be related to ambient air temperature (Zoratti et al., 2015).

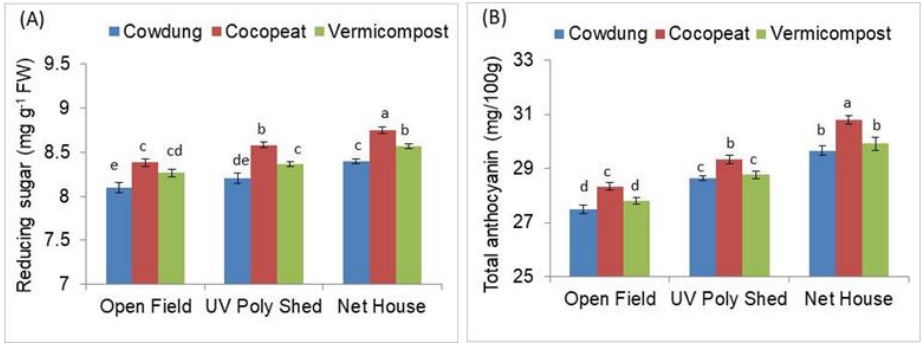

Figure 4. Average content of reducing sugar (mg g FW^{-1}) (A) and total anthocyanin (mg 100 g FW^{-1}) (B) of strawberry grown with different growing media under different shade houses. Mean±S.E (n=15). Different letters mean significant differences between the treatments according to Duncan's multiple range test ($p<0.05$).

3.5. ANOVA Analysis

The treatments had highly significant effect on yield and some quality traits i.e., reducing sugar, ascorbic acid and titratable acidity. However, growing media had no significant effect on pH and anthocyanin content (Table 3).

Table 3. Analysis of variance (F-value) for yield and different biochemical parameters of strawberry as affected by shed house, growing media and their interaction.

Parameters	Treatments	SS	df	MS	F
Average fruit weight (kg)	Shade house	34903.49	2	17451.74	5.75*
	Growing media	58100.55	2	29050.27	14.07**
	Shade house x growing media	103651.92	8	12956.49	58.68**
	Error	3973.81	14	283.84	
	Total	107625.73	26		
Total soluble solid (^{0}Brix)	Shade house	2.00	2	1.00	1.65NS
	Growing media	6.50	2	3.25	7.80*
	Shade house x growing media	10.50	8	1.31	3.93*
	Error	6.00	14	.42	
	Total	16.50	26		
pH	Shade house	.03	2	.02	3.42*
	Growing media	.02	2	.01	1.93 NS
	Shade house x growing media	.09	8	.01	2.34 NS
	Error	.08	14	.01	
	Total	.17	26		
Titratable acidity	Shade house	.04	2	.02	3.65*
	Growing media	.05	2	.026	4.42*
	Shade house x growing media	.16	8	.020	10.13**
	Error	.03	14	.003	
	Total	.19	26		
Ascorbic acid	Shade house	241.13	2	120.56	10.62**
	Growing media	220.86	2	110.43	9.05**
	Shade house x growing media	486.46	8	60.81	40.47**
	Error	27.04	14	1.93	
	Total	513.51	26		
Reducing sugar	Shade house	.46	2	.23	8.42*
	Growing media	.54	2	.27	11.12**
	Shade house x growing media	1.02	8	.13	21.97**
	Error	.105	14	.007	
	Total	1.129	26		
Anthocyanin	Shade house	22.825	2	11.41	17.76**
	Growing media	3.575	2	1.78	1.23NS
	Shade house x growing media	26.819	8	3.35	5.28*
	Error	11.424	14	.82	
	Total	38.243	26		

* $p<0.05$, ** $p<0.01$, NS: Not significant.

4. Conclusion

Strawberry plants responded better to the cocopeat-based growing medium than to the other growing media used (cowdung and vermicompost) across all growing shade features, while net house proved superior for reproductive growth and quality traits. The number of flowers, fruit, individual fruit weight, and fruit output of strawberry plants did not improve significantly when using vermicompost-based growing media. Based on findings obtained from the study cocopeat growing media and net house shade may be advised to obtain better quality and yield for strawberry.

Acknowledgment

The present work was financially supported by the National Agricultural Technology Program Phase-II Project (NATP-2), Bangladesh (project ID: 152).

Conflict of Interest

The authors declare that they have no conflict of interest.

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JOURNAL OF AGRICULTURAL PRODUCTION

ISSN: 2757-6620

https://prensip.gen.tr

RESEARCH ARTICLE

The Effect of Zeolite (Clinoptilolite) as a Feed Additive and Filter Material for Freshwater Aquariums

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ARTICLE INFO

Article History
Received: 23.02.2023
Accepted: 23.05.2023
First Published: 30.06.2023

Keywords
Feed additive
Filter material
Water parameters
Zeolite

ABSTRACT

Ammonia, which occurs as a natural result of aquaculture in production facilities, has a negative effect on the quality of aquaculture water and aquatic organisms. In this study, it was aimed to determine the effect of zeolite in fish feed and in water, which has the ability to adsorb ammonium, on ammonia removal. In the study, 12 different experimental groups were organized by creating 0, 2, and 10% ratios for fish feed (G1-G3), groups that zeolite only in water (G4, G5), and combinations (zeolite and/or in water/in feed) of 0, 7, and 20 g/L amounts to water (G6-G12). When NH_3 and TAN data of G1-G3 were examined, it was determined that although there was no statistical difference, it decreased proportionally with the increase in the amount of zeolite in the feed. The difference between water temperature, dissolved oxygen, pH, NH_3 and TAN values in G4 and G5 groups was found to be insignificant. The dissolved oxygen, pH, NH_3 and TAN values between the groups (G6-G12) were statistically different. As a result, it was determined that 10% addition of zeolite into the feed decreased the TAN values by 37%, and the addition of 10% into the feed and 20 g/L into the water decreased the TAN values by 45%. When the results are evaluated from another point of view, considering the economy and ease of use, it is concluded that 2% zeolite in feed and/or 7 g/L in water can be recommended for aquatic species with high tolerance to ammonia values.

Please cite this paper as follows:

Öz, M., & Aral, O. (2023). The effect of zeolite (clinoptilolite) as a feed additive and filter material for freshwater aquariums. *Journal of Agricultural Production*, *4*(1), 39-46. https://doi.org/10.56430/japro.1255407

1. Introduction

Water quality is a critical factor in the breeding of aquatic organisms. Therefore, water quality should be monitored in order to achieve optimum production in the cultivation of aquatic organisms and to ensure the growth and survival of the organisms, to provide optimum conditions that vary according to the species. The concentration of total ammonia nitrogen (TAN) is the most important parameter limiting water quality in aquaculture (Shalaby et al., 2021).

Fish metabolism and the protein in unconsumed fish feed cause ammonia to build up in the water. The amount of ammonia in the water increases with water temperature and pH. Most of the NH_4^+ converts to NH_3 when the temperature and pH are high (under alkaline conditions). According to Maulini et al. (2022), ammonia in the molecular form (NH_3) is more harmful than the ionic form (NH_4^+). To avoid harming aquatic creatures, ammonia (NH_3) content should be lowered, especially in re-circulating aquaculture systems (RAS) like aquariums.

High organic matter content in the effluent of aquaculture facilities can promote or increase eutrophication and algae bloom and cause serious problems for the aquatic ecosystem. It is usually characterized by an increase in dissolved nitrogen and phosphorus content from unconsumed feed residues and metabolic wastes of fish. Ammonia is the main nitrogenous waste produced by fish through metabolism (Lazzari & Baldisserotto, 2008). More than 90% of waste materials in

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aquaculture pass into the water through unconsumed nutrients and fish feces.

As reported in YSI (2010), the main source of ammonia is simply fish excrement. stool rate; It is affected by the feeding rate and the amount of protein in the feed used. While the bait protein is broken down in the fish's body, some of the nitrogen is used to create energy and the unused portion is excreted through the gills as ammonia. If this ammonia in the water is too much, aquatic creatures will be damaged.

The main methods used to remove ammonium from wastewater are chemical, biological and physical treatment. Much literature on ammonium removal by adsorption has focused on natural minerals because of their high safety, good adsorption capacity, and cost effectiveness (Şahin et al., 2019; Öz et al., 2021). In addition, natural adsorbents are harmless for fish that can be used in aquaculture. There are many ways to reduce ammonia (NH_3) levels in the aquatic environment, including interrupting the feed temporarily, adding new water, reducing fish density, and venting the aquaculture system (Maulini et al., 2022).

Dangerous short-term levels of toxic un-ionized ammonia which are capable of killing fish over a few days start at about 0.6 mg/L (ppm). Chronic exposure to toxic un-ionized ammonia levels as low as 0.06 mg/L (ppm) can cause gill and kidney damage, reduction in growth, possible brain malfunctioning, and reduction in the oxygen-carrying capacity of the fish (Durborow et al., 1997).

In aquaculture, it is reported that clinoptilolite-type zeolites are used to remove excess ammonia. The utilization of zeolite to regulate ammonia levels has the potential to enhance the efficacy of aquaculture practices. Furthermore, it is possible to mitigate the pollution issues arising from the incorporation of ammonia in fish discharge waters into the receiving water environment (Mumpton & Fishman, 1977; Terlizzi, 1996; Emadi et al., 2001; Peyghan & Azar Takamy, 2002; Karadağ et al., 2006; Sirakov et al., 2015; Skleničková et al., 2020; Öz et al., 2021).

The structural features and functions of clinoptilolite are affected by many factors (such as the characteristics of the mining area and the characteristics of the environment in which they will be used). Therefore, according to the area (environment) where it will be used; In order to evaluate the potential of the zeolite, much more detailed research is required to determine its technical properties and to provide the conditions for the purpose of use.

With this research, the effects of zeolite, which is used as a feed additive and filter material, in aquatic environments such as aquarium was determined. The addition of zeolite to water has been observed to result in a reduction of elevated ammonia levels, an increase in oxygen levels, and the restoration of pH balance. The addition of zeolite to fish feed has been found to have a potentially beneficial impact on fish metabolism and the elimination of metabolic waste (Ghiasi & Jasour, 2012; Ibrahim et al., 2016). Presence of cations such as Ca_2^+, Mg_2^+ and Na_2^+ in the feed solution reduced the clinoptilolite adsorption capacity to about 11.68 mg NH_4^+g/zeolite (Ashrafizadeh et al., 2008). In addition, at the end of this study, if the zeolite added to the feed and water has a negative factor in balancing the water parameters (such as the rivalry of sodium, calcium, potassium ions in the feed with ammonium), data on these can be obtained. With this study, it is thought that the zeolite added to the feed will reduce the ammonia release in the feed, and the water parameters will be kept at an ideal level for fish farming and at the same time, the waste water quality will increase. In this study, it was aimed to add zeolite to water and fish feed at different rates to provide suitable conditions in water parameters for aquaculture.

2. Materials and Methods

2.1. Feed Materials

Feed raw materials were obtained from a local feed company. Trial feeds were prepared from these raw materials. Zeolite (clinoptilolite) was not added to the control feed (G1), while 2% (G2) and 10% (G3) zeolite were added to the other two feeds, respectively. It was ensured that the chemical content of the feeds was the same in all groups. The amount of nutrients used in the study and the nutritional components of the feeds are given in Table 1.

Table 1. Formulation and chemical composition of experimental diets (g/100g).

	Experimental Feeds		
	Without zeolite (Control)	**2% zeolite**	**10% zeolite**
Nutrients			
Fish meal	24	24	24
Soybean	21	21	21
Corn protein	4.5	4.5	4.5
Sunflower seeds	22	23	26
Semolina meal	22.5	19.5	8.5
Fish oil	4	4	4
Vitamin-mineral premix	2	2	2
Zeolite	0	2	10
Nutrient composition (dry matter %)			
Moisture	9.94	6.82	8.72
Crude protein	40.47	40.30	39.75
Crude oil	6.66	6.91	6.96
Crude ash	6.45	8.37	10.43
Crude celulose	1.78	1.85	2.01
Starch	16.19	12.28	10.34
Nitrogen free extract	35.27	34.18	31.09

2.2. Zeolite Material

The type of zeolite used as feed and filter material in the research is the West-Anatolian clinoptilolites, which are Turkey's most important zeolite source in terms of the size of the reserves and their potential for use.

The filter material (trade name FILTER-CLINO) and the zeolite used as a feed additive (trade name NAT-MIN 9000) are the same, the difference between them is the crushed sizes. Zeolites were obtained from the manufacturer in 100 microns to be added to the feed, and 1-3 mm in size as filter material. NH_4^+ exchange capacity of the zeolite used in the research; is between 1.6-2.1 meq/g (1.8 meq/g on average) for natural products, Bet surface area 40.79 m^2/g (Bilgin, 2017). The zeolites (1-3 mm) were washed with tap water until the turbidity was removed and dried at 105 ºC (Şahin et al., 2018a).

The zeolite (100 microns) was added to the raw materials of pellets and mixed by hand until the ingredients were homogeneity. Each formulated diet was mixed to prepare each of the experimental diets. Warm water was added with continuous mixing. The paste-like passed through the meat mincer to produce pellets (1-2 mm in diameter), then the feeds were dried in the drying cabinet at 60 degrees for 10 hours. The experimental diets are: Use the commercial control diet (0% zeolite), adding a diet with 2% zeolite and adding a diet with 10% zeolite (Abdulathem & Al-Rudainy, 2021).

2.3. Experimental Design

In the research, 12 aquariums of 60x45x50 cm (three same division) were used. The volume of water used in aquariums is 20 liters. A hand-held field and laboratory device named YSI Professional Plus was used to measure the water parameters (Water temperature, ammonium, dissolved oxygen, pH) of the groups.

Three separate experiments were conducted in this study. In the first experiment, the effects of adding zeolite to the feed, adding zeolite to the water in the second experiment, and adding zeolite to the feed and/or water in the third experiment were investigated.

The experiment was carried out in three designs. In the first design, the effects of zeolite additives only in feed; in the second design, the effects of zeolite additives only in water and in the third design, the effects of zeolite additives in feed and/or water were determined.

In the first experiment, there were three groups; control without zeolite (G1), 2% dietary zeolite (G2) and 10% dietary zeolite in feed (G3).

In the second experiment, there were two groups with 7 g/L (G4) and 20 g/L (G5) zeolite in the water without feed.

In the other seven experimental groups in the third design, feed without zeolite and water without zeolite (G6), feed without zeolite, 7 g/L zeolite in water (G7), 2% zeolite in feed, 7 g/L in water (G8), 10% zeolite in feed and 7 g/L zeolite in water (G9), feed without zeolite and 20 g/L zeolite in water (G10), 2% zeolite in feed and 20 g/L zeolite in water (G11), 10% zeolite in feed and 20 g/L zeolite in water (G12) were added. In the experiment, the effects of zeolite on water parameters were examined for a week.

24 hours before the start of the research, the aquariums of all groups were filled with 20 liters of water and aerated using an air stone, and all groups were aerated equally during the 7-day experiment. Aquarium water temperature values were obtained by heating the environment of the experimental setup with a room-type air conditioner. In the study, the contact surface of the zeolite with water was increased by dispersing the zeolites in the porous net bags on the aquarium floor in a way that they would not clump together. The amount of zeolite determined for the research groups was placed on the aquarium floor in a single bag.

After placing the zeolite bags in the amounts determined in the research plan within the aquariums, 10 grams of feed was distributed equally to all aquarium floors in a way that it would not clump together. The next day, water quality parameters such as oxygen, pH, temperature, and ammonium were determined, data were started to be collected and the measurements were repeated every day at 24-hour intervals.

As a result of the research carried out for seven days, the effects of the presence of zeolite in two different amounts of feed and/or water on the aquatic environment were determined.

2.4. Data Analysis

Total Ammonia Nitrogen (TAN) and NH_3 values; were calculated using NH_4^+-N, water temperature and pH values which measured by YSI Professional Plus Multiparameter (Chow et al., 1997; EPA, 1999; Emerson et al., 1975; Jorgensen, 2002; YSI, 2007). In a previous study, it was reported that the results obtained from the Nessler method were similar and reliable to the results obtained from the traditional electrode method (Prajapati, 2014). The ammonia and TAN were calculated as given below (Purwono et al., 2017):

$$pK(NH_3) = \frac{2726.3}{273+℃} + 0.0963 \quad (1)$$

$$NH_3\text{-}N = 10^{(pH-\mathrm{pK}(NH_3))}\ x\ NH_4^+\text{-}N \quad (2)$$

$$TAN = NH_3\text{-}N + NH_4^+\text{-}N \quad (3)$$

2.5. Statistical Analysis

Analysis of variance was performed to determine whether the difference between water parameters was significant at the beginning of the study and it was determined that the difference between the groups was not statistically significant (P>0.05).

Statistical analysis of the results obtained in the research was made with the "Minitab Release 15 for Windows" package

program. Parametric (ANOVA) tests were used in the data when the prerequisites of the analysis of variance were met, and non-parametric tests (Kruskal-Wallis) were applied when they were not. The results were given as mean±standard error (mean±SE) and the margin of error in the experiment was chosen as 0.05.

3. Results

The water quality parameters determined at the beginning and end of the study are presented in Table 2, Table 3, and Table 4.

When the data of G1, G2 and G3 presented in Table 2 are examined, the water temperature parameters are statistically indifferent. It was determined that the dissolved oxygen and pH values were statistically different and higher in the G3 supplemented with 10% zeolite than in the other two groups. When NH_3 and TAN data were examined, it was determined that although there was no statistical difference, it decreased proportionally with the increase in the amount of zeolite in the feed.

At the end of the experiment, the mean highest TAN concentration in the G1 group, which did not contain any zeolite in the feed, was 5.99 mg/L, and the lowest TAN concentration was 3.77 mg/L in the G3 group. It was determined that 10% zeolite addition into the feed decreased TAN values by 37%.

