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The bioavailability of ampicillins in some animals

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ABSTRACT

Ampicillin, a partially synthetic derivative of penicillin, has been widely used in both human and veterinary medicine for a long time. As a result of its limited ability to be absorbed into the bloodstream when taken orally by humans, precursor chemicals such bakampicillin, pivampicillin, and talampicillin have been created. Although ampicillin is widely used in veterinary treatment practice, the applications of ampicillin esters in animals have not gone beyond scientific trials and have not found widespread use. Since there are not many antibiotic options authorized for use in poultry, in our review, the pharmacokinetic properties of ampicillins, especially oral bioavailability, in some animal species will be mentioned and the need for studies that will examine the bioavailability of pre-ampicillins will be emphasized.

Keywords: ampicillins, poultry, bioavailability, horse

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Introduction

In contemporary poultry farming, various medications, primarily antibiotics, are used for the treatment and control of infectious diseases in winged animals. Antibacterial drug classes commonly preferred in poultry diseases include penicillins, aminoglycosides, macrolides, lincosamides, phenicols, tetracyclines, sulfonamides, and fluoroquinolones (Sumano and Gutierrez Olivera, 2010).

Use of ampicillin in horses and poultry

Ampicillin, being a member of the aminopenicillin group, functions by interacting with penicillin-binding proteins (PBPs) localized in the bacterial cell wall membrane. This interaction prevents the synthesis of peptidoglycan, which is crucial for the bacterium's resilience in the external environment. Inactivation of PBPs hinders the cross-linking of peptidoglycan chains necessary for the formation of the bacterial cell wall, thereby impeding cell wall synthesis in susceptible bacteria. Consequently, the bactericidal effect of ampicillin leads to the demise of the bacteria (Bolme et al., 1976; Goren et al., 1981; Demain and Solomon, 1983; Sumano Lopez and Gutierrez Olivera 2010; Landoni and Albarellos, 2015). Due to its broad antibacterial spectrum, ampicillin is utilized in poultry for the treatment of secondary infections in chronic respiratory diseases caused by Escherichia coli, Pasteurella multocida, and Salmonella spp. Additionally, it is employed for the treatment and control of necrotic enteritis caused by Clostridium perfringens (Ensley and Janssen, 1981; Sumano Lopez and Gutierrez Olivera, 2010; Landoni and Albarellos, 2015). Ampicillin is susceptible to hydrolysis by beta-lactamases secreted by certain bacteria (Demain and Solomon, 1983). In animals, a small portion of ampicillin is converted to the main inactive metabolite, penicilloic acid, in the organism (Tsuji, 1983). It is

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generally indicated that ampicillin binds to serum proteins in animals to a low extent (Villa et al., 1994). In horses, ampicillin is not significantly metabolized, but it is rapidly eliminated through glomerular filtration and renal tubular secretion. A small fraction of ampicillin (<4%) in horses can enter the enterohepatic cycle and be eliminated via bile after intravenous administration (Horspool et al., 1992). The binding of ampicillin to serum proteins in horses is reported to be less than 10% (Dürr, 1976). In poultry, oral absorption generally occurs rapidly. Ampicillin exhibits a wide distribution in body fluids and tissues. The primary elimination route is through the urinary tract, with minimal amounts being excreted in bile (Sumano Lopez and Gutierrez Olivera, 2010).

Ampicillin, being a member of the beta-lactam group, exhibits time-dependent antibacterial activity. In other words, initiating treatment with high concentrations in the target site does not contribute to the rate of bacterial death. In recent years, the approach of correlating pharmacokinetic and pharmacodynamic parameters used in predicting the success of antibacterial therapy (PK/PD) emphasizes the importance of maintaining effective concentrations in the infection site for a certain duration (T>MIC) for beta-lactam antibiotics (Yıldırım, 2008).

Pharmacokinetic properties of ampicillin in some animal species

The oral bioavailability of ampicillin in humans can vary between 39-54%, as reported by Bolme et al. (1976). However, data in avian species show significant differences. In a study focused on broilers, it was indicated that the oral bioavailability of ampicillin is around 30% (Sumano Lopez and Gutierrez Olivera, 2010). Another study revealed that in healthy 6-day-old chicks, the oral bioavailability of ampicillin was approximately 55%, whereas in those with coccidiosis, it was considerably lower at 14% (Kandeel, 2014).