The difference between water temperature, dissolved oxygen, pH, NH3 and TAN values in G4 and G5 groups was found to be insignificant (Table 3) (P>0.05).

When the data of G6, G7, G8, G9, G10, G11 and G12 groups presented in Table 4 were examined, the water temperature parameters were statistically indifferent. It was determined that the dissolved oxygen, pH, NH_3 and TAN values between the groups were statistically different.

The pH values of the experimental groups were higher in all zeolite groups (except the G7 group) than in the G6 group without zeolite.

The average highest TAN concentration was 7.19 mg/L in the G6 group, which had no zeolite in its feed and water, and the lowest TAN concentration was 3.93 mg/L in the G12 group, which contained 10% zeolite in its feed and 20 g/L zeolite in its water at the end of the experiment. Adding 10% zeolite to feed and 20 g/L to water decreased TAN values by 45%.

Table 2. G1, G2 and G3 groups at the beginning and end of the study, mean temperature (ºC), dissolved oxygen (mg/L), pH, NH_3 (mg/L) and TAN (mg/L) values (mean±SE).

	Zeolite amount		Parameters									
			Temperature (ºC)		Dissolved oxygen (mg/L)		pH		NH_3 (mg/L)		TAN (mg/L)	
Groups	**In feed (g/g)**	**In water (g/L)**	**BE**	**EE**	**BE**	**EE**	**BE**	**EE**	**BE**	**EE**	**BE**	**EE**
G1	-	-	22.00± 0.00^a	22.65± 0.08^a	6.71± 0.14^a	6.16± 0.11^a	8.44± 0.03^a	8.19± 0.05^a	0.04± 0.00^a	0.57± 0.11^a	0.34± 0.00^a	5.99± 0.93^a
G2	2	-	22.03± 0.03^a	22.74± 0.09^a	6.41± 0.09^a	6.34± 0.11^a	8.37± 0.01^a	8.28± 0.04^a	0.03± 0.00^a	0.50± 0.09^a	0.33± 0.00^a	4.71± 0.71^a
G3	10	-	22.00± 0.00^a	22.69± 0.08^a	6.69± 0.15^a	6.60± 0.21^b	8.35± 0.04^a	8.34± 0.06^b	0.03± 0.00^a	0.47± 0.08^a	0.33± 0.00^a	3.77± 0.54^a

BE = Beginning of the experiment, EE = End of the experiment, G1 = No zeolite in feed - No zeolite in water, G2 = 2% zeolite in feed - No zeolite in water, G3 = 10% zeolite in feed - No zeolite in water. Values are expressed as mean ± standard error (n=3), Different letters on the same line indicate that the differences between groups are significant (P<0.05).

Table 3. G4 and G5 groups at the beginning and end of the study, mean temperature (ºC), dissolved oxygen (mg/L), pH, NH_3 (mg/L) and TAN (mg/L) values (mean±SE).

	Zeolite amount	Parameters									
		Temperature (ºC)		Dissolved oxygen (mg/L)		pH		NH_3 (mg/L)		TAN (mg/L)	
Groups	**In water (g/L)**	**BE**	**EE**	**BE**	**EE**	**BE**	**EE**	**BE**	**EE**	**BE**	**EE**
G4	7	21.53± 0.17^a	23.08± 0.25^a	7.80± 0.13^a	7.34± 0.15^a	8.34± 0.00^a	8.46± 0.01^a	0.02± 0.00^a	0.15± 0.01^a	0.22± 0.00^a	1.12± 0.05^a
G5	20	21.30± 0.00^a	23.02± 0.26^a	7.76± 0.11^a	7.33± 0.19^a	8.40± 0.05^a	8.44± 0.01^a	0.02± 0.00^a	0.17± 0.01^a	0.22± 0.00^a	1.37± 0.07^a

BE = Beginning of the experiment, EE = End of the experiment, G4 = No feed - 7 g/L zeolite in water, G5 = No feed - 20 g/L zeolite in water. Values are expressed as mean ± standard error (n=3), Different letters on the same line indicate that the differences between groups are significant (P<0.05).

Table 4. G6, G7, G8, G9, G10, G11 and G12 groups at the beginning and end of the experiment, mean temperature (ºC), dissolved oxygen (mg/L), pH, NH_3 (mg/L) and TAN (mg/L) values (mean±SE).

Zeolite amount			Parameters									
			Temperature (ºC)		Dissolved oxygen (mg/L)		pH		NH_3 (mg/L)		TAN (mg/L)	
Groups	**In feed (g/g)**	**In water (g/L)**	**BE**	**EE**	**BE**	**EE**	**BE**	**EE**	**BE**	**EE**	**BE**	**EE**
G6	-	-	21.57± 0.03^{a}	23.25± 0.25^{a}	7.54± 0.13^{a}	6.07± 0.05^{b}	8.39± 0.03^{a}	8.27± 0.04^{bc}	0.02± 0.00^{a}	0.79± 0.15^{a}	0.22± 0.00^{a}	7.19± 1.16^{a}
G7	-	7	21.30± 0.00^{a}	23.18± 0.27^{a}	7.75± 0.02^{a}	6.45± 0.12^{ab}	8.39± 0.02^{a}	8.23± 0.03^{c}	0.02± 0.00^{a}	0.42± 0.08^{b}	0.22± 0.00^{a}	4.83± 0.71^{b}
G8	2	7	21.60± 0.00^{a}	23.17± 0.25^{a}	7.80± 0.12^{a}	6.66± 0.10^{a}	8.43± 0.04^{a}	8.35± 0.03^{ab}	0.02± 0.00^{a}	0.59± 0.09^{ab}	0.22± 0.00^{a}	4.98± 0.60^{b}
G9	10	7	21.63± 0.20^{a}	23.16± 0.25^{a}	7.92± 0.06^{a}	6.89± 0.15^{a}	8.46± 0.04^{a}	8.38± 0.03^{a}	0.03± 0.00^{a}	0.59± 0.08^{ab}	0.23± 0.00^{a}	4.83± 0.56^{b}
G10	-	20	21.37± 0.09^{a}	23.07± 0.26^{a}	7.78± 0.10^{a}	6.57± 0.11^{a}	8.42± 0.02^{a}	8.28± 0.03^{bc}	0.02± 0.00	0.54± 0.08^{b}	0.22± 0.00^{a}	5.20± 0.63^{b}
G11	2	20	21.53± 0.07^{a}	23.21± 0.25^{a}	7.55± 0.03^{a}	6.77± 0.11^{a}	8.45± 0.02^{a}	8.37± 0.03^{a}	0.03± 0.00^{a}	0.49± 0.07^{b}	0.23± 0.00^{a}	4.09± 0.42^{b}
G12	10	20	21.37± 0.12^{a}	23.16± 0.24^{a}	7.77± 0.12^{a}	6.83± 0.11^{a}	8.40± 0.04^{a}	8.41± 0.04^{a}	0.02± 0.00^{a}	0.51± 0.06^{b}	0.22± 0.00^{a}	3.93± 0.35^{b}

BE = Beginning of the experiment, EE = End of the experiment, G6 = No zeolite in feed - No zeolite in water, G7 = No zeolite in feed - 7 g/L zeolite in water, G8 = 2% zeolite in feed - 7 g/L zeolite in water, G9 = 10% zeolite in feed - 7 g/L in water zeolite, G10 = No zeolite in feed - 20 g/L zeolite in water, G11 = 2% zeolite in feed - 20 g/L zeolite in water, G12 = 10% zeolite in feed - 20 g/L zeolite in water. Values are expressed as mean ± standard error (n=3), Different letters in the same column indicate that the differences between groups are significant (P<0.05).

When NH_3 daily change values were examined, it was determined that after the 5th day in G1, G2 and G3 groups, it was lower in zeolite groups compared to the control (G1) group and ammonia values decreased as the amount of zeolite in the feed increased (Figure 1). When the daily NH_3 change in the groups with zeolite in both feed and water was examined, it was determined that after the 4th day, the experimental group without zeolite contained higher NH_3 values than the experimental groups with different ratios of zeolite in their feed and/or water. On the last day of the experiment, this difference was determined with the highest value in G6, which did not contain any zeolite, and the lowest value in the G12 group, which contained 10% zeolite in feed and 20 g/L zeolite in water (Figure 2).

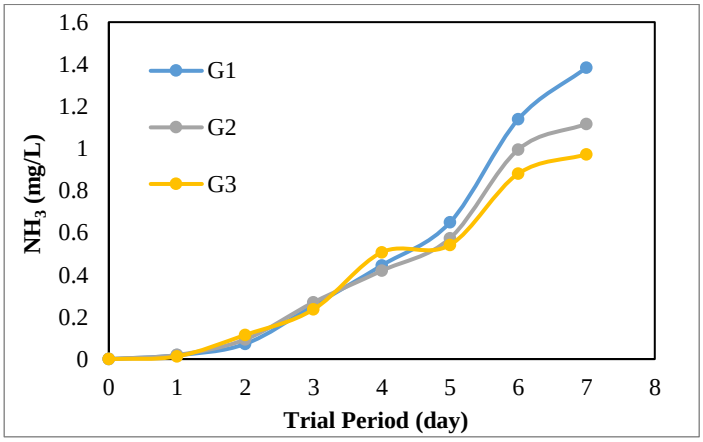

Figure 1. Daily change in NH_3 values of G1, G2 and G3 groups.

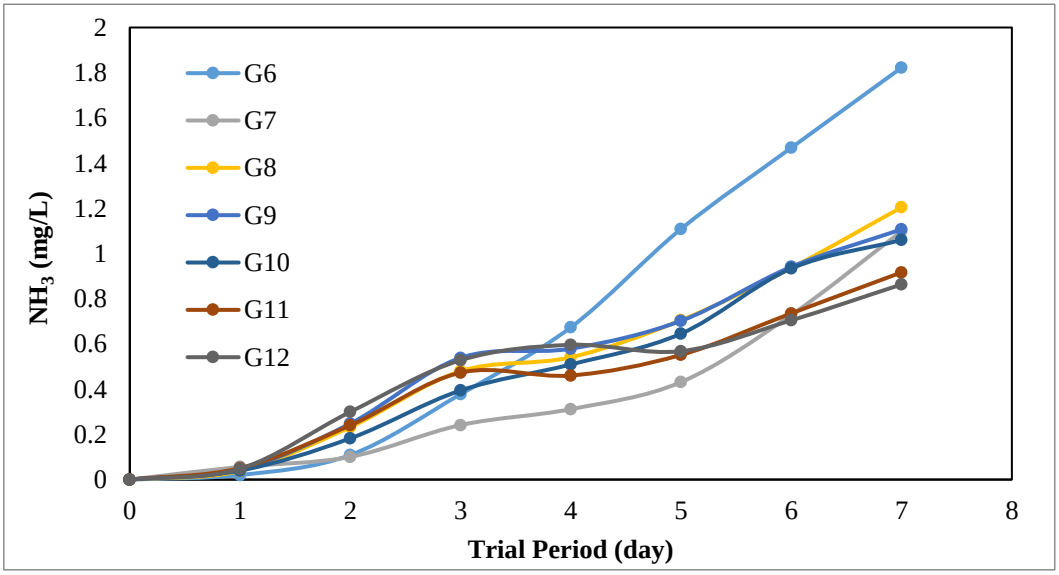

Figure 2. Daily change in NH_3 values of G6, G7, G8, G9, G10, G11 and G12 groups.

4. Discussion

In this study, it was determined that pH values increased in most of the groups (G2, G3, G4, G5, G8, G9, G10, G11 and G12) with zeolite added to the feed and/or water in comparison with the groups without zeolite addition (G1 and G6). This finding regarding pH is supported by other studies in which the ammonium retention capacity of clinoptilolite in various concentrations is investigated (Mazeikiene et al., 2008; Zabochnicha-Swiatek & Malinska, 2010; Öz et al., 2017; Şahin et al., 2018a; Şahin et al., 2018b; Şahin et al., 2019).

Temperature and pH values of the water environment are the most important factors affecting the NH_3/NH_4 ratio. One unit increase in pH values increases the availability of NH_3 10 times (Durborow et al., 1997). In this study, when the effect of pH increase in zeolite groups on NH_3 change, which is important for aquaculture, was examined (Table 3), it was determined that it was not at a level that would adversely affect the reduction of NH_3 values. It was founded that NH_3 remained at lower values in zeolite groups, especially in increasing ammonia concentrations (after the 4th day). Similar to this result, Zhang and Perschbacher (2003) found in their study that although the pH ratio increased by 0.10 units (from 7.85 to 7.95) in the zeolite experimental group, there was no statistical difference compared to the control group. Although the pH values in the experimental groups were affected by the addition of zeolite, they remained within the limits suitable for fish farming in all experimental groups.

As a result of this study, when the daily changes of NH_3 values were examined, it was determined that there was a significant decrease in ammonia values in the groups with zeolite added after the 4th day compared to the control groups (G1 and G6). It was found to be similar to Zain et al. (2018) study. The inclusion of 2.5% natural zeolite in the diet reduced the ammonia excretion rate of rainbow trouts by 24% compared to the control group.

Dissolved oxygen concentration was higher in G4 and G5 groups than in all other groups. This difference is due to the fact there is no factor, such as unconsumed feed, that will cause oxygen consumption in the environment. The lowest value of dissolved oxygen was determined from the G1 and G6 groups that did not contain zeolites in their feed and water (Tables 2 and 4). As the zeolite content in feed and water increased, the oxygen ratio generally increased. As a result, dissolved oxygen was positively affected by the addition of zeolite into water, feed or both feed and water. In this respect, the results were found to be similar to Berka (1986), Bower and Turner (1982), Sing et al. (2004), Zain et al. (2018), Shalaby et al. (2021). The sum of the NH_3 and NH_4^+ values in equilibrium depending on the pH and temperature values in the water is expressed as TAN. The use of zeolites in feed, water or both feed and water contributes to an effective reduction of TAN values in water. Among the zeolite groups, TAN values are lower as the amount of zeolite increases. However, the lack of statistical difference between the groups showed that even low zeolite amounts in the study could be used effectively.

Despite the beneficial and promising effects of zeolite as an additive in various animal feeds (Valpotić et al., 2017), there is enough research on its use for aquaculture. It seems more likely that clinoptilolite could be used as a filter, for transporting live fish, and as a fish feed additive, especially to remove ammonia (Zhou & Boyd, 2014; Ghasemi et al., 2018).

When these results are evaluated, ammonia tolerance values of fish species are under the influence of many factors and variables. In the case of much more sensitive aquatic species, it may be recommended to prefer higher values such as 10% zeolite addition to the feed and 20 g/L zeolite into the water where lower ammonia values are determined. When the results are evaluated from another point of view, considering the economy and ease of use, it is concluded that 2% zeolite in feed and/or 7 g/L in aquarium water can be recommended for aquatic species with high tolerance to ammonia values. Similar to this study, Ibrahim et al. (2016) evaluated the effect of natural zeolite (clinoptilolite) as a feed additive in fish diets on the growth performance, genetic characteristics and health status of freshwater Nile tilapia fish (*Oreochromis niloticus* L.). In the study, in parallel with the amount of zeolite added to the feed, an increase was determined in the ash values in the feed, similar to the previous studies (Ibrahim et al., 2016; Tekeşoğlu & Ergün, 2021; Al Amir et al., 2022). The amount of ash is related to the mineral matter content, and the zeolite (clinoptilolite) also has mineral substance content. This may be a factor that positively affects the quality of fish feeds. By increasing the nutritional value of the feed, it can be effective both on fish growth and on the protection of water conditions.

As a result, it has been determined that the addition of zeolite to the feed or water has a positive effect for the control of important water parameters such as pH, oxygen and TAN in freshwater aquaculture systems.

Acknowledgment

This research was produced from doctorate thesis. We would like to thank TÜBİTAK, which supported this thesis with the project numbered 108O307 within the scope of the Rapid Support Program, to the project team that carried out the project, to SİBAL A.Ş., which we saw their support in the supply and analysis of feed raw materials and ENLİ MADENCİLİK A.Ş. for helping in the supply of raw materials and zeolite raw materials.

Compliance with Ethical Standards

The study protocol was approved by ethics committee of Sinop University (Decision number 2007/13) and experiments were carried out in accordance with the ethical guidelines and

regulations declared by the Sinop University and the international principles of laboratory animal use and care.

Conflict of Interest

The authors declare that they have no conflict of interest.

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JOURNAL OF AGRICULTURAL PRODUCTION

ISSN: 2757-6620

https://prensip.gen.tr

RESEARCH ARTICLE

The Effect of Vitamin C and E Supplementation into Drinking Water on Carcass Characteristics, Meat Quality and Intestinal Microflora During Pre-Slaughter Feed Withdrawal in Broiler Chickens

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ARTICLE INFO

Article History
Received: 09.04.2023
Accepted: 23.05.2023
First Published: 30.06.2023

Keywords
Broiler
Feed withdrawal period
Intestinal microflora
Meat quality
Slaughter characteristics

ABSTRACT

This study investigates the effects of adding vitamin C and E to the drinking water on carcass characteristics, meat quality and intestinal microflora populations in broiler chickens during the 10-h pre-slaughter feed withdrawal (FW) period. As study materials, forty male broilers at the age of 42 days were used. The broilers were randomly divided into four groups: Control (non-vitamin, NV), vitamin C (1000 mg/L, VC), vitamin E (500 mg/L, VE) and vitamin combination (1000 mg/L VC+500 mg/L VE, VCE). In the study, vitamin additions didn't affect carcass characteristics, visceral weights and the pH values of the digestive system (P>0.05). The addition of VC and VE increased the weight of the Bursa of Fabricius, and the addition of VE increased the weight of thymus (P<0.05). Additions of vitamin decreased tendency of carcass contamination (P<0.01) and increased pH_{45min} and pH_{24h} of thigh meat and pH_{24h} of breast meat (P<0.05, P<0.01, P<0.01, respectively). While a* color intensity of breast and thigh meat increased with all vitamin supplements, L* and b* values of thigh meat decreased (P<0.01). Vitamin supplements, especially VE, reduced the drip loss of breast and thigh meat (P<0.05) and the pathogenic microorganism populations of intestinal contents (P<0.01). As a result, it is thought that the addition of 500 mg/L vitamin E to the drinking water of broiler chickens exposed to the pre-slaughter fasting period will be beneficial to improve meat quality and reduce intestinal pathogenic microorganism load. However, more extensive experimental studies are needed.

Please cite this paper as follows:

Kaya, H. (2023). The effect of vitamin C and E supplementation into drinking water on carcass characteristics, meat quality and intestinal microflora during pre-slaughter feed withdrawal in broiler chickens. *Journal of Agricultural Production*, *4*(1), 47-55. https://doi.org/10.56430/japro.1280038

1. Introduction

The quality of chicken meat, which has an important place in meeting animal protein needs all over the world, is affected by pre-slaughter management. The pre-slaughter management, which covers all the activities and processes that the broilers are exposed to pre-slaughter, begins with the feed withdrawal (FW). The pre-slaughter FW is done to reduce the content of the digestive system and to prevent fecal contamination and carcass contamination during transport and slaughter (Petrolli et al., 2016). It has been reported that the 10 h pre-slaughter FW period is sufficient to maintain the balance between weight loss, meat quality and carcass contamination (Xue et al., 2021). However, taking longer pre-slaughter processes causes stress to the broiler chickens, negatively affecting both animal welfare and meat quality and causing economic losses (Pan et al., 2018). Oxidative stress occurring before slaughter can lead to poor meat quality by promoting oxidative reactions in meat after slaughter (Nawaz & Zhang, 2021), oxidizing lipids and proteins, causing undesirable changes in sensory properties such as color, taste, texture, and nutritional value (Attia et al.,

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2017; Mazur-Kuśnirek et al., 2019). Therefore, the oxidative state of broiler chickens during slaughter is very important for quality poultry meat production (Zeferino et al., 2016; Pan et al., 2018).

There is a good balance in the intestinal bacterial flora of healthy animals (Yadav & Jha, 2019). However, it is reported that stress-causing factors such as transportation (Bello et al., 2018), stocking density (Yu et al., 2021), environmental temperature (Calik et al., 2022) and fasting (Kayan & Açıkgöz, 2020; Xue et al., 2021) can disrupt this balance (Wickramasuriya et al., 2022). Stress, which weakens immunity in chickens, can activate certain pathogenic microorganisms suppressed in the gut (Zhao et al., 2017; Mishra & Jha, 2019), causing a disruption of the floral balance (Burkholder et al., 2008) and a devastating effect on the intestinal immune barrier (Zhao et al., 2017). Burkholder et al. (2008) reported that broiler chickens exposed to pre-slaughter FW (24 hours) or heat stress (HS, 30 °C) increased their susceptibility to intestinal pathogens. Nawaz and Zhang (2021) pointed out that stress stimulates intestinal bacteria by affecting intestinal epithelial cells.

The fact that the maintenance and management conditions such as FW, catching and transportation applied to the broilers in the pre-slaughter period are suitable for animal welfare affect the taste and quality of the meat positively (Pan et al., 2018). Therefore, studies have recently focused on identifying effective ways to reduce the pre-slaughter stress response of broilers and improve post-slaughter meat quality and gut microbiota (Karacay et al., 2008; Kop-Bozbay & Ocak, 2015; Petrolli et al., 2016). It is well known that endogenous antioxidant factors such as vitamin C and vitamin E reduce some adverse effects caused by oxidative stress (Attia et al., 2017). It has been reported that vitamin C is effective in relieving multiple stress factors with its immunomodulatory, antimicrobial and strong antioxidant properties (Gan et al., 2020).

It has been reported that the addition of vitamin E, which is a powerful antioxidant, antimicrobial and immune system supporter (Dalia et al., 2018; Calik et al., 2022), to broiler diets slows down oxidative stress, reduces drip loss, and improves meat quality (Souza et al., 2007; Attia et al., 2017; Ghasemi-Sadabadi et al., 2022). Vitamin C and vitamin E, which have strong antioxidant properties, are reported to have a synergistic effect in reducing the negative effects of stress (Attia et al., 2017). There are limited studies evaluating the effects of adding vitamin C and vitamin E alone or in combination to chicken diets on pre-slaughter multiple stress. This study was carried out to investigate the effects of 1000 mg/L vitamin C (VC), 500 mg/L vitamin E (VE) and 1000 mg/L vitamin C +500 mg/L vitamin E (VCE) supplementations into drinking water during the 10 h pre-slaughter feed withdrawal period on slaughter characteristics, meat quality and intestinal microflora populations of male broiler chickens.

2. Materials and Methods

2.1. Animals and Experimental Design

40 healthy male broiler chicks, aged 42 days, obtained from a commercial enterprise, were randomly divided into 4 treatment groups, each of which was housed in an individual compartment. These are follows; NV (non-vitamin): birds were given drinking water during the 10 h fasting period, VC: birds were given drinking water with 1000 mg/L VC during the 10 h FW period, VE: birds were given drinking water with 500 mg/L VE during the 10 h FW period, and VCE: birds were given drinking water with 1000 mg/L VC+ 500 mg/L VE during the 10 h fasting period. In the study, artificial light and ad-libitum water were provided to the chickens during the 10-hour fasting period (from 22:00 in the evening to 08:00 the next day). Feed withdrawal time was planned individually for each broiler so that the pre-slaughter fasting period did not exceed 10 hours (Xue et al., 2021). The determined amounts of vitamins obtained from a commercial company were added to the water that the broilers would drink in 10 hours (Aviagen, 2009).

2.2. Sample Collection and Determination of Parameters

In broiler chickens sacrificed by cervical dislocation method, the weights of carcass, thigh meat, breast meat and visceral organs were relatively calculated by dividing live weight. pH values of the crop, proventriculus, gizzard, meat and intestine were measured with a calibrated pH meter (MARTINI Mi 150 pH / Temperature Laboratory Bench Meter) with buffer solutions of 4.00, 7.00 and 10.00 (Ohkawa et al., 1979). Two different pH values for breast and thigh meat, 45 minutes (pH_{45min}) and 24 h after death (pH_{24h}), were measured 3 times in 3 locations, and the average values were taken as the final result. The pH decline within 24 h after slaughter was calculated as a percentage (Pan et al., 2018).

$$\text{pH decline (\%)} = [(pH_{45min} - pH_{24h})/pH_{45min}] \times 100 \quad (1)$$

To detect drip loss, breast and thigh fillets (approximately 5 cm×3 cm×2 cm) were weighed after cutting (W_1), hung in a 50 mL cold storage tube at 4°C for 24 h, and the surface liquids were wiped off and reweighed (W_2). Drip loss was calculated as a percentage of weight loss during storage (Xue et al., 2021).

$$\text{Drip loss (\%)} = [(W_1 - W_2)/W_1] \times 100 \quad (2)$$

Color intensities (L*, a* and b*) in breast and thigh meats were determined using a Konica Minolta colorimeter (Chroma Meter, CR-400, Minolta Konica, Japan). Color intensities were determined according to the specifications given by the International Commission on Illumination CIELAB (Commission Internationale de I'e Clairage) based on three-dimensional color measurement (CIE, 1986). According to

these criteria; L* (=0 black, =100 white (darkness/lightness)), a* (= +60 red, = -60 green) and b* (= +60 yellow, = -60 blue) indicate different color intensities. The colorimeter was calibrated using a special whiteboard before measurement, and the tip of the measuring head was placed flat on the middle surface. Measurements from 3 different sessions were averaged for each sample. For the microbiological analysis of the contents of the small intestine, the samples taken from 4 animals from each group in sterile conditions were stored at –80ºC until the day of analysis. Total mesophilic aerobic bacteria (*TMAB*) and *Coliform* (Harrigan, 1998), *E. coli* (ISO 16649-2, 2001) and *Salmonella spp*. (Andrews & Hammack, 2011) populations were determined in the microbiology laboratory.

Table 1. Contamination tendency scale (Xue et al., 2021).

Grade	Volume of feces excretion (ml)
0	0
1	0-2
2	2-4
3	4-6
4	6-8
5	>8

Contamination tendency was assessed and subjectively recorded in slaughtered animals after peeling and plucking, before dissection. After the chickens were pressed 3 times in a row by applying a uniform force on the abdomen, faecal shedding was observed by three observers, with a value of 0 indicating no faecal discharge, and a value of 5 denoting maximum faecal discharge, evaluated with a scale numbered from 0 to 5 (Table 1). The mean of these observations was determined as the contamination tendency.

2.3. Statistical Analysis

The data obtained from the study were analyzed with the "Statistical Package for Social Sciences" (IBM SPSS Statistic 25) statistical program. One-way analysis of variance (ANOVA) was applied to determine the statistical calculations of carcass characteristics, meat quality, digestive system pH and intestinal microflora and the importance of the difference between the mean values of the groups. Duncan's multiple comparison test was used to control the significance of the difference between the groups. Differences between groups in intestinal *Salmonella spp.* were evaluated with the "chi-square test" and a $P<0.05$ level was considered statistically significant.

3. Results and Discussion

3.1. Carcass Characteristics and Visceral Weights

Table 2 shows that the addition of vitamins to the drinking water of broilers exposed to pre-slaughter FW period has no effect on carcass characteristics and some visceral weights. The results of previous studies (Rathgeber et al., 2007; Karacay et al., 2008; Kop-Bozbay & Ocak, 2015; Petrolli et al., 2016; Güler et al., 2019) and current study results were similar. On the other hand, Kayan and Açıkgöz (2020) reported that the addition of 0.1% mixed organic acid into drinking water during the 6 or 12 h pre slaughter FW period reduced carcass and breast meat yield and thigh weight. It has been reported that the addition of VC (200 mg/kg), VE (100 mg/kg) or VCE to HS-administered chicken diets did not affect liver and heart weights, but the addition of VE increased the weight of carcass and abdominal fat (Attia et al., 2017). Zeferino et al. (2016) reported that the addition of VC (257 to 288 mg/kg) and VE (93 to 109 mg/kg) did not affect carcass, thigh and breast yields and abdominal fat, heart and liver weights in chickens under HS, similar to the results of the present study. Also, Yu et al. (2021) determined that the addition of 200 mg/kg VC to chicken diets under high stocking density (HSD) stress did not affect breast meat yield. It has been reported that the addition of 250, 500 and 1000 g/ton VC + citric flavonoids did not affect the yield of carcass, breast, thigh and abdominal fat in chicken diets under HS (Peña et al., 2008), and that the addition of 100 and 200 mg/kg VE to carcass, heart, liver and does not affect abdominal fat yield (Mazur-Kuśnirek et al., 2019).

Table 2. The effect of adding vitamin to the drinking water during the pre-slaughter FW period on carcass characteristics and visceral weights of broiler chickens (% of live body weight).

Parameters (%, BW)	Treatment Groups					
	NV	VC	VE	VCE	Average	p-*value*
Hot dressing	71.58±0.31	70.98±0.29	71.67±0.24	71.67±0.53	71.47±0.18	0.470
Cold dressing	71.00±0.33	70.61±0.31	71.27±0.24	71.37±0.50	71.06±0.18	0.449
Thigh dressing	26.64±0.42	26.44±0.58	26.06±0.21	26.90±0.39	26.51±0.21	0.553
Breast dressing	22.67±0.39	22.48±0.62	21.93±0.47	23.31±0.66	22.59±0.275	0.371
Heart	0.43±0.00	0.44±0.01	0.44±0.01	0.44±0.01	0.44±0.06	0.667
Kidney	0.03±0.00	0.02±0.00	0.02±0.00	0.03±0.00	0.03±0.00	0.224
Abdominal fat	1.41±0.06	1.47±0.05	1.43±0.06	1.53±0.08	1.46±0.03	0.610
Liver	1.82±0.03	1.72±0.06	1.81±0.06	1.76±0.06	1.78±0.03	0.547
Spleen	0.103±0.00	0.105±0.01	0.106±0.00	0.114±0.01	0.107±0.00	0.556
Bursa fabricus	$0.40±0.00^{c}$	$0.50±0.00^{b}$	$0.62±0.00^{a}$	$0.45±0.00^{bc}$	0.49±0.02	0.000
Thymus	$0.05±0.00^{b}$	$0.05±0.00^{b}$	$0.07±0.00^{a}$	$0.06±0.01^{b}$	0.06±0.00	0.020

NV, Non vitamin-Control; VC, 1000 mg/L water Vitamin C; VE, 500 mg/L water Vitamin E; VCE, Combination of VC (1000 mg/L water) and VE (500 mg/L water). $^{a-c}$; Means in the same row with different superscripts differ significantly ($P < 0.05$). Values are given as mean±standard deviation.

In this study, bursa weight increased with both VC and VE additions (P<0.01), while thymus weight increased only with VE addition (P<0.05) (Table 2). It was reported that adding 200 mg/kg Vit C or 300 mg/kg Vit E to the broilers diets reared under HS (Gharieb & Moursi, 2013), 125 mg/kg Vit E (Habibian et al., 2014) increased the weights of thymus and bursa, similar to current study results. In another study, it was reported that VE supplementation at 100 and 200 mg/kg did not affect the weight of the thymus but increased the weights of the bursa and spleen in chickens reared under thermoneutral (TN) conditions (Singh et al., 2006). On the other hand, Niu et al. (2009), Dalia et al. (2018) and Attia et al. (2017) reported that the addition of different doses of VC or VE to broiler diets did not affect thymus and bursa weights, contrary to the current study. Regarding the effects of VC and VE additions on the weights of the lymphoid organ thymus and bursa, the reason for the discrepancy between the results of the present study and other studies is not yet fully understood. However, it has been reported that lymphoid organ weights can be a reliable indicator of stress (Rosales, 1994) and that stress can cause lymphoid organ atrophy (Moberg, 2000). In this context, it is thought that the addition of VE and VC with antioxidant effects to the drinking water of chickens found in the pre-slaughter FW period may be effective in suppressing stress by affecting lymphoid organ weights. It has been reported that the addition of VE in poultry increases the T-helper/T-cytotoxic lymphocyte ratio and the percentages of T-helper lymphocytes in the spleen and thymus (Erf et al., 1998) and improves the immune response in rats (Moriguchi et al., 1993).

3.2. Meat Quality

The effects of VC, VE or VCE additions to drinking water during the 10 h pre-slaughter FW period on thigh and breast meat quality characteristics are shown in Table 3. The additions of vitamin increased the pH_{45min} and pH_{24h} values of thigh meat (P<0.05, P<0.01, respectively) but did not affect the pH decline rate (P>0.05). Addition of VC, VE or VCE to the drinking water of broilers during pre-slaughter FW period resulted in decreased thigh meat L* and b* color intensities and drip loss, and increased a* color intensity (P<0.01). With the results of this study, Lin et al. (2007), Serdaroğlu and Öztürk (2011), Güler et al. (2019) and Pan et al. (2018) reports are compatible. In poultry production, stress caused by various environmental factors during pre-slaughter accelerates post-slaughter glycolysis and leads to lactic acid accumulation, thereby reducing meat pH (Lin et al., 2007). The decrease in meat pH initiates protein denaturation, which has an effect on the color and water holding capacity of the meat, resulting in pale and low water holding capacity meat production (Serdaroğlu & Öztürk, 2011). This condition is called pale, soft and juicy (pale, soft and exudative; PSE) meat (Barbut, 1998).

Table 3. The effect of adding vitamin to drinking water during the pre-slaughter FW period on meat quality of broiler chickens.

Parameters	Treatment Groups					
	NV	VC	VE	VCE	Average	p-*value*
Thigh meat						
pH_{45min}	6.16±0.06^{b}	6.28±0.03^{a}	6.32±0.06^{a}	6.31±0.02^{a}	6.27±0.02	0.016
pH_{24h}	5.67±0.03^{b}	5.84±0.04^{a}	5.89±0.03^{a}	5.88±0.02^{a}	5.82±0.02	0.000
pH decline (%)	7.90±0.61	7.01±0.76	6.77±0.37	6.88±0.43	7.14±0.28	0.483
L*	50.49±0.25^{a}	48.84±0.31^{b}	48.41±0.87^{b}	46.65±0.27^{c}	48.60±0.32	0.000
a*	4.01±0.10^{c}	4.71±0.12^{b}	5.49±0.22^{a}	5.62±0.08^{a}	4.96±0.12	0.000
b*	7.98±0.18^{a}	6.15±0.20^{b}	5.54±0.30^{b}	5.67±0.24^{b}	6.33±0.19	0.000
Drip loss (%)	7.67±0.27^{a}	6.53±0.47^{b}	5.76±0.29^{b}	6.44±0.44^{b}	6.60±0.21	0.010
Breast meat						
pH_{45min}	6.10±0.03	6.18±0.04	6.21±0.02	6.19±0.03	6.17±0.02	0.057
pH_{24h}	5.76±0.03^{b}	5.86±0.03^{a}	5.91±0.03^{a}	5.86±0.03^{a}	5.85±0.02	0.007
pH decline (%)	5.63±0.37	5.14±0.49	4.93±0.26	5.27±0.48	5.24±0.20	0.677
L*	47.74±0.56	48.01±0.52	48.65±0.18	48.07±0.61	48.12±0.25	0.628
a*	2.56±0.18^{b}	4.02±0.23^{a}	3.82±0.17^{a}	3.53±0.39^{a}	3.48±0.15	0.001
b*	6.22±0.26^{a}	5.48±0.18^{b}	5.68±0.10^{b}	6.27±0.18^{a}	5.91±0.11	0.010
Drip loss (%)	6.26±0.30^{a}	5.34±0.41^{b}	4.57±0.26^{b}	4.91±0.27^{b}	5.27±0.18	0.04

NV, Non vitamin-Control; VC, 1000 mg/L water Vitamin C; VE, 500 mg/L water Vitamin E; VCE, Combination of VC (1000 mg/L water) and VE (500 mg/L water). $^{a-d}$; Means in the same row with different superscripts differ significantly (P<0.05). Values are given as mean±standard error.