For laying hens and broilers, the direct administration of ampicillin trihydrate at a dose of 7 mg/kg in the crop resulted in undetectable drug levels in the plasma. After administration of a 70 mg/kg dose, very low plasma concentrations (an average of 1 µg/ml) were observed within the first 2 hours. Furthermore, at doses of 20, 30, 40, and 50 mg/kg, the plasma Cmax values were found to be 1.6, 3.4, 4.7, and 9.4 µg/ml, respectively. The study emphasized the rapid absorption and elimination of ampicillin (Goren et al., 1981).

The pharmacokinetic profile of ampicillin was examined in different parrot species. In the first stage, oral ampicillin trihydrate equivalent to 175 mg/kg was given to four of six Amazon parrots and 150 mg/kg to the remaining two. After a one-month waiting period, IM ampicillin sodium 100 mg/kg was administered to all parrots. In the second phase of the study, five blue Naped parrots were first administered oral ampicillin trihydrate at 150 mg/kg, and one month later, a single dose of 100 mg/kg IM ampicillin sodium was administered (Ensley and Janssen, 1981). The study showed that the drug level in plasma reached peak levels within two hours following oral ampicillin administration, and in a short time in those administered IM ampicillin; It has been emphasized that it reaches peak levels in plasma within thirty minutes (Ensley and Janssen, 1981). In a study investigating the pharmacokinetic parameters of ampicillin and another aminopenicillin, amoxicillin, using a similar design in pigeons, ten pigeons were initially administered a single intravenous (IV) dose equivalent to 100 mg/kg ampicillin sodium. After a 3-week waiting period, a single oral dose of 100 mg/kg ampicillin trihydrate was given. Six weeks later, a single intramuscular (IM) dose of ampicillin sodium equivalent to 100 mg/kg was administered. The authors of the study reported that the oral bioavailability of ampicillin in pigeons was 26%, and the Cmax value in parrot species administered IM at a dose of 100 mg/kg of Ampicillin sodium was 3-4 times higher than in pigeons administered 100 mg/kg IM ampicillin sodium (Dorrestein et al. al., 1987).

In a study conducted on ducks, pharmacokinetic parameters were investigated after a single dose of intravenous (IV), intramuscular (IM), subcutaneous (SC), and oral administration of ampicillin. A total of 120 ducks were divided into four equal groups. After administering ampicillin orally at an equivalent dose of 20 mg/kg through IM, IV, SC, and oral routes, blood samples were collected at 0.15, 0.30, 1, 2, 3, 4, 5, 6, 8, 12, 16, and 24 hours, and plasma was obtained. Subsequently, ampicillin concentrations were determined from the plasma samples. The study revealed that compared to IV administration, the intramuscular bioavailability was 91.11%, oral bioavailability was 17.78%, and subcutaneous bioavailability was 62.22% (Poapolathep et al., 2001).

In a study involving chickens (6-9 months old) and turkeys (6-8 weeks old), a single intramuscular dose of 25 mg/kg ampicillin sodium was administered to 31 chickens. After application, serum concentration peaked at 4.6 µg/ml at 30 minutes, dropped to 0.75 µg/ml two hours later, and was undetectable in serum at 6 hours. In the same study, the second group of 17 chickens received a direct esophageal administration of ampicillin trihydrate at a dose of 25 mg/kg. The highest concentration was reported as 0.6 µg/ml, and

antibiotic serum levels were very low at 8 hours. In the third group, consisting of 10 chickens, a single dose of 50 mg/kg ampicillin trihydrate was directly administered to the esophagus. After four hours, the animals were slaughtered, and ampicillin concentrations in their internal organs were determined. The concentrations were found to be 0.36 µg/ml in the liver, 1.48 µg/ml in the kidneys, 0.18 µg/ml in the spleen, 0.32 µg/ml in the lungs, and 0.20 µg/ml in the muscle tissue, respectively. The authors of the study emphasized the low oral absorption of ampicillin in poultry (Ziv et al., 1979).