In this study, it was found that vitamin supplements increase the pH values of thigh meat, decrease drip loss and improve the a* color parameter, which is consistent with the reports of Souza et al. (2007) and Serdaroğlu and Öztürk (2011). On the other hand, the results of the current study on the color density and pH values of thigh meat differ from the results of Imik et al. (2012), Kop-Bozbay and Ocak (2015) and Karacay et al. (2008). It is thought that the reason for this situation is due to the different types and doses of additives added to the drinking water of chickens.

Additions of vitamin to drinking waters of broilers during the 10 h pre-slaughter FW period did not affect breast meat pH_{45min}, pH decrease rate and L* color intensity (P>0.05), but pH_{24h} and drip loss decreased (P<0.01, P<0.01, respectively) (Table 3). Breast meat's a* color intensity increased with all vitamin supplements (P<0.01), while b* color intensity decreased with VC and VE (P<0.01). These results are consistent with the results of Petrolli et al. (2016) and Kop-Bozbay and Ocak (2015). While Petrolli et al. (2016) reported that the addition of 200 mg/L VE to the water of broilers during the 12 h pre-slaughter FW period did not affect the L* value of breast meat, Kop-Bozbay and Ocak (2015) reported that addition of 3 g/L glucose, sucrose to the drinking water during the 10 h pre-slaughter FW period did not affect the pH_{1h} and L* values of breast meat, but the a* color intensity increased by addition of starch. On the other hand, the results of the present study are partially compatible with the results of Zhang et al. (2022) and Karacay et al. (2008). It is reported that the addition of guanidineacetic acid to the drinking water of broilers exposed to transportation stress for 3 hours after the 8-hour pre-slaughter FW period did not affect the breast meat's pH_{45min} and a* and b* color densities, but increased pH_{24h} and decreased L* color density and drip loss (Zhang et al., 2022), and the addition of sucrose to drinking water during the 10 h pre-slaughter FW period did not affect the breast meat's L* and b* color intensities, but increased the a* value (Karacay et al., 2008). In addition, there are studies reporting that the addition of different levels of VC, VE or VCE to chicken diets under stress has no effect on breast meat's pH and color parameters (Peña et al., 2008; Zeferino et al., 2016; Attia et al., 2017; Mazur-Kuśnirek et al., 2019; Yu et al., 2021). However, Imik et al. (2012) reported that VC addition decreased breast meat's pH and a* value and increased L* and b* values, while Zhang et al. (2013) reported that VE addition decreased L* and b* color intensity and increased a* value. It was reported that the quality defect PSE can be determined by examining pH_{24h} values lower than 5.8 and L* values higher than 52 together (Barbut, 1998). Accordingly, it can be said that the incidence of PSE meat is low since the pH_{24h} values of thigh and breast meat detected in all vitamin supplemented groups in the current study were above 5.8 and L* values were below 52. The results of the current study, which found that breast meat's drip loss was reduced, were similar with the results of Mazur-Kuśnirek et al. (2019), but different from the results of Peña et al. (2008), Zeferino et al. (2016) and Zhang et al. (2013).

3.3. Digestive System pH, Intestinal Microflora and Contamination Tendency

It was determined that the addition of VC, VE and VCE to the drinking water of broilers during the pre-slaughter FW period did not affect the pH values of crop, proventriculus, gizzard and intestinal contents (P>0.05), but reduced the tendency of carcass contamination (P<0.01) (Table 4). Kayan and Açıkgöz (2020) reported that the addition of organic acid to the drinking water during the 6 or 12 hour pre- slaughter FW period did not affect the pH values of crops, gizzards and proventriculus. On the other hand, Hinton et al. (2000) reported that the feed material in the crop was depleted 6 hours after the chickens were prevented from accessing the food, while Kayan and Açıkgöz (2020) reported that the pH of the gizzard and bezel stomach decreased as the FW extended from 6 hours to 12 hours. Depletion of feed material in the crop lowers the lactic acid concentration, leading to an increase in pH and thus a decrease in its ability to inhibit the growth of enteropathogens (Hinton et al., 2002). The main purpose of the pre-slaughter FW period in of broiler chickens production is to prevent carcass contamination (*Salmonella*, *Campylobacter*, etc.) and to produce hygienic chicken meat by ensuring that the digestive system is empty. It is reported that pre-slaughter fasting period and transportation time are associated with the contamination tendency, which is one of the indicators of carcass pollution, and the increasing contamination tendency increases processing costs and reduces profitability (Menconi et al., 2014). In addition, it is important to control the rate of carcass contamination during slaughter since carcass contamination is a serious public health problem and adversely affects carcass production and meat quality (Xue et al., 2021). In this respect, the fact that the addition of VC, VE or VCE to the water during the 10 h pre-slaughter FW period reduces the tendency of carcass contamination (P<0.01), is seen as an important result in terms of both hygienic chicken meat production and processing cost.

All of the vitamin additions to the drinking water of broilers during the 10-hour pre-slaughter FW period decreased the pathogenic microorganism population (P<0.01) in the intestinal contents (Table 4). The addition of VE was more effective at attenuating *TMAB*, *Coliform* and *E. coli* populations than other groups, while the addition of VCE was more effective than the control and VC additions (P<0.01). Oxidative stress damages the intestinal microflora and leads to an increase in pathogenic bacteria (Mishra & Jha, 2019). Dietary antioxidants can help alleviate the negative effects of various stress factors on microbiota by regulating the intestinal microflora (Yang et al., 2020). It is reported that the addition of VE (250 mg/kg) to broilers' diets housed under HS improves antioxidant status, alters intestinal microorganism population and functions, and alleviates the negative effects of stress (Calik et al., 2022). However, there are also studies reporting that the addition of VE at different doses (up to 200 mg/kg) to chickens' diets does not affect the intestinal pathogenic microorganism (*Coliform*, *TMAB*, *E. coli*) population (Scocco et al., 2017; Dalia et al., 2018; Ghasemi-Sadabadi et al., 2022). Mandal et al. (2005) reported that the intestinal *Coliform* count was reduced by supplementation of 150 mg/kg of VC or 300 mg/kg of VE in broilers during the 8 h pre-slaughter FW period. It has been observed that the addition of 50 ml/L VC reduces the intestinal *E. coli* count (Nosrati et al., 2017) and the addition of 300

mg/kg VC reduces both the intestinal *Coliform* and *E. coli* counts (Hajati et al., 2015). On the other hand, during 6 h pre-slaughter FW period the addition of maltodextrin did not affect the *TMAB* count of the crop content, but weakened the *E. coli* and *Coliform* bacteria population (Rathgeber et al., 2007), the addition of 0.1% organic acid during the 6 or 12 h pre-slaughter FW period did not affect the intestinal *Coliform* count (Kayan & Açıkgöz, 2020).

Table 4. The effect of adding vitamin to drinking water during the pre-slaughter FW period on the pH values of the digestive system and intestinal microflora of broiler chickens.

Parameters	Treatment Groups					
	NV	**VC**	**VE**	**VCE**	**Average**	**p-*value***
Crop pH	5.05±0.27	5.52±0.09	5.70±0.17	5.21±0.11	5.37±0.09	0.057
Proventriculus pH	3.54±0.85	3.74±0.09	3.63±0.12	3.78±0.06	3.67±0.05	0.556
Gizzard pH	3.01±0.05	3.21±0.06	2.93±0.06	3.17±0.24	3.08±0.06	0.363
Intestine pH	6.45±0.09	6.47±0.12	6.53±0.08	6.65±0.05	6.52±0.05	0.382
Contamination tendency (ml)	1.63 ± 0.29^{a}	0.80 ± 0.19^{b}	0.90 ± 0.17^{b}	0.40 ± 0.10^{b}	0.93±0.12	0.001
***TMAB* (log kob/gr)**	7.03 ± 0.02^{a}	6.85 ± 0.02^{b}	4.58 ± 0.04^{d}	4.90 ± 0.01^{c}	5.84±0.29	0.000
***Coliform* (log kob/gr)**	7.13 ± 0.01^{a}	6.94 ± 0.02^{b}	4.32 ± 0.05^{d}	6.71 ± 0.02^{c}	6.27±0.29	0.000
***E. coli* (log kob/gr)**	7.02 ± 0.01^{a}	6.80 ± 0.02^{b}	4.21 ± 0.03^{d}	4.99 ± 0.01^{c}	5.76±0.31	0.000

NV, Non vitamin-Control; VC, 1000 mg/L water Vitamin C; VE, 500 mg/L water Vitamin E; VCE, Combination of VC (1000 mg/L water) and VE (500 mg/L water); *TMAB*, Total Mesophilic Aerobic Bacteria. $^{a-d}$; Means in the same row with different superscripts differ significantly (P<0.05). Values are given as mean±standard error.

Table 5. The effect of adding vitamin to drinking water during the pre-slaughter FW period on intestine *Salmonella spp*. populations of broiler chickens.

Groups	Positive	Negative	Total
NV[a]	4	0	4
VC[a]	4	0	4
VE[b]	0	4	4
VCE[a]	3	1	4
Total	11	5	16

(X^2)=12.509 P=0.006. NV, Non vitamin-Control; VC, 1000 mg/L water Vitamin C; VE, 500 mg/L water Vitamin E; VCE, Combination of VC (1000 mg/L water) and VE (500 mg/L water). $^{a-b}$; Differences between the groups shown with different superscripts are statistically significant (P < 0.05).

In order to determine the statistical effects of vitamin additions to the drinking water of broilers during the pre-slaughter FW period on *Salmonella spp.* in the intestinal content, the groups were compared according to the X^2 test and a significant difference was observed (P<0.05). It was determined that the difference between the groups was due to the VE group (P<0.05). Accordingly, it can be said that the addition of VE to the drinking water of broilers during the pre-slaughter FW period reduces the risk of *Salmonella spp*. compared to other groups. It is thought that the addition of vitamin E, which has a strong antioxidant effect (Dalia et al., 2018; Calik et al., 2022), to the drinking water of broiler chickens in the pre-slaughter FW period may have affected the intestinal microflora by reducing intestinal oxidation stress (Attia et al., 2017; Ghasemi-Sadabadi et al., 2022). It has been emphasized that adding 30 IU/kg VE to the diets of layer chickens exposed to Salmonella Enteritidis (SE) reduces the stress symptoms caused by SE and can be recommended to improve poultry health and production performance by controlling Salmonella infection (Liu et al., 2019). Also, Dalia et al. (2018) observed that adding 100 mg/kg VE to chickens' diets reduced the number of *Salmonella spp.*

4. Conclusion

The results of this study show that the addition of VC or VCE, especially 500 mg/L VE, to the drinking water of broilers during the pre-slaughter FW period may be a good alternative, as it improves meat quality and reduces intestinal pathogenic microorganism populations. It is thought that more studies with more different doses are needed to fully determine the effects of vitamin C and E supplementation in the drinking water of broiler chickens during the pre-slaughter FW period.

Acknowledgment

The author would like to thank Şeyda Merve KARATAŞ (Asst.) for helping at different stages of the project.

Compliance with Ethical Standards

The study was carried out after the approval of the Animal Experiments Local Ethics Committee of Kafkas University (KAÜ-HADYEK/ 2023-040).

Conflict of Interest

The authors declare that they have no conflict of interest.

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JOURNAL OF AGRICULTURAL PRODUCTION

ISSN: 2757-6620

https://prensip.gen.tr

RESEARCH ARTICLE

Effect of a Natural Adsorbent Mixture (Zeolite and Leonardite) on the Reduction of Ammonia Caused by Fish Feed

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ARTICLE INFO

Article History
Received: 29.03.2023
Accepted: 06.06.2023
First Published: 30.06.2023

Keywords
Ammonia
Aquaculture
Leonardite
Zeolite

ABSTRACT

In this this research, the utilization of zeolite (clinoptilolite) and leonardite mix, which are the natural adsorbents that can be used to provide optimum water conditions for aquaculture, was investigated. Three groups with 3 replications were formed and a commercial aquarium fish feed having 47.5% crude protein was added as the ammonia factor in three different concentrations (0.2 g feed/500 ml tap water, 0.4 g/500 ml tap water, 0.6 g/500 ml tap water). Ammonia increases resulted from 3 different amounts of feed were monitored for 7 days. At the end of this period, the adsorbent mixture, which has water-regulating properties, was added to the experimental groups at a ratio of 1:2 (clinoptilolite:leonardite) to remove ammonia, which is harmful for aquatic organisms, and ammonia decreases was determined at regular intervals. NH_3 value reached its highest (0.7 mg/L) at the end of the stage where the ammonia values from the feed were measured. After this period, it started to decrease with the addition of natural adsorbents (mixed clinoptilolite-leonardite) and the lowest ammonia value was determined at the end of the 6th measurement (0.07 mg/L). As a result of this study, it was determined that the clinoptilolite:leonardite mixture has a positive influence on ammonia removal in freshwater aquariums.

Please cite this paper as follows:

Şahin, D. (2023). Effect of a natural adsorbent mixture (zeolite and leonardite) on the reduction of ammonia caused by fish feed. *Journal of Agricultural Production*, *4*(1), 56-62. https://doi.org/10.56430/japro.1273000

1. Introduction

Water parameters such as temperature, pH, dissolved oxygen, ammonia, etc. are the parameters that should be in optimal conditions for sustainable aquaculture (M. Öz et al., 2017; Ü. Öz et al., 2017). Among these parameters, ammonia exists in two forms in water as ammonia (NH_3-N) and/or ammonium ion (NH_4-N). Changes in the pH and temperature of the water affect the presence of ammonium and ammonia (Aly et al., 2016). In aquaculture systems, removal of excess nitrogenous compounds is a mandatory process. Because especially ammonia (NH_3) is harmful to many aquatic organisms (Lin et al., 2023). It is possible to remove this harmful factor from the environment with mechanical, biological, and chemical treatments. To achieve this, adsorbent materials such as zeolite, diatomite, bentonite, and leonardite, etc. are used (Şahin et al., 2018; Öz et al., 2022; Şahin, 2022).

Naturally abundant and mined zeolites are crystalline hydrated alumina silicates of alkali and alkaline earth metals. Clinoptilolite type zeolite can be used for many processes such as molecular sieve, cation exchange and adsorption (Rodrigues et al., 2007; Mazeikiene et al., 2008; Şalcıoğlu, 2022). Scientific studies have determined that zeolite has high efficiency in ammonia removal from wastewater and freshwater (Aly et al., 2016; Ghasemi et al., 2018; Skleničková et al., 2020). Furthermore, the use of zeolite as a dietary supplement or to remove ammonia from water has more useful

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for aquaculture (Öz et al., 2016; Şahin et al., 2019; Aly et al., 2020; Öz et al., 2021).

Leonardite emerges during coalification and has a humic acid content ranging in 40-85%. Leonardite is also referred to as a completely natural organic matter which contains carbon, macro and micro nutrients, in addition to high levels of humic acids, and has not reached the coal form. It is of significant economic value due to the high content of humic acids (İstanbulluoğlu, 2012). It is black-brown, appears like compacted soil, and can be easily crumbled by hand. Its pH varies between 3 and 5. It has a low solubility in water (Engin & Cöcen, 2012).

Unconsumed feed, fish excrement, and metabolic wastes discharged through gills in recirculated aquaculture units such as aquarium systems accumulate and cause ammonia formation in the water. Among the objectives of this study is to investigate the amount of ammonia that results from unconsumed feed. The other aim, on the other hand, is to investigate the use of natural adsorbent materials, i.e., zeolite (clinoptilolite) and leonardite, to maintain ammonia accumulation below the limit values.

2. Materials and Methods

2.1. Experimental Setup

The study was carried out in two different periods. Both periods were designed to consist of 3 groups with 3 replications. Crude protein values in the feeds of aquarium fishes vary between 28-30% and 45-50% depending on the nutritional requirement of the species (Khan & Maqbool, 2017). In experimental 1, commercial fish feed that contains 47.5% protein was used in 3 different concentrations (0.2 g feed/500 ml tap water, 0.4 g/500 ml tap water, 0.6 g/500 ml tap water) as the ammonia source (Şahin, 2022). The ammonia concentration resulting from unconsumed feeds in aquariums was determined in the first 7-day period. In the second period created subsequently, a mixture of clinoptilolite and leonardite (1:2 ratio), which has soil and water regulating properties, was added to suitable ammonia level, the excess amount of which is harmful to aquarium creatures. Table 1 shows the properties of the adsorbents investigated for this research. Analyzes were carried out by Central Research Laboratory, Kastamonu University. The pH was calculated following by according to Tokat (2019).

Table 1. Characteristics and chemical composition of clinoptilolite[a] and leonardite[b].

Clinoptilolite[a]			
	%		
SiO_2	78.41	SiO_2/Al_2O_3	5.67
Al_2O_3	13.83	BET Surface Area	34.316 m^2/g
MgO	1.646	pH	8.08
K_2O	2.372		
CaO	3.885		
Na_2O	1.042		
Fe_2O_3	1.414		
P_2O_5	0.058		
Leonardite[b]			
	%		
SiO_2	13.68	SiO_2/Al_2O_3	1.93
Al_2O_3	7.07	BET Surface Area	12.253 m^2/g
MgO	0.11	pH	3.15
K_2O	0.454		
CaO	0.323		
Na_2O	<0.014		
Fe_2O_3	1.238		
P_2O_5	0.055		

[a]Clinoptilolite was provided by Rota Mining Corporation, Manisa, Türkiye. [b]Leonardite was provided by Kütahya Chemistry, Kütahya, Türkiye.

In the second period, some water parameter values were determined as a result of 6 separate measurements conducted at regular intervals after the introduction of clinoptilolite:leonardite (1:2) mixture to the environment. Water criteria (dissolved oxygen, NH_4, pH, temperature) were determined by using a multiparameter instrument (YSI Professional Plus Series). A total of 3 g of water-regulating material was used in each replication in a way to contain 1 g of clinoptilolite type zeolite in 500 ml and 2 g of leonardite in 500 ml.

2.2. Data Analysis

NH_3 and TAN values in the study were calculated from NH_4^+, water temperature, and pH values (Emerson et al., 1975; Chow et al., 1997; EPA, 1999).

The TAN removal efficiencies (%) in the study were showed according to Alshameri et al. (2017):

$$\text{Removal efficiency (\%)} = \frac{C_0 - C_e}{C_0}\ x\ 100 \quad (1)$$

Where; C_0 (mg/L): Initial ammonium concentration, C_e (mg/L): Equilibrium ammonium concentration.

Adsorption continues until an equilibrium is established between the concentration of the substance deposited on the adsorbent surface and the concentration of the substance remaining in the solution. Mathematically, this equilibrium is explained by adsorption isotherms. The most commonly used isotherms are the Freundlich and Langmuir equations (Fu et al., 2020).

The Freundlich parameters are calculated using as:

$$q_e = K_f C_e^{1/n} \quad (2)$$

Where; qe(mg/g) is the amount adsorbed at equilibrium, C_e: The remaining unadsorbed concentration of the adsorbate at equilibrium (mg/L), K_f: Freundlich constant, n: Constant (n>1).

The Langmuir parameters are calculated using as:

$$q_e = \frac{q_{max} K_d C_e}{1 + K_d C_e} \quad (3)$$

Where; q_{Max}: Maximum adsorbing capacity of the adsorbent (constant), K_d: Langmuir adsorption constant.

2.3. Statistical Data Analysis

Statistical analyzes were performed using Minitab Statistical Software version 17 on Windows operating system. Data are presented as mean ± standard error (SE). One-way analysis of variance (ANOVA) and subsequent Tukey's HSD post-hoc test were employed to compare different experimental groups. A 95% confidence interval was used.

3. Results

3.1. Experimental Period

Water temperature, dissolved oxygen, pH, and ammonium values were determined as 21.4±0.02 °C, 0.45±0.01 mg/L, 8.38±0.01, and 0.1±0.01 mg/L, respectively at the beginning of the experiments. It was determined that there was no difference between the groups (P>0.05).

In the present work, 3 different concentrations (0.2 g, 0.4 g, 0.6 g) of aquarium fish feed having 47.5% protein were added to 500 ml tap water for all experimental groups. At the end of the first period, mean water temperature (°C) and oxygen values (mg/L) were 19.14±0.23, 18.88±0.23, 18.83±0.23 and 0.19±0.01, 0.19±0.01, 0.17±0.01 for treatments a, b, and c, respectively (P>0.05). The pH values were determined as $7.81±0.05^a$, $7.69±0.07^{a,b}$, and $7.57±0.07^b$ for treatments a, b, and c, respectively (P<0.05). Difference in pH values increased depending on the feed amount. The level of NH_4 increase caused by different feed ratios in the groups at the end of the 1st period is shown in Figure 1. At the end of this period, TAN values (mg/L) were calculated and determined as 6.09±1.03, 9.08±1.55, and 9.62±1.74 for treatments a, b, and c, respectively (P>0.05). The increase in TAN values was directly proportional to the amount of feed.

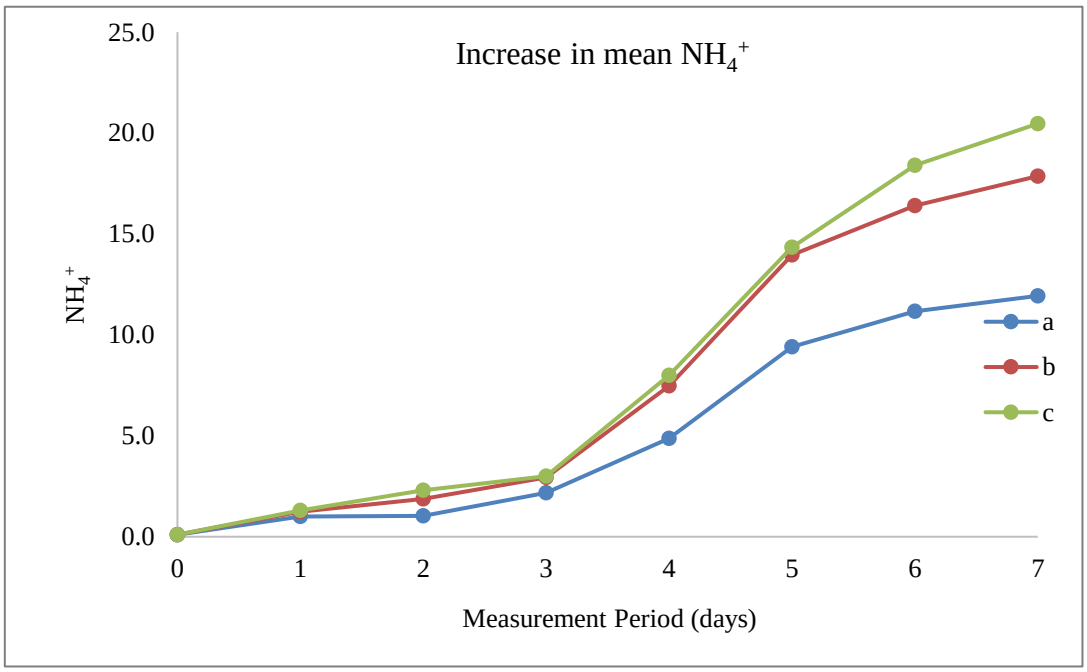

Figure 1. Increase in mean NH4+ at the end of the first period.

Water temperature (°C), dissolved oxygen (mg/L) and pH values obtained from measurements taken at regular intervals after the second experiment stage within a day were 20.31±0.18, 19.97±0.18, and 19.87±0.18; 0.23±0.01, 0.23±0.01, and 0.22±0.01; and 7.54±0.04, 7.48±0.03, and 7.52±0.02 for groups a, b, and c, respectively (P>0.05). NH_4 values (mg/L) were determined as 7.84±0.24^a, 13.07±0.40^b, and 16.62±0.42^c for groups a, b, and c, respectively (P<0.05).

It was determined that TAN removal efficiency significantly differed between all treatments as shown in Figure 2 (P<0.05).

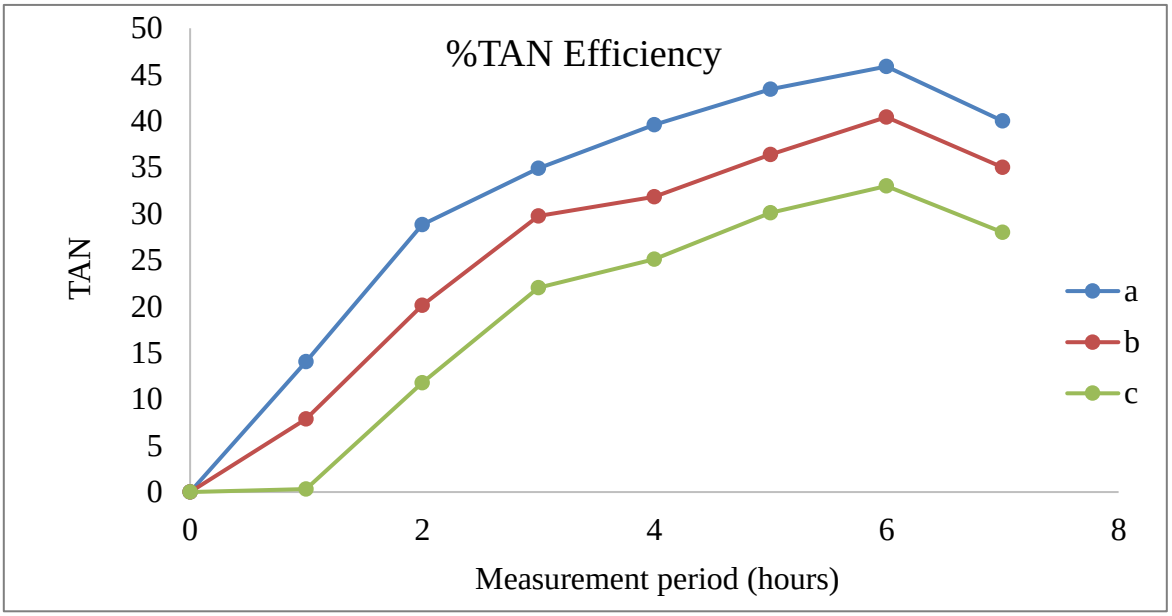

Figure 2. TAN removal efficiencies in groups a, b, and c throughout 6 measurements.

This experiment showed that clinoptilolite and leonardite can beneficially improve aquaculture water conditions by reducing increased levels of NH_3 and TAN.

With the increase in the amount of feed and the amount of TAN resulting from the feed, the retention efficiency of the adsorbents also increased.

In this study, it was shown that NH_3 values reached their peak at the end of the first period in which the ammonia values originating from the feed were determined, and started to decrease with the addition of clinoptilolite and leonardite to the water in the second period (Figure 3).

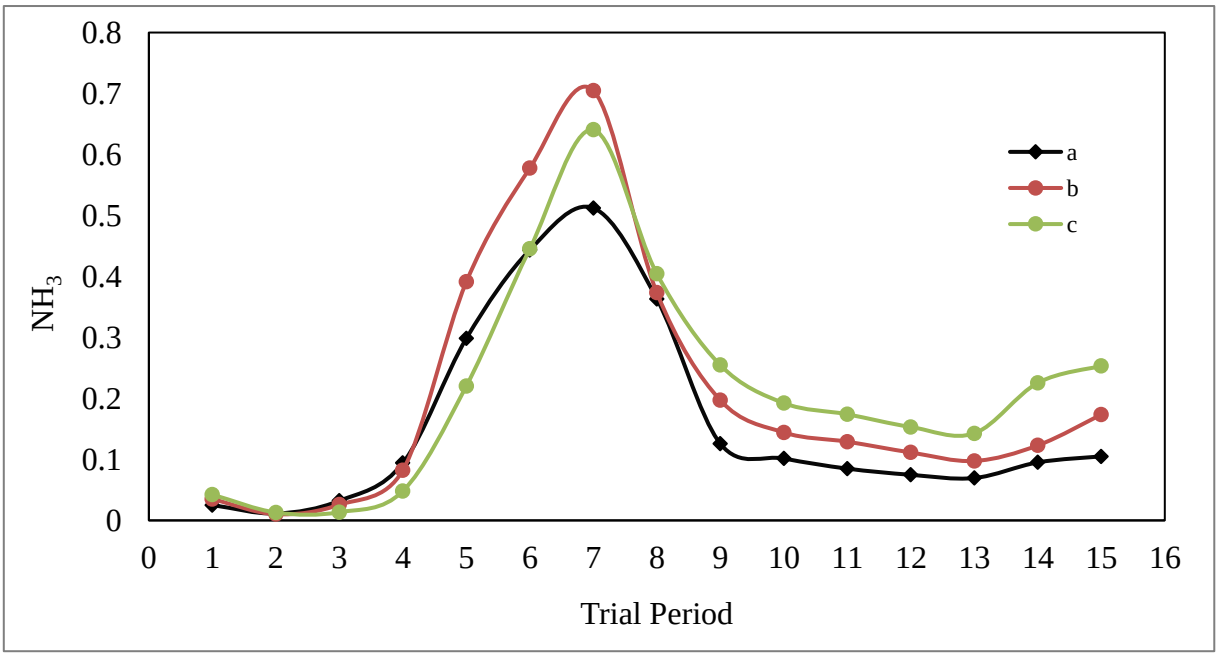

Figure 3. Changes in NH_3 values throughout the 15 measurements.

In the study examining the ammonia removal from different feed amounts, it was determined that the correlation coefficient value (R^2=0.984) found for Freundlich in the ideal group a, was higher when compared to the R^2 value (0.937) found for Langmuir. This showed that the experimental data were suitable for the Freundlich isotherm.

4. Discussion

Fish feed and excrement are the main wastes in aquarium waters. The majority of nitrogenous wastes in aquaculture also stems from unconsumed feed and excrement (Manguti et al., 2021). In this study, clinoptilolite and leonardite were used at high ammonium concentrations to keep ammonia levels at the desired level. At the end of the first period of the experiment, NH_4 values resulting from 47.5% protein feed for 7 days in treatments a, b, and c reached 12.2, 18.4, and 20.6 mg/L, respectively. Similar to this study, Kibria et al. (1997) reported that the amount of NH_4 was approximately over 20 mg/L after using 45% protein feed for 7 days. Şahin (2022) determined that the NH_4 values resulting from the feed that contains 35% protein were 3.53, 5.40, and 6.13 mg/L at the end of 7 days. The difference between the present study and Şahin (2022)'s study can be attributed to different amount of protein content of the feed.

Molecular ammonia (NH_3) and ammonium ion (NH_4^+), both of which are forms of soluble ammonia, coexist in equilibrium in water. Their relative concentrations are affected by pH and temperature. Higher pH and temperature values favor the formation of toxic molecular ammonia. Moreover, higher pH values result in more ionized ammonia, which raises toxicity (Purwono et al., 2017). The clinoptilolite used in this research can effectively adsorb NH4 from water. At the end of the experiment, in which the use of leonardite (another natural adsorbent) together with zeolite has a positive influence on ammonia removal, agrees with the studies of Öz et al. (2016) and Şahin et al. (2019). Chammui et al. (2014) and Terdputtakun et al. (2017) demonstrated that leonardite is more beneficial in removing unwanted compounds in aquatic environments. Furthermore, similar to this study, it was reported by Zengin (2013) and Şahin (2022) that the use of a clinoptilolite-leonardite mixture was effective.

Since nitrogen is one of the fundamental components of life, nitrogen molecules are necessary for the survival of living things (Şahin et al., 2019). There are two types of ammonia in water: unionized ammonia (NH_3) and ionized ammonium (NH_4^+). Ammonia (NH_3) is particularly toxic to fish. In aquaculture, NH_4^+ concentrations typically range from 1 to 5 mg/L. Aquaculture water has substantially lower NH_4^+ concentrations than municipal water (Jorgensen, 2002). When the NH_3 values observed in this study were examined, it was found that ammonia values from feed increased up to 0.77 mg/L and the highest value was reached in group c with the highest amount of feed, at the end of 7 days. With the addition of adsorbent, this value decreased to 0.1 mg/L, which is an acceptable value for aquaculture.

In this study, clinoptilolite and leonardite, which are utilized both as filtration material (Zengin, 2013; Şahin et al., 2018; Öz et al., 2021) and feed additive (Kanyılmaz et al., 2015; Turan & Turgut, 2020; Şahin, 2022), were used.

As a result of this research, it was concluded that it is possible to maintain nitrogenous compounds, which accumulates in the environment as natural wastes, within the desirable limits for aquaculture by using natural materials; zeolite and leonardite. Overall, when the effects of zeolite and clinoptilolite are evaluated together, it was determined that it does not have a harmful influence and the water parameters were within the appropriate values for aquaculture.

Conflict of Interest

The author declares no conflict of interest.

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JOURNAL OF AGRICULTURAL PRODUCTION

ISSN: 2757-6620

https://prensip.gen.tr

RESEARCH ARTICLE

Exopolysaccharide from *Rhodococcus pyridinivorans* ZZ47 Strain: Evaluation of Biological Activity and Toxicity

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ARTICLE INFO

Article History
Received: 01.06.2023
Accepted: 19.06.2023
First Published: 30.06.2023

Keywords
Acute toxicity
Anti-angiogenic activity
Antibiofilm activity
Exopolysaccharide
Genotoxicity
Rhodococcus pyridinivorans

ABSTRACT

Microbial polysaccharides are extracellular polymeric macromolecules excreted in microorganisms. These are widely used in food, cosmetic and pharmaceutical industries. One of them, exopolysaccharides (EPS), plays important role against the factors such as phage attack, antibiotics, toxic compounds or osmotic stress. Recently, this natural polymer has received great attention due to their therapeutic potential. The purpose of the study was to evaluate biological activity and potential toxicity of EPS from *Rhodococcus pyridinivorans* ZZ47 strain isolated from nature. EPS has no genotoxic effect on *Salmonella typhimurium* TA98, TA102, and TA1537 strains by Ames Test. No death occurred with single dose oral toxicity test of EPS and LD_{50} value of it is calculated by >2000 mg/kg in mice. The EPS showed antibiofilm activity on different bacteria. In addition, EPS demonstrated dose-dependent anti-angiogenic properties by HET-CAM test. In conclusion, the isolated EPS has antioxidant activity with no genotoxicity and the biological activities of the polymer indicated that it may be suitable for use in different sectors and industrial applications.