The data concerning the oral bioavailability of ampicillin with specified studies in avians indicate variable outcomes. Due to the generally low and inconsistent oral bioavailability of ampicillin in both humans and animals, prodrug forms of ampicillin have been developed for human use. Ampicillin prodrugs, also referred to as esters of ampicillin, are typically administered orally and are rapidly hydrolyzed to ampicillin by nonspecific esterases in the digestive system, following absorption. The conversion to ampicillin can occur during absorption from the gastric and intestinal mucosa, depending on the specific ampicillin prodrug. It is emphasized that these prodrugs lack intrinsic antimicrobial activities (Demain and Solomon, 1983).

Among the prodrugs designed for use in human medicine, such as pivampicillin, bakampicillin, hetacillin, and talampicillin, there is a lack of relevant data on their oral bioavailability in avian species.

Ampicillin esters

The chemical structure of ampicillin has been modified to develop numerous prodrug forms. These drugs, released into the market by enhancing characteristics such as bioavailability, water solubility, and chemical stability, have been utilized in the practice of human medicine for years (Demain and Solomon, 1983).

Hetacillin

Hetacillin is obtained by condensation with acetone of ampicillin. Its oral bioavailability in humans is approximately 38%. Apart from the increased chemical stability, there is no pharmacokinetic superiority of ampicillin over amoxicillin (Demain and Solomon, 1983). In a study, oral absorption levels of hetacillin were examined in rats, rabbits and dogs and it was determined that hetacillin showed a similar absorption compared to the same dose of ampicillin (Gubert et al., 1987)

Pivampicillin

Pivampicillin is the pivaloyloxymethyl ester of ampicillin. Due to its high solubility in water, it is predominantly utilized in the form of its hydrochloride salt. Following oral administration in humans, it undergoes hydrolysis in the gastrointestinal tract via nonspecific esterases to yield formaldehyde and ampicillin. The oral bioavailability in humans is significantly higher than that of ampicillin, ranging from 82% to 92% (Demain and Solomon, 1983). In horses, the oral bioavailability of pivampicillin has been determined as 30.9% under fasting conditions and 35.9% under fed conditions (Ensink et al., 1992). The effectiveness of ampicillin in the treatment of equine infectious diseases is directly associated with the concentration reached at the site of infection and the duration of exposure. These two factors are dependent on the physicochemical properties of the antibiotic, the route of administration, and the applied dosage (Prescott and Baggot, 1988). In another study, different doses of ampicillin and pivampicillin were administered to horses, and the concentrations of ampicillin in plasma and pulmonary epithelial lining fluid (PELF) were determined. The results were correlated with the minimum inhibitory concentration (MIC) of common respiratory pathogens in horses. Following a single intravenous dose of 15 mg/kg ampicillin and a single intragastric dose of 19.9 mg/kg pivampicillin, the drug concentrations in the pulmonary epithelial lining fluid (PELF) and plasma of horses were investigated. Following intravenous administration, the elimination of ampicillin is reported to be rapid, with the drug being undetectable in the plasma 12 hours after administration in three out of six horses. Pivampicillin, when administered to fed horses, has been found to have an oral bioavailability of 36%. The degree of penetration of ampicillin into the pulmonary epithelial lining fluid (PELF) was determined by the ratio of the area under the concentration-time curve for PELF to that for plasma in the 0-12 hours post-intravenous administration period, resulting in a ratio of 0.40. This ratio was found to be 1.00 when oral pivampicillin was administered. In a study conducted in horses, orally administered pivampicillin was emphasized to provide clinically significant drug concentrations in the PELF for at least 12 hours (Winther et al., 2012).

Bacampicillin

Bacampicillin is the acid-stable ethoxycarbonyloxymethyl ester of ampicillin. Esterification of the carboxyl group on the thiazolidine ring of ampicillin reduces polarity and increases lipid solubility at physiological pH, leading to only minimal decrease in water solubility. This results in increased oral absorption. The ester is completely hydrolyzed by esterases in enterocytes and plasma. It exhibits a similar oral bioavailability to pivampicillin in humans.

Following oral administration, bacampicillin in humans is absorbed almost entirely by passive diffusion (Demain and Solomon, 1983). In a study conducted in horses, the absolute bioavailability was found to be 39% (Ensink et al., 1996).