Please cite this paper as follows:

Taşkaya, A., Güvensen, N. C., Güler, C., Şancı, E., & Karabay Yavaşoğlu, N. Ü. (2023). Exopolysaccharide from *Rhodococcus pyridinivorans* ZZ47 strain: Evaluation of biological activity and toxicity. *Journal of Agricultural Production*, *4*(1), 63-71. https://doi.org/10.56430/japro.1307611

1. Introduction

The legal discharge of man-made or natural substances threatens public health because of their toxicity (Ahmad et al., 2023). These environmental pollutants, dyes, heavy metals, herbicides and pesticides, cause chronic diseases by transferring to food (Bello et al., 2018). Microbiota existing in nature works to eliminate environmental pollutants. In many studies, it is showed that increased tolerance to toxic pollutants and improved degradation capabilities of bacterial biofilm generally (Chaisuwan et al., 2020 & Ahmad et al., 2023). This biopolymeric matrix obtains stability and refuge to the cells in a biofilm and the main structure of this component is exopolysaccharides (EPS). However, the role of EPS goes

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beyond providing protection to microbial cells under stress and appears as a biomaterial in many (Matho et al., 2022). Biofilms can happen on nearly all type surfaces such as food processing surfaces, food and food packaging surfaces, water and food related surfaces. EPS, one of the most commonly used biomaterials, is carbohydrate polymers that can be produced from many plants, fungus, algae and bacteria (Botelho et al., 2014). EPSs have the ability to produce a significant number of microorganisms (bacteria, fungi, yeast) belonging to different taxonomy. The yeast and yeast-like fungi which include the genera of *Candida*, *Cryptococcus*, *Pichia*, *Sporobolmyces*, *Trichosporon*, *Lipomyces* and *Rhodotorula* have been described to produce EPS in the laboratory scale under submerged culture conditions (Ramirez, 2016). While predominantly vegetable polysaccharides were used (Hussain et al., 2017), the industry began using these biopolymers, with the introduction of microbial EPS as well as the benefits to human health (Gürleyendağ, 2006). Microbial EPS are regular, branched or unbranched structures with a high molecular weight of ionic or non-ionic biopolymer capable of dissolving in water and whose repetitive units are merged with glycosidic bonds (Erdoğdu, 2018). Commercially produced microbial EPSs have film-forming properties, emulsifying, thickening, structure-modifying, gelling capacities, and/or biological activities. It has been reported by studies that EPS material is widely used in the food industry, cosmetics, pharmaceutical and biomedical industries. Examples used in all these industries include, alginate, pullulan, glucans, bacterial cellulose, dextran, succinoglycan, xanthan gum and levan (Madhuri & Prabhakar, 2014 & Zhaoa et al., 2017). In the table below, various microorganisms, usage areas and production conditions from which Microbial EPSs are obtained are given (Erdoğdu, 2018).

Table 1. Various microorganisms, usage areas and production conditions from which microbial EPS is obtained

Microbial strains	EPS	Substrates	EPS concentrations (gL^{-1})	Production conditions	Areas of use
Acetobacter xylinum	Cellulose	Fructose Glucose	7-23.6	pH=4-5; 30 °C; 40 h	It is used as a dressing material in the bandaging of wounds, as a binder in high-quality acoustic-diaphragm membranes, ceramic powders and salts, etc.
Pseudomonas aeruginosa	Alginate	Xylose	0.4	30-37 °C; 1 bar 72 h	Food industry, pharmacology etc.
Leuconostoc sp.	Dextran	Sucrose	8.17	pH=5.5; 35 °C 1 bar	Coating of antibiotics, textile industry, blood volume enhancer
Agrobacterium	Curdlan	Glucose/sucrose	5.02-76	pH=7.5; 30 °C	Oligosaccharide, tertiary oil recovery, gelling agent in foods

EPS is used as a biomaterial suitable for medical and biological use due to its chemical structure and physical properties. By looking at the biological activities of EPS produced from different microorganisms in many studies, its applicability as a biomaterial is being investigated with many in vitro and in vivo studies. Basic physical and chemical properties, in vitro antioxidant properties and biological activities such as antiproliferative properties of EPS produced from *R. pyridinivorans* ZZ47 strain isolated from nature. The antioxidant properties of EPS with DPPH and hydroxyl radical elimination have been determined in our previous studies. The proliferation of EPS on HT-29 and MCF-7 cell lines was determined by performing the MTT test. In this study, the genotoxicity, acute toxicity, anti-angiogenesis and anti-biofilm activities were examined in the same EPS material.

2. Materials and Methods

2.1. Bacterial Strain and Culture Media

Rhodococcus pyridinovorans strain ZZ47 has been isolated from a biofilm that is a problem in a wastewater treatment plant. Activation of the *R. pyridinovorans* strain was done in Tryptic Soy Broth medium. The activated strain was optimized by referring to previous studies (Erdoğdu, 2018)

2.2. Growth Curve of Rhodoccocus pyridinivorans ZZ47

Bacterial growth was defined by measuring optical density at 600 nm. *Rhodococcus pyridinivorans* bacteria suspension was diluted in TSB in order to obtain a suitable initial OD for the experiments (0.01 to 0.05 at 600 nm). Triplicates of bacteria were grown in TSB at 37 ˚C in Erlenmeyer flasks shaken at 200 rpm and OD measurements were performed for 24 h with both spectrophotometers (Thermo Multiscan Microplate Spectrophotometer) until stationary phase was reached. The aerobic bacteria, inoculated culture tubes were incubated in duplicate in a water bath at 37 ˚C. The OD was measured using the experimental manifold at 0, 1, 2, 4, 6, 8, 10, 12, 22 and 24 h (Castellane et al., 2017 & Maia et al., 2016).

2.3. Isolation and Purification of EPS

Wang et al. (2015) studies were optimized and EPS isolation was performed. Purification of the *R. pyridinovorans* strain, and precipitation of cells and proteins in the culture

medium were performed according to Güvensen et al., (2022). EPS material, which was left to dry in the lyophilizer, was kept in the lyophilizer until it dried. The dried EPSs were pulverized in sterile air. The purified EPSs were stored in sterile plastic Eppendorf tubes under refrigerator conditions at -4 ˚C.

2.4. Yield Rate Calculation of EPS

The yield rate was calculated by weighing the wet weight after production and the dry weight after the lyophilizer of EPS produced from the harvest, which was made from the EPS producer *R. pyridinovorans*. The % EPS wet and dry yield rate was calculated with the formula below.

(%) Wet EPS yield rate = 100 ml culture x wet EPS weight (g) / culture in total volume (ml)

(%) Dry EPS yield rate = 100 ml culture x dry EPS weight (g) / culture in total volume (ml)

2.5. Biological Activities of EPS

2.5.1. Anti-biofilm activity determination

In order to determine the antibiofilm activity, Venkatesh et al. (2016)'s method has been modified. The reaction mixture was prepared using a 96-well microplate. 180 µl of TSB and 10 µl of biofilm-forming pathogenic bacteria culture were placed in each well, for a total of 200 µl. 10 µl EPS at different concentrations (0.025, 0.050, 0.1 mg/ml) was added to each well. Bacteria used for monitoring biofilm removal; *Salmonella typhimurium* CCM 583, *Aeromonas hydrophila* ATCC 19570, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Shigella dysenteriae* ATCC 11835, *Staphylococcus aureus* ATCC 6538/P and *Bacillus subtilis* CCM 99. After the wells were prepared, they were incubated at 37°C for 48 h. At the end of the incubation, the reaction mixture was removed from the wells and washed with 200 µl of PBS. Biofilms were fixed with 50 µl of methanol and incubated for 10 minutes. After removal of methanol, it was washed again with PBS. After staining the biofilms with 0.2% crystal violet, they were washed with deionized water (dH2O). The microplates were left to dry in the crystal violet and 100 µl of acetic acid (0.5 M) was added. The absorbance of the microplates at 570 nm was measured. The % removal was calculated with the formula below.

(%) = Control-Test/Control x 100

2.5.2. Anti-angiogenic activity (HET-CAM test)

In order to determine the anti-angiogenic effects of EPS, a chorioallantoic membrane model was applied on fertilized chicken eggs (Krenn & Paper, 2009). Leghron white chicken eggs used for HET-CAM analysis were purchased from Izmir Veterinary Control Research Institute. Fertilized eggs were incubated in a horizontal position at 37.5±1 °C in 70±2% humidity through 7 days. On the seventh day, CAM was analyzed by cutting a window (2 cm^2) on one side of the egg. Normal developing embryos were included in the assay; malformed or dead embryos were excluded. Eggs were divided into three groups. 0.9% NaCl (300 µl) was used as the negative control, Suramin (50 µg/pellet) was used as the positive control and EPS in different concentrations (2, 1 and 0.5 mg/ml) were used. After the experiment, the eggs were numbered and photographed. The opening in the egg shells was covered with lab film and the eggs were incubated for 24 hours. All compounds were tested in triplicate at different intervals.

After the incubation, eggs were photographed. Angiogenesis scores and anti-angiogenic effects of the compounds on CAM were evaluated according to the scoring system given in Table 2. Finally, the results obtained are calculated by placing them in the formula given below.

Average score = [numbers of egg (score 2) x 2 + number of egg (score 1) x 1] / [total number of eggs (score 0, 1, 2)].

According to this system, a score < 0,5 indicated that there was no anti-angiogenic effect, 0,5-1 indicated a low anti-angiogenic effect, and > 1 indicated a powerful anti-angiogenic effect.

Table 2. Score values used in the evaluation of HET-CAM.

Score	Effect	Impression
< 0.5	No effect	Normal embryo development. There is no change according to the surrounding capillaries. No hemorrhage, vascular lysis or coagulation was detected.
0.5 - 1	Low	The area without the covers is low or the density of the capillary is reduced in a specific area. The effects are not more than 2 times the field of matter
> 1	High	There is space without capillaries. Normal embryo formation is not observed.

2.6. Toxicity of EPS

2.6.1. Genotoxicity assay (AMES test)

Salmonella typhimurium TA98, TA102 and TA1537 strains were used to determine the genotoxic potential of EPS according to Maron and Ames (1983). The design of experiment was performed using Araclor 1254 (CAS 48586) Sigma Aldrich (Germany) induced rat liver S9. Strain-specific positive control chemical Sodium azide (10 µg/ml) was used as strain-specific positive control agent.

For this experiment, the bacteria cultures were incubated at 37 °C shaking incubator (at 100 rpm continuously) for 12-14 hours using growth medium [Xenometrix (PMM-GMOO, LOT: K05672P)]. Subsequently, 100 µl cultures and 100 µl EPS (final concentrations at 2000 µg/plate) were added to 2 ml melted top agar supplemented with 0.5 mM histidine and 0.5 mM biotin. In the presence of S9 activation was added to the suspension of tested strains. The tubes were shaken and then

inoculated on the previously prepared Minimal Glucose Agar (MGA) petri dishes, which were kept at 37 °C for half an hour and heated. The petri dishes were incubated for 48 hours at 37 °C and after incubation, colonies observed in petri dishes were counted and the number of his+ revertants were determined. The experiment was done in triplicate.

2.6.2. Single dose toxicity test

This study was approved by the Ege University, Local Ethical Committee of Animal Experiment (2022-033). To determine potential single dose acute toxicity of EPS, 6 BALB/c female mice (8-10 weeks old, 20-25 g range) were used within the scope of OECD Guideline 423 Acute Oral Toxicity (Acute Toxic Class Method). Dosing was started as 300 mg/kg body weight due to the lack of literature information about the substance. The test substance was administered in such a way that the maximum fluid volume that could be administered at one time did not exceed 1 ml/100 g body weight. Doses were prepared shortly before administration. The test substance was administered orally as a single dose. Before dosing the animals will be fasted overnight, after the fasting period the animals are weighed and the test substance administered. After administration of the test substance, the mice were not given food for 3 hours. The test substance at a dose of 300 mg/kg was administered to 3 female mice. According to the clinical and death findings of the animals, the dose administration order specified in the Guideline was followed by either a 300 mg/kg dose again or a higher dose (2000 mg/kg). 3 female mice were used each time and a minimum of 24 hours was allowed between dosing of each animal. Animals were checked 30 minutes and 24 hours after test substance administration. The animals were followed for 14 days and after this period the animals were euthanized.

2.7. Statistical Analysis

Statistical analysis of the results was applied to SPSS version 25.0 (IBM Corp., Armonk, New York, USA). Values were expressed as mean ± SEM.

3. Results and Discussion

Due to bacteria live in harsh conditions, they have various protective molecules to survive their environment. One of them, EPS, which is high molecular weight carbohydrate polymers secreted by bacteria into extracellular environment, plays crucial defensive role against biotic and abiotic stress factors such as phage attack, antibiotics, toxic compounds, heat or osmotic stress (Limoli et. al., 2015 & Angelin & Kavitha, 2020). The EPS, which has synthesized by bacteria homo- or heteropolysaccharides structure, has biocompatible, biodegradable and non-toxic properties. In addition, it has invaluable biological activities such as anticancer, antioxidant, antiviral, anti-biofilm, and immunomodulatory activities (Angelin & Kavitha, 2020 & Barcelos et al., 2020 & Mohd Nadzir et al., 2021). Thanks to these features, it has great interest food, cosmetic, pharmaceutical and biomedical industries. Among them, in biomedical applications, it is used many aims such as a scaffold, drug carrier, diagnostic agent and surgical sealant (Mohd Nadzir et al., 2021). For these purposes, EPS is extracted from various bacteria. Bacterial EPS production has many advantages such as defined and reproducible rapidly, higher and quality production, and well-known isolation methods. Also, produced EPS amount is depending on several factors; these are bacteria strain, medium composition and culture conditions (Moscovici, 2015 & Barcelos et al., 2020 & Mohd Nadzir et al., 2021). Determination of potential toxic effect of produced EPS is extremely important particularly in terms of usability in food and health areas. With the preliminary studies obtained from *R. pyridinivorans*, high efficiency extraction of EPS material was achieved and purification and production conditions were optimized (Güvensen et al., 2022). The yield rate of our EPS material, whose production conditions were optimized, was calculated. For this purpose, the yield rate was calculated by weighing the wet weight after production and the dry weight after the lyophilizer of EPS from the 6th harvest, in this work. The wet and dry EPS ratios obtained from the culture in total (10 ml x 176 = 1760 ml) are given in the table below (Table 3).

Table 3. The wet and dry EPS ratios.

EPS	EPS amount obtained from 1760 ml culture	100 ml EPS yield rate from culture
Wet EPS	30.13 g	% 1.71
Dry EPS	17.84 g	% 1.01

The growth curve of *R. pyridinivorans*, the wild strain isolated from activated sludge, was formed by OD adjustment considering the study of Castellane et al. (2017) and the experiment was performed in 3 replications and doublets. The OD measurements of this bacterium made every hour are shown in the table. The graph formed according to the results of the OD measurements shows the growth curve of our strain (Fig. 1).

With our preliminary studies obtained from *R. pyridinivorans*, high efficiency extraction of EPS material was achieved and purification and production conditions were optimized. In addition, high film forming, viscosity and gelling capacity were determined and physicochemical characterization of EPS was revealed. Studies have shown that it has high antibiofilm activities (Fig. 2).

Time/OD	OD1	OD2	OD3	Average
0	0,231	0,231	0,231	0,231
1	0,226	0,281	0,202	0,2363333
2	0,301	0,306	0,298	0,3016667
3	0,448	0,446	0,45	0,448
4	0,622	0,611	0,631	0,6213333
5	0,821	0,822	0,825	0,8226667
6	0,916	0,912	0,906	0,9113333
7	0,936	0,941	0,939	0,9386667
8	0,945	0,946	0,952	0,9476667
9	0,945	0,951	0,951	0,949
10	0,951	0,949	0,952	0,9506667
11	0,951	0,953	0,955	0,953
12	0,953	0,957	0,955	0,955
13	0,958	0,965	0,962	0,9616667
14	0,957	0,958	0,961	0,9586667
15	0,956	0,957	0,956	0,9563333
16	0,976	0,981	0,978	0,9783333
17	0,995	0,997	0,991	0,9943333
18	0,999	0,985	0,992	0,992
19	0,987	0,988	0,986	0,987
20	0,992	0,991	0,991	0,9913333
21	0,995	0,997	0,992	0,9946667
22	0,997	0,989	0,991	0,9923333
23	0,986	0,987	0,987	0,9866667
24	0,995	0,994	0,995	0,9946667
	0,995	0,994	0,995	0,9946667

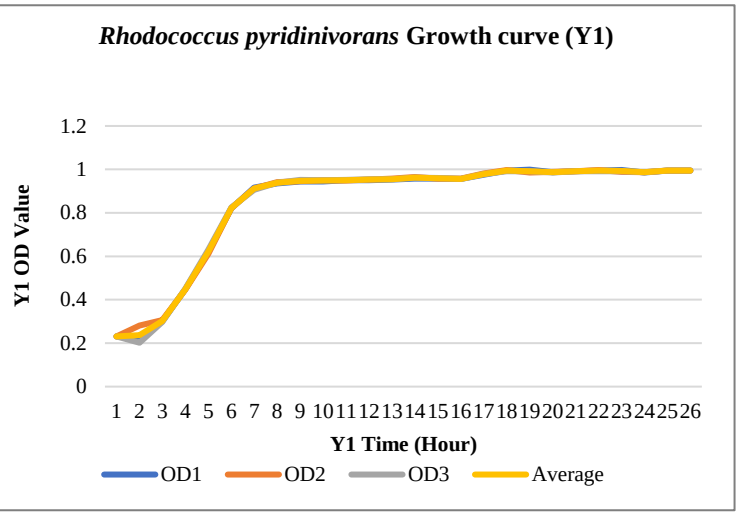

Figure 1. *Rhodococcus pyridinivorans* Growth curve (Y1).

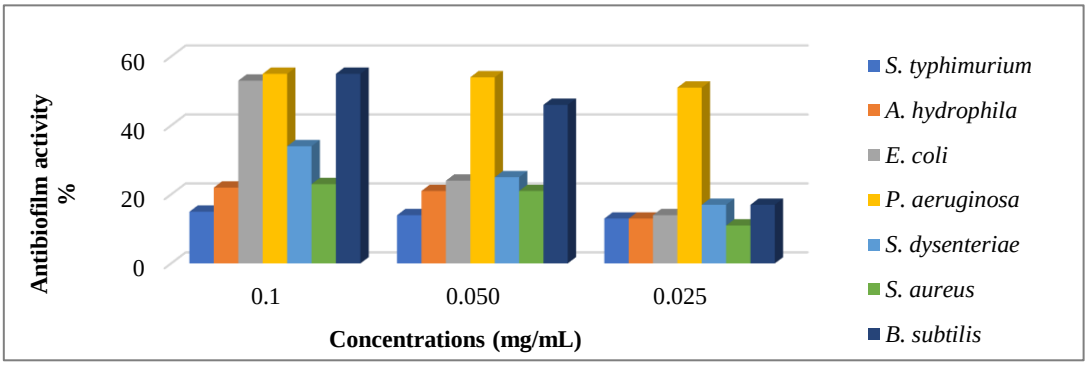

Figure 2. Percent antibiofilm activities.

R. pyridinivorans showed antibiofilm activity between 11-55%. It was observed that biofilm removal was highest in *B. subtilis* and *P. aeruginosa* bacteria at a concentration of 55% at a concentration of 0.1 mg/ml. Again, at the same concentration, it is 53% in *E. coli*. The lowest activity was seen in *S. aureus* at a rate of 11% at 0.025 mg/ml. It was then observed in *S. typhimurium* at a rate of 13% at 0.025 mg/ml. Thanks to the antibiofilm activity of EPS, it will be possible to delay microbial spoilage and extend shelf-life in foods. In addition, our natural strain, R. *pyridinivorans* ZZ47, isolated from activated sludge, was identified by conventional and molecular techniques. Our strain has been registered in GenBank with accession number AF173005. The originality of the study is increasing in terms of toxicity studies of *R. pyridinivorans* ZZ47, which is our national isolate, in order to bring it using in different sectors.

Anti-angiogenic therapy is one kind of popular and common strategy for cancer treatment. Since cancer cells have high energy consume to realize proliferation and metastasis, they need to new blood vessels. Angiogenesis is a complex mechanism the formation of new blood vessels, and several molecules play a role in the phenomenon (Oguntade et al., 2021 & Al-Husein et al., 2012). In our study, the EPS was showed strong and weak anti-angiogenic activity at 2 mg/ml and 1 mg/ml, respectively. On the other hand, no anti-angiogenic effect was seen at 0.5 mg/ml. Hence, we were demonstrated that the EPS has dose-dependent anti-angiogenic properties by HET-CAM test (Table 4, Figure 3). In parallel with our results, an EPS isolated from *Bacillus velezensis* OM03 has inhibited nearly 95% vascularization at 400 µg/ml concentration by chick CAM. In addition to this, this EPS has significant cytotoxic effect on PA-1 (human ovarian teratocarcinoma) and SKOV-3 (human ovarian cancer) cells without damage normal human ovarian surface epithelial cell line T1074, and has triggered apoptosis of PA-1 cells (Chirakkara & Abraham, 2023). In another study, EPS from *Lactobacillus acidophilus* with antioxidant properties has inhibited tumor angiogenesis related genes expressions and upregulated anti-angiogenic genes in colon cancer cell lines (Deepak et al., 2016).

Table 4. Anti-angiogenic effect results of EPS.

Concentration (mg/ml)	Score	Anti-angiogenic effect
2	1.33 ± 0.47	Strong
1	0.66 ± 0.23	Weak
0.5	0.33 ± 0.23	No
Suramin (50 µg/pellet)	1.32 ± 0.05	Strong
NaCl (%0.9)	0.10 ± 0.05	No
Water	0.10 ± 0.05	No

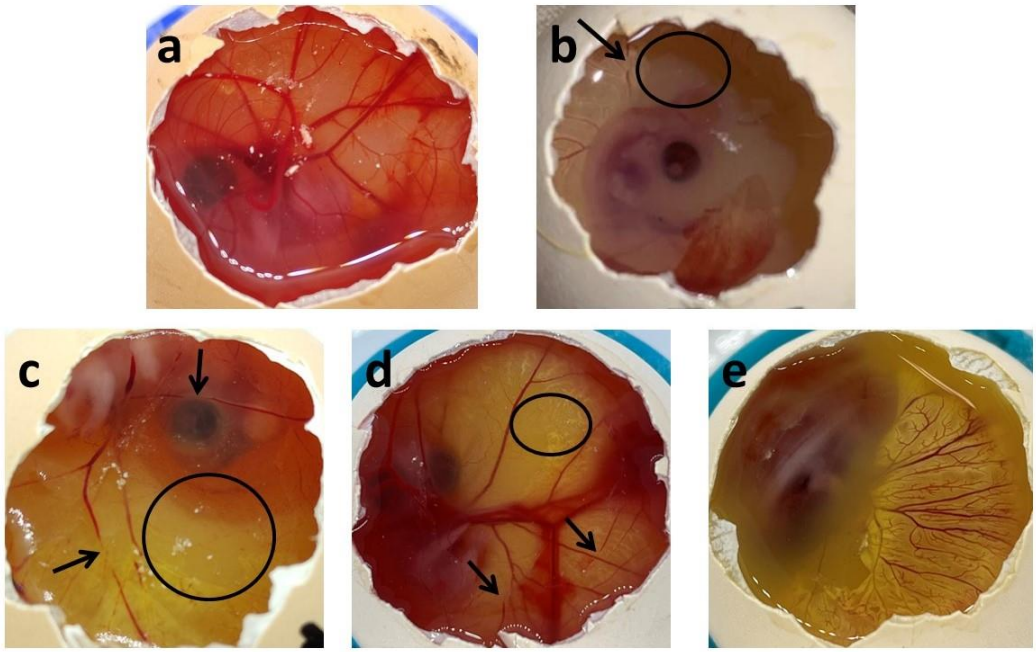

Figure 3. Anti-angiogenic properties results of EPS by HET-CAM test. a. NaCl (negative control); b. Suramin (positive control); c. 2 mg/ml EPS; d. 1 mg/ml EPS; e. 0.5 mg/ml EPS administration. Arrow shows prevented vessel formation. Circle shows area without capillaries.

In recent years, natural products are very attractive research area to find a solution against especially in cancer. Among them, many studies are carried out with bacterial EPS due to it has various biological effect particularly cytotoxic effect. Recently, novel exopolysaccharide EPSR4, isolated from *Bacillus subtilis* strain AG4, has showed remarkable cytotoxic effect on T-24 (bladder carcinoma), A-549 (lung cancer) and HepG-2 (hepatocellular carcinoma) cancer cells and IC_{50} values were 244 µg/ml, 148 µg/ml and 123 µg/ml, respectively. Also, it has strong antioxidant activity cause scavenging DPPH and hydrogen peroxide free radicals (Abdel-Wahab et al., 2022). In a study, EPS, produced from *Rhodococcus erythropolis* HX-2, has demonstrated significant cytotoxic effect on A-549, SMMC-7721 liver cancer cells and HeLa cervical cancer cells without harm to L929 normal cells (Hu et al., 2020). In our previous studies, EPS *R. pyridinivorans* ZZ47 was showed low cytotoxic activities on human colorectal adenocarcinoma (HT-29) and human breast adenocarcinoma (MCF-7) cells (Güvensen et al., 2018 & Güvensen et al., 2022). Although several cytotoxicity tests have performed for different bacterial-source EPS in the literature, there are not genotoxicity test enough. In present study, revertant colonies numbers of EPS treatment were not exceed spontaneous revertant numbers on *S. typhimurium* TA98, TA102 and TA1537 strains (Table 5). Therefore, EPS has not genotoxic effect. Likewise, in a recent study showed that EPS_{KC27L}, isolated from *Lactobacillus salivarius* KC27L, has not genotoxic effect by chromosome aberration, sister chromatid exchange, micronucleus, and comet assays (Yildiz et al., 2023).

Table 5. Revertant colonies numbers by AMES test after EPS administration.

Treatment	Concentration	Revertant colonies numbers (Mean±SD)					
		TA 98		TA 102		TA 1537	
		+ S9	- S9	+ S9	- S9	+ S9	- S9
EPS	2 mg/ml	37±23	17±4	200±5	170±6	33±4	19±3
Distilled water	100 µl	17±4	30±9	130±32	115±20	6±5	6±4
Sodium azide	10 µg	1600±42*	1720±55*	1590±72*	1150±85*	3360±54*	485±35*

*Deviation from the number of spontaneous revertant colonies.

During the single-dose toxicity test, no adverse reactions such as death, general appearance or behavior were observed in the mice. There was no weight loss in the body weight between before and end of the study (22,63±0,17 g. and 25,21±1,56 g, respectively). After a single dose toxicity study, organ (heart, lung, liver and kidney) weights and relative organ weights of the mice are presented in Table 6. Rodents show variation in many clinical chemistry values and therefore reference values cannot be specified for many parameters. However, in the evaluation made by considering the reference values specified for the BALB/cByJ breed (Loeb and Quimby, 1999), according to Total protein (TP) and Albumin (ALB) values, which are indicators of general physical condition, the general health status of the animals was good (Table 7). At the end of the single dose toxicity study, LD_{50} value of the EPS was determined as >2000 mg/kg in BALB/c. In parallel our results, Pinto et al. (2016) showed that bacterial cellulose, which is one kind of cellulosic exopolysaccharide obtained from sugarcane molasses, has not acutely toxic effect on rats with no cytotoxic or genotoxic effect on cells.

Table 6. Acute toxicity test result organ weights and relative organ weights (mean±SD).

	Heart	Lung	Liver	Kidney
Organ weight (g)	0.17±0.01	0.23±0.03	1.48±0.02	0.44±0.03
Relative organ weight	0.006	0.008	0.049	0.015

Table 7. Effect of EPS on biochemical parameters of mice.

Biochemical Parameters	EPS	Ref. range*
Total Protein (g/dL)	4.1	4.4-7.6
ALB (g/dL)	2.2	2.7-4.9
ALP (U/L)	<14	-
GLU (mg/dL)	283	114-279
TBIL (U/L)	1.9	0.5-1.1
TCHO (mg/dL)	67	80-219
ALT (U/L)	203	-
GGT (U/L)	<10	-
Ca (mg/dL)	<4.0	8.5-10.9
IP (mg/dL)	7.2	-
CRE (mg/dL)	0.39	0.2-0.7
BUN (mg/dL)	21.5	-
GLOB (g/dL)	1.9	-

* BALB/cByJ reference range (Loeb & Quimby, 1999).

In conclusion, this study showed that our EPS material, which has antibiofilm, antiangiogenic and antioxidant activities, does not have genotoxicity and acute toxic effects. Due to the biological activities of the polymer it may be suitable for use in different industries such as pharmaceutical, diagnostic, therapeutic and food. Also, thanks to the antibiofilm activity of EPS, it will be possible to delay microbial spoilage and extend shelf-life in foods. It is thought that EPS can activate the defense system more thanks to its antioxidant effect and prevent metastases due to its anti-angiogenic effect. In addition, although promising results have obtained for the EPS, further toxicological studies are required to elucidate the potential of it for use in cancer therapy.

Conflict of Interest

The authors declare that they have no conflict of interest.

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JOURNAL OF AGRICULTURAL PRODUCTION

ISSN: 2757-6620

https://prensip.gen.tr

RESEARCH ARTICLE

Fungal and Bacterial Bioagents Efficiency on the Control of Potato Pest *Phthorimaea operculella* via Ingestion or Contact

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ARTICLE INFO

Article History
Received: 12.06.2023
Accepted: 22.06.2023
First Published: 30.06.2023

Keywords
Bacillus spp.
Beauveria bassiana
Biological control
Phthorimaea operculella
Solanum tuberosum

ABSTRACT

Potatoes are one of the most important food products in the world and considered a main human nutrition sources source. Potato tuber moth (*Phthorimaea operculella* Zeller (PTM) (Lepidoptera: Gelechiidae)) causes remarkable economic losses to important crop, both in field and under storage conditions In this study, the insecticidal efficiency of the following bioagents: *Brevibacillus brevis* (FD-1), *Bacillus atrophaeus* (FD 17), *Bacillus sphaericus* (FD 49), *Bacillus cereus* (FD 63), *Vibrio hollisae* (FD 70), *Bacillus thuringiensis* subsp. *kenyae* (FDP 8) bacteria strains and *Beauveria bassiana* fungal isolate (ET 10), were evaluated on their efficacy to control *P. operculella*, under controlled conditions. In addition to insecticidal efficacy evaluations, analyses were also carried out to determine the differences between bioagents action mode: (1) uptake (ingestion as a gastric poison) and (2) contact. For (1), 20 larvae were fed on the tubers immersed in these suspensions to analyze efficacy by ingestion; for (2), suspensions of $1x10^8$ CFU/ml of bacteria and $5.7x10^5$ conidia/ml of fungus were prepared and sprayed to 20 larvae. FD-63 (91.67%) and FD-17 (88.33%) taken up by diet gave the most effective results against the pest.

Please cite this paper as follows:

Dadaşoğlu, F., Tozlu, E., Tozlu, G., Tatar, M., & Kotan, R. (2023). Fungal and bacterial bioagents efficiency on the control of potato pest *Phthorimaea operculella* via ingestion or contact. *Journal of Agricultural Production*, *4*(1), 72-80. https://doi.org/10.56430/japro.1313505

1. Introduction

Potato (*Solanum tuberosum* L.), which belongs to the Solanaceae family, is one of the most important food crops in the world (Khorrami, 2018). According to FAO (2019) data, 370.4 million tons of potatoes were produced on 17.3 million hectares of land worldwide. 28.3% of the world's potato cultivation areas are in China, 12.1% in India and, 6.9% in Russia, and these three countries account for 47.3% of the world potato production. In 2019, 91.8 million tons of potatoes were produced in China, 50.1 million tons in India, and 22.07 million tons in Russia (FAO, 2019). According to 2019 production data, 4 million 980 thousand tons of potatoes were produced in 141 thousand hectares of land in Turkey (TUIK, 2020).

There are some important insect species and nematodes as well as fungal, bacterial and viral disease factors that cause

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economic losses in potato production both in the field and in storage conditions (GTHB, 2016). The potato tuber moth (PTM), *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae) is known to be one of the potato pests causing significant economic losses by damaging the crop in the field and the warehouse (Tsedaley, 2015; Rondon, 2010). This pest probably first appeared in the tropical highlands of South America (Graf 1917), and was later recorded as an important and widespread potato pest in 90 countries in Africa, Asia, Central and South America with tropical and subtropical climates (Sporleder et al., 2004; Jensen et al., 2005; Lacey et al., 2010). Its hosts primarily include potatoes and tomatoes (*Lycopersicon esculentum* (Miller)), other cultivated plants belonging to Solanaceous genus such as tobacco (*Nicotiana tabacum* L.), eggplant (*Solanum melogena* L.) and bell peppers (*Capsicum annuum* L.) and wild species belonging to the genera of *Solanum*, *Datura*, *Nicotiana*, *Fabina*, *Hyoscyamus*, *Physalodes*, *Lycium* and *Nicandra* (Schaub & Kroschel, 2018).

PTM females lay their eggs on leaves and exposed tubers near the eye, while their larvae dig tunnels during feeding, causing approximately 100% damage to both unharvested tubers in the field and post-harvest potato tubers held in storage (Visser, 2005; Sporleder et al., 2008). In addition, the larvae enter the leaflets, form transparent bubbles, injure the leaves, and then pass into the root tissue, causing the death of the plant (Sabbour & Raheem, 2015). However, it is stated that the damage to the green parts is not economically significant, and the main damage takes place in the warehouse as a result of the larvae's feeding on the tubers. Potatoes' edible and seed properties deteriorate in contaminated warehouses, causing loss of quality and weight (Okello et al., 2017). The extent of the damage increases rapidly when several generations develop during the storage period (Sharaby et al., 2019).

To date, various control methods have been utilized to prevent and control the damage of PTM (Clough et al., 2010). The high adaptability of PTM to daily and seasonal changes, its high reproductive potential and its survival even in extremely hot conditions pose difficulties in the control of this pest (Doğramacı & Tingey, 2008). In addition, it has developed resistance to insecticides such as pyrethroid, carbamate and organophosphate, posing challenges to chemical pest control (Kroschel, 2006; Clough et al., 2008; Hafez, 2011; Tsedaley, 2015; Yan et al., 2019). In warehouses, timing is critical to ensure that newly hatched PTM larvae are killed before they enter the potato tubers and tunnel through (Tsedaley, 2015). The risks of chemicals for the environment, animal and human health have also driven awareness about the environment and the search for alternative control methods. Biological control, which is one of these methods, is an important control method that is environmentally friendly, has side effects on human and animal health; ensures the protection of the existing natural balance; does not cause resistance; does not cause residue problems in nutrients and does not pollute soils as well as groundwater. Studies have been conducted using entomopathogens, natural enemies, essential oils and plant extracts to reduce the damage caused by PTM.

In this study, insecticidal effects on *Phthorimaea operculella* larvae of *Brevibacillus brevis* (FD 1), *Bacillus atrophaeus* (FD 17), *B. sphaericus* (FD 49), *B. cereus* (FD 63), *Vibrio hollisae* (FD 70), *B. cereus* (FDP 8) bacterial strains and *Beauveria bassiana* (ET 10) fungal isolate (Saraç et al., 2011; Tozlu et al., 2011; Dadasoglu et al., 2013; Dadasoglu et al., 2014; Tozlu et al., 2020a,b; Tozlu et al., 2021), which were found to be effective on various pests in previous studies, were tested.

2. Materials and Methods

2.1. Insect, Bioagent Bacterial and Fungal isolates

Potato tubers contaminated with PTM larvae wintering during the larva period under climate conditions of Erzurum (Turkey), were obtained during the survey studies conducted at the time of potato harvest in September 2019, brought to Atatürk University Faculty of Agriculture, Department of Plant Protection Pest Systematics laboratory and stored in a 30×45×30 cm desiccator at 25±2 ºC under 65%±5 proportional humidity and 16-h light: 8-h dark conditions by providing daily fresh food and humidity check (Figure 1).