Talampicillin

Talampicillin is the phthalidyl ester of ampicillin. Its absorption rate is higher compared to ampicillin, and when administered orally to humans, serum concentrations 2.5 to 3 times higher than the equivalent amount of ampicillin are achieved (Demain and Solomon, 1983). In a study conducted in horses, oral bioavailability was found to be 23% (Ensink et al., 1996).

Conclusion

However, the time-consuming nature of these studies, the economic burden they pose, and the lack of guaranteed success have directed researchers towards prolonging the lifespan and efficacy of existing drugs rather than developing new ones (Çolak, 2009). Therefore, we believe that investigating the pharmacokinetic properties of ampicillin esters, which exhibit high bioavailability in humans compared to ampicillin, after a single oral dose in various animal species will contribute to the literature and scientific knowledge.

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Characterization of virulent *Escherichia coli* in healthy pet dog feces: Implications for public health

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ABSTRACT

The characterization of *Escherichia coli* that colonizes pets is necessary to maintain animal health and to reduce the chance of transmission to owners. In this study, we investigated the incidence of potentially virulent *E. coli* inhabiting healthy pet dogs as a risk of infection to pet owners. Antibiotic-resistant *E. coli* isolated from freshly passed dog feces were whole-genome sequenced using Illumina chemistry and classified into pathogenic lineages using pathogen-specific markers. The antimicrobial resistance genes (ARGs), virulence-associated genes (VAGs), and plasmids were respectively predicted using the ResFinder, VirulenceFinder, and PlasmidFinder. Of the 32 isolates, 13 carried resistance genes such that four, six, and 11 contained β-lactam (*bla*TEM), aminoglycoside [*aac-6*(*Ib7*)/*ant-3*(*Iia*)/*aph-3*(*Ib*)/*aph-6*(*Id*)] and tetracycline (*tet*) resistance genes, respectively. The IncF plasmids were most prevalent (n=12, 38.71%) but the highly self-conjugative IncN plasmids occurred simultaneously with the plasmid-borne [quinolones (*QnrS1*/*QnrB7*) and sulfonamide (*sul3*)] ARGs in ≥ 2 *E. coli*. One *E. coli* each was classified as avian pathogenic *E. coli*, atypical enteropathogenic *E. coli*, enterotoxigenic *E. coli*, Shiga toxin-producing enteroaggregative *E. coli*, and enteroaggregative *E. coli*. Pet feces should be carefully handled because they contain virulent and drug-resistant *E. coli*.

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Introduction

Escherichia coli resident in the gastrointestinal tract of humans and animals are mostly commensal (Majowicz et al., 2014) except for certain lineages that cause diarrhea (Puño-Sarmiento et al., 2013) and extra-intestinal infections (Manges et al., 2019). These pathogenic strains cause different intestinal and extra-intestinal diseases by using their virulence genes which affect several cellular processes. *Escherichia coli* infections can be challenging when pathogenic strains acquire antibiotic-resistant characteristics since this may herald a scenario where pan-resistant pathogenic lineages could emerge in the shared environment between humans, animals, and plants (Ikhimiukor et al., 2022), and this can increase the chance of human acquisition of infections that could be untreatable.

Keeping pet animals such as dogs is common in several households worldwide. The cross-infection of pet owners and diarrheic dogs by *E. coli* in households lacking appropriate animal-waste management systems (Penakalapati et al., 2017) has suggested the need to identify the lineages of the bacterium that are responsible for human infection (Robins-Browne et al.,

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2016; Ashbolt et al., 2018; LeCuyer et al., 2018). Little is known of the *E. coli* lineages that are responsible for shared infections between humans and dogs, besides extraintestinal *E. coli* and uropathogenic *E. coli* (LeCuyer et al., 2018; Manges et al., 2019).