Figure 1. Original field collected and laboratory stored potato tuber showing seve infestation and damaged by *Phtorimaea operculella* larvae.

The fungal and bacterial isolates considered in this study had previously demonstrated effective biocontrol in different pests as shown in Table 1. Bacterial biocontrol isolates were grown in Nutrient Broth (NB; Difco) containing 15% glycerol at -80ºC in the Culture Collection in Atatürk University Faculty of Agriculture Plant Protection Department Plant Clinical Laboratory, while fungal biocontrol isolate was stored in oblique Potato Dextrose Agar (PDA; Difco) medium at +4ºC in Atatürk University Faculty of Agriculture Plant Protection Department Mycology Laboratory (Table 1).

Table 1. Bacterial strains and fungal isolate used in the study.

Bacterial strains					
Strains	**Isolated from**	**MIS* Identification results**	**S****	**HR*****	**Reference**
FD 1	*Malacosoma neustria*	*Brevibacillus brevis*	0.625	-	Tozlu et al. 2011
FD 17	*Yponomeuta evonymella*	*Bacillus atrophaeus*	0.459	-	Tozlu et al. 2011
FD 49	*Culex* sp.	*Bacillus sphaericus*	0.681	-	Dadaşoğlu et al. 2016
FD 63	*Yponomeuta evonymella*	*Bacillus cereus*	0.241	-	Tozlu et al. 2011
FD 70	*Melolontha melolontha*	*Vibrio hollisae*	0.476	-	Tozlu et al. 2019
FDP 8	*Bemisia tabaci*	*Bacillus cereus*	0.652	-	Tozlu et al. 2011
Fungal isolate					
Isolate	**Isolated from**	**ITS Identification Result**	**S****	**ITS 1 sequences******	**Reference**
ET 10	*Sphenoptera antiqua*	*Beauveria bassiana*	0.99	GB KY806126	Tozlu et al. 2017

***MIS:** Microbial Identification System, ****S:** Similarity index, *****HR:** Hypersensitivity test, **-:** Negative reaction, ******GenBank Accessed Number.

2.2. Preparation of the Bacterial Suspension

The tested bacterial strains were cultured in 4 phases in Nutrient Agar (NA) medium at 30 ºC for 24 hrs to obtain fresh cultures. A single bacterial colony taken from these cultures in sterile loops was inoculated into Erlenmeyer flasks with 300 ml of NB medium and incubated for 24 hours at 250 rpm and 27 °C in a horizontal shaker incubator. The bacterial density of the resulting aqueous culture was adjusted to $1x10^8$ CFU/ml by BIOLOG turbidimeter and transferred to sterile spray vials.

2.3. Preparation of the Fungal Conidial Suspension

Conidia production was achieved by incubation of *B. bassiana* ET 10 isolate in Sabourth Dextrose Agar (SDA) medium at 25 ºC, 80% humidity for 2-3 weeks. Then, a stock suspension was prepared by washing the surface of the culture into bottles containing sterile water with 0.2 ml/l Tween-80 solution (Quesada-Moraga et al., 2006). Using a hemocytometer, the concentration of conidia suspension to be applied was found to be $5.7x10^5$ conidia/ml.

2.4. Insecticidal Effect Test in Controlled Conditions

Within the scope of the study, blotting paper was placed on the bottom of each petri dish (12 cm). In order to determine the stomach poison effect of entomopathogens, potato tuber pieces cut in a rectangular shape (1.5×7×5 cm) were placed in petri dishes after dipping them into bioagent bacteria and fungus suspensions for 5 minutes (Figure 2). 20 PTM larvae were placed in each petri dish under a binocular microscope. To determine their efficacy by contact, potato tuber pieces cut in the same sizes were placed in petri dishes without any application, 20 PTM larvae were placed under a binocular microscope, and bioagent suspensions were sprayed on the larvae. Petri dishes were then kept under controlled conditions at 25±2 °C, 65-70% RH and a 16:8 (light:dark) photoperiod. The number of dead larvae was recorded regularly every 24 hours and the death rates were determined by the formula below.

$$Mortality\ rate\ (\%) = \frac{100\ x\ the\ number\ of\ dead\ adults\ in\ treatment}{Total\ adult\ in\ treatment} \quad (1)$$

In the study, a sterile NB medium was used as negative control and commercial chemical Red Sunny WP (25% Diflubenzuron) was used a positive control. The trial was performed in triplicate for each combination on the same day.

2.5. Data Analysis

Analysis of variance was applied to the values related to the results obtained, and the differences between the means were compared to the LSMeans Student test at a significance level of $P<0.01$. Data analyses were performed using the statistical software package JMP IN (SAS Institute, Cary, NC, .0% PC version).

3. Results

According to the results obtained, it was determined that all bacterial and fungal isolates had a pesticidal effect at varying rates. While there was no death in the negative controls, the death rate in the positive control was 100%. In the first 24 hours of the study, over 20% of death was detected in FD-17 and FD-63 isolates, and 10% of death were in FD-49 isolates, which were all applied to potato pieces. Treatments with FD-17 and FD-49 isolates, which had the highest death rate in the 24- hour evaluation, in the 48 hours the results were 61.67% and 56.67%, respectively. These two isolates gave the best results in all periodic counts. In the counts, death rates for FD-17 and FD-49 isolates were respectively as follows: 68.33% and 81.67% at 72 hours; 83.33% and 86.67% at 96 hours; 88.33% and 91.67% at

120 hours; 96.67% for both at 144 hours and 100% for both at 168 hours (Figure 2). In the study, the standard deviation values in the application to potato pieces varied 0.00 (FD-1, FD-17, Red Sunny WP (25% Diflubenzuron), FD-63, FD-49, F 70), 2.89 (FDP-8, ET 10), 25.17 (NB) and in the sprayed 0.00 (ET 10, F-70, FD-63, FD-17, Red Sunny WP (25% Diflubenzuron)), 2.89 (FDP-8, FD-49, NB), 5,77 (FD-1) according to the 240 hours (Figure 2).

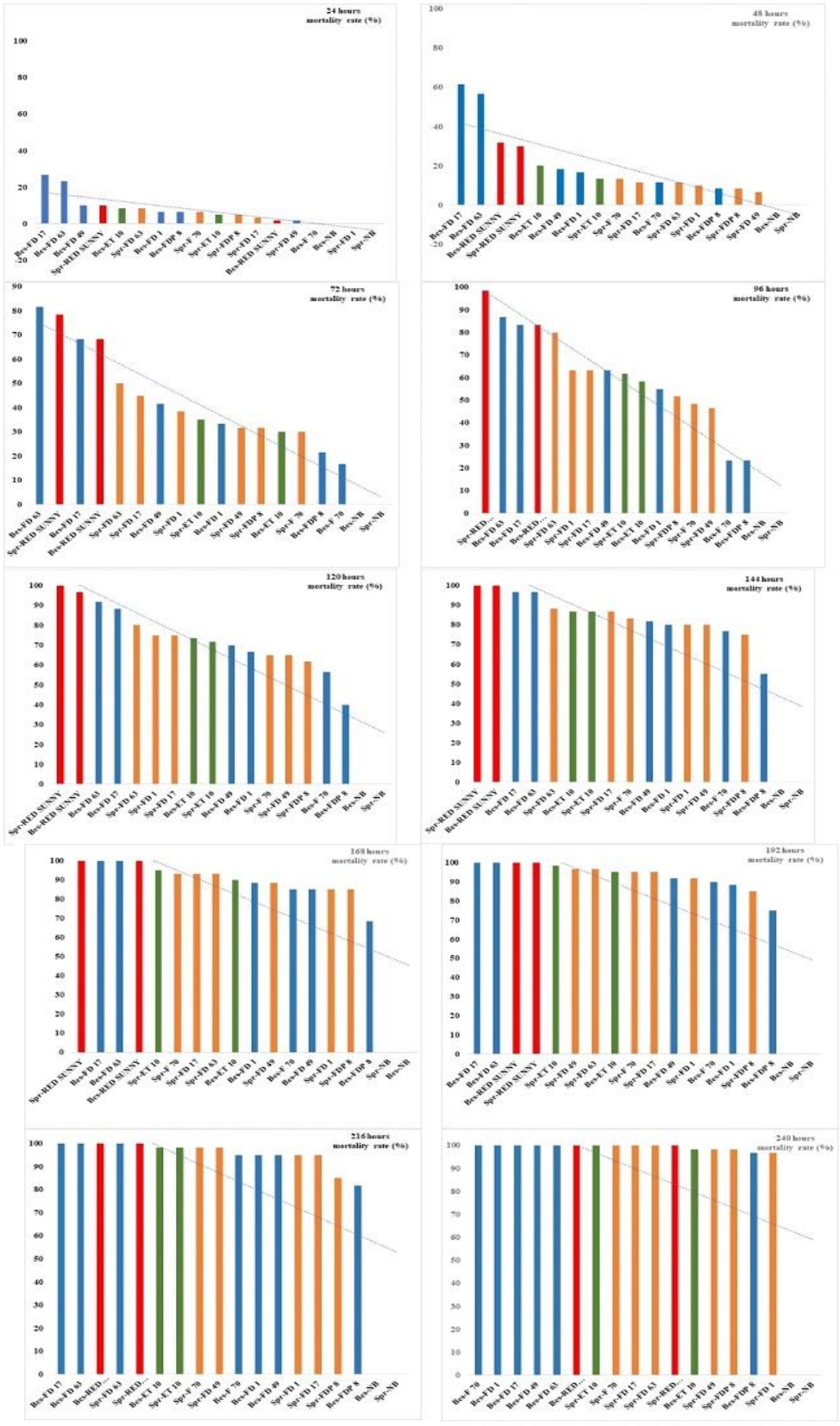

Figure 2. Percentages of mortality of *Phthorimaea operculella* larvae in response to some entomopathogenic bacterial strains and fungal isolate by hours (Bes= Ingeston; Spr= Contact).

4. Discussion

Entomopathogens are among the most influential factors regulating pest populations. Many biopesticides are commonly used worldwide in the biological control of pests in greenhouse products, ornamental plants, stored products, forest products and horticultural products (vegetables and fruit) (Lacey et al., 2001). There are many studies on the use of entomopathogenic bacteria and fungi in biological pest control (Inglis et al., 2001; Wraight et al., 2001; Lacey et al., 2001; Vestergaard et al., 2003; Copping, 2004; Aslantas et al., 2008; Khachatourians & Sohail, 2008; Lacey et al., 2010).

Among bacteria, species belonging to the genus *Bacillus* are among the most important species in biological control. Among these species, especially *Bacillus thuringiensis*, *B. brevis*, *B. cereus*, *B. circulans*, *Bacillus megaterium* and *B. subtilis* are known to be used for biotechnological and industrial applications (Xu & Côté, 2003; Rooney et al., 2009). Furthermore, *B. thuringiensis* has also been reported to be used against *P. operculella* in various parts of the world to reduce pesticide use and create a rich natural enemy complex in the fields (Alvarez et al., 2005; Chandel et al., 2020).

In a study investigating the means to biologically control the larvae of PTM in the tubers, 960 g of sand was mixed with 40 g of *B. thuringiensis* subsp. *kurstakii* and 96% effective control were achieved (Kroschel & Koch, 1996). Imam and Ghiet (2019) evaluated insecticide properties against PTM larvae of *B. thuringiensis* isolates and *Artemisia judaica* extract and reported that the death rates of PTM larvae caused by 1.25 and 10 CFU/ml of *B. thuringiensis* were in the range of 14-58%, respectively and those caused by 125 and 1000 ppm of *Artemisia judaica* were in the range of 34-76%, respectively. Göktürk et al. (2018)'s study, on the other hand, investigated the insecticidal effect against nymphs and adults of *Ricania simulans* of 10 bacteria and 1 fungus isolates, including *B. brevis* CP 1, FD 1, *B. thuringiensis* FDP 1, *Bacillus thuringiensis* subsp. *kenyae* FDP 8, FDP 42, *Bacillus thuringiensis* subsp. *kurstakii* FDP 41, BAB 410, *Bacillus subtilis* EK 7, *Pseudomonas chlororaphis* NEM 28 and *B. sphaericus* FD 49 and *B. bassiana* ET 10, reported that *B. thuringiensis* subsp. *kenyae*, *B. brevis*, and *B. sphaericus*were effective against nymphs, while *B. thuringiensis* subsp. *kurstakii*, *P. chlororaphi*, and *B. brevis* were effective against adults. In addition, they noted that the mortality rate varied in the range of 19.58 to 42.08% in nymph applications and 6-18% in adult applications. Tozlu et al. (2019) investigated the insecticidal effect against nymphs of *Halyomorpha halys* of *Brevibacillus*, *Bacillus*, *Pantoea*, *Vibrio*, *Pseudomonas*, and *Beauveria* under controlled conditions and determined that bacterial isolates were 75-100% effective and *B. bassiana* fungus isolate was 76.19% effective. They also reported that bacterial isolates of *B. cereus* and *Pantoea agglomerans* had a success potential of 100%. Tozlu et al. (2020a) determined that bacterial isolates of *Bacillus pumilus* TV 67C, *B. brevis* CP 1, and *B. megaterium* TV 91C against *Pseudaulacaspis pentagona* caused death in the range of 41.68 to 89.04%. Similarly, in the current study, the isolates of *B. brevis* FD 1 (100%-96.67%), *B. atrophaeus* FD 17 (100%-100%), *B. sphaericus* FD 49 (100%-98.33%), *B. cereus* FD 63 (100%-100%)- FDP 8 (96.67%-98.33%) and *V. hollisae* FD 70 (100%-100%) species were effective against PTM at varying rates.

Fungi as well as bacteria are used in biological control against pests (Lacey & Neven, 2006). Entomopathogenic fungi that colonize plants as endophytes have lethal and non-lethal pathological effects on insect pests (Mutune et al., 2016; Zhang et al., 2021). *B. bassiana*, which is one of the most widely and extensively studied entomopathogenic fungi species, is the active ingredient of many products currently in use and under development worldwide (Tangtrakulwanich et al., 2014). The use of *B. bassiana* in Integrated Pest Management (IPM) programs is of great importance due to its environmentally friendly nature, bio-persistence, and ability to kill pests at various developmental stages of their life cycle (Kumar & Sultana, 2015). The spores of *B. bassiana* adhere to the cuticle of the insect, germinate, and the formed hyphae multiply by penetrating the body of the insect. Attacked insects die after about 3-5 days, and infected cadavers serve as a spore source for the secondary spread of the fungus. Previous studies reported various death rates in a variety of pest groups to which *B. bassiana* was applied (Sahab & Sabbour, 2011; Abdel-Raheem et al., 2015).

Sabbour and Raheem (2015) tried to determine the efficacy of *Beauveria brongniartii* and *Nomuraea riley* against PTM and reported that the number of eggs per female PTM decreased significantly compared to the control group under laboratory conditions, while the number of adults decreased in their applications in field plots, thus the yield of potatoes increased. Similarly, Zhang et al. (2021) determined that the survival of the larvae of *P. operculella* fed on potato plants inoculated with *B. bassiana* and the number of eggs laid by the females were quite low, and they noted that *B. bassiana* could be a potential biological control agent in the control of this species.

Zeleke et al. (2015) showed that *B. bassiana* significantly reduced PTM larvae infestation of both leaves and tubers, compared to *M. anisopliae* and *V. lecanii* and concluded that the number of larvae and damage to leaves significantly decreased with increasing fungus concentration levels. In addition, many researchers demonstrated that *B. bassiana* and *M. anisopliae* isolated from *P. operculella* larvae caused death of larvae (Yuan et al., 2017; Yuan et al., 2018; Gao, 2018). Hui-guo et al. (2018) reported a death rate of 90.3% ± 2.1 for PTM larvae treated with *B. bassiana* (1×10^7 conidia mL^{-1}) and further argued that *B. bassiana* not only has a high pathogenicity against PTM larvae but also causes non-lethal effects such as shortening the development of a generation,

reducing the fertility of the female offsprings and affecting the population parameters. Khorrami et al. (2018) tested the efficacy of *M. anisopliae* IRAN 2252, *Nomura earileyi* IRAN 1020C and *Paecilomyces tenuipes* IRAN 1026C isolates against PTM larvae and eggs, and suggested that 1×10^3 conidia/ml of *N. rilei* gave the most effective results and that individual fungus applications reduced adult emergence and first generation progeny of PTM. Based on their data, they stated that these entomopathogenic fungi have lethal effects on eggs and larvae of *P. operculella* and recommended using these fungi in integrated pest management (IPM) programs.

Tozlu et al. (2017) isolated *B. bassiana* ET 10 isolate, the effect against PTM of which was investigated in the current study, from *Sphenoptera antiqua* larvae in a previous study and tried to determine its effect on many pests. In one of these studies, they applied 10^6, 10^7, and 10^8 spore suspensions of ET 10 against *S. parreyssii* and reported death rates as 82.72%, 83.95%, and 90.12%, respectively (Tozlu et al., 2017). Tozlu et al. (2019) reported a death rate of 76.19% for *Halyomorpha halys* nymphs treated with *B. bassiana* at 264 hours. In another study, Tozlu et al. (2020b) noted a death rate of %100 for *Icerya purchasi* nymphs treated with *B. bassiana and* 80% for the adults. Similarly, at the end of the current study, it was established that *B. bassiana* ET 10 fungal isolates showed various levels of efficacy (98.33%-100%) against PTM.

5. Conclusion

In the study, it was determined that environmentally friendly control agent entomopathogens can be used successfully as an alternative to chemicals in the control of *P. operculella*. Considering that the efficacy of the microorganisms used in these controls may change under field conditions, the efficacy of entomopathogens should also be tested under field conditions.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Kasumyan, A. O., & Døving, K. B. (2003). Taste preferences in fishes. *Fish and Fisheries*, *4*(4), 289-347. https://doi.org/10.1046/j.1467-2979.2003.00121.x

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