Studying the incidence of pathogenic *E. coli* strains in healthy pet dogs is crucial (Manges et al., 2019) to enhance our understanding of the lineages responsible for shared infections between pet owners and their dogs. Previous studies that performed whole genome sequencing (WGS) of dog-derived *E. coli* either prioritized extended spectrum β-lactamases (ESBLs) investigations (Tudu et al., 2022) or weighed in on the possible role of animal feeds in the spread of resistance by pet animals (Mounsey et al., 2022). We hypothesized that certain lineages of *E. coli*, in this context, healthy pet dog-derived *E. coli* that can resist antibiotics may have evolved genetic traits to persist sufficiently to initiate infections in their host. Since carriage of these genetic traits, otherwise called virulence genes, is the basis upon which the lineages are classified, the current investigation aimed to determine the likely incidence of known *E. coli* pathotypes in freshly passed fecal samples of healthy dogs to deduce possible infection occurrence, and to know whether these pathotypes are reservoirs of mobile resistance traits.

Materials and methods

Selection of drug-resistant *Escherichia coli* for whole genome sequencing: Previously, we described drug-resistant *E. coli* isolated from fecal samples of apparently healthy dogs in Ibadan, Nigeria (Falodun et al., 2022), adhering to established guidelines (Sherwin et al., 2003) and the National Health Research Ethics Committee of Nigeria (NHREC, 2007). Briefly, fresh fecal samples (n=60) collected from dogs into sterile sample bottles were processed to isolate *E. coli* on Eosine Methylene Blue and MacConkey agars. *Escherichia coli* were identified with *uidA* primer through a polymerase chain reaction while confirmed isolates were tested against 10 antibiotics using the Kirby-Bauer disc diffusion method. Selected antibiotics are among the most commonly administered agents in veterinary practices in Nigeria (Olowe et al., 2015, Ogbolu et al., 2020). The ESBL *E. coli* phenotypic detection was performed using the double disc synergy test following CLSI (2021) while isolates showing ESBL phenotype were genotyped for ESBL *blaSHV*, *blaTEM*, and *blaCTX-M* using multiplex PCR. In this study, we retrieved 31 drug-resistant *E. coli* isolates from our previous study (Falodun et al., 2022) and included one additional *E. coli* isolate (INOF032) from the same study making the number of organisms examined equal to 32. All the isolates were subjected to whole genome sequencing using Illumina technology. The sequences generated were inspected for specific diarrheagenic virulence markers. These markers were used to classify the organisms into specific pathogenic lineages.

Extraction of DNA, preparation of library, and whole genome sequencing: The isolates' total genomic DNA was extracted using a Wizard DNA extraction kit (Promega). The extracted genomic DNA was quantified using a dsDNA Broad Range fluorometric assay (Invitrogen). The preparation of DNA libraries was done using the NEBNext Ultra II FS DNA library kit for Illumina (New England Biolabs). Libraries were sequenced at Wellcome Sanger Institute, Cambridge, United Kingdom, on the Illumina HiSeq 2500 instrument (Illumina, San Diego, CA, USA) using 2 × 250 bp paired-end reads.

Bioinformatic analyses of the sequences: Raw reads were trimmed using TrimmomaticPE, and assessed for quality using FastQC, while individual FastQC reports were aggregated into a single report file using MultiQC (Ewels et al., 2016). To further assess the quality of the sequences, reads were assigned taxonomic markers using Kraken (Wood et al., 2015) and these markers were quantified using Bracken (Lu et al., 2017). Based on the quality of the sequence, reads were assembled into contigs using Spades v3.9.0 (Bankevich et al. (2012). The genomes were annotated using Prokka v1.12 (Seemann, 2014) and reads were mapped to a reference genome (*E. coli*_042) to generate a multi-fasta alignment file. The IQTree's Model Selection option was used to determine a best-fit model (GTR+F+ASC+R4) to produce the phylogenetic tree by the IQ-tree (Trifinopoulos et al., 2016), and the tree was visualized using the Microreact tool (Berghain et al., 2018).

Assessment of whole genome sequences for resistance, virulence, and plasmid markers: The sequences were assessed for ARGs, virulence genes, and plasmid replicons using the Centre for Genomic Epidemiology Resfinder (Clausen et al., 2018), VirulenceFinder (Malberg et al., 2020), and PlasmidFinder (Carattoli and Hasman, 2020). The sequence types (STs) were determined with ARIBA using Achtman's multi-locus sequence typing scheme (Clausen et al., 2018). The phylogenetic grouping of the genomes was done by the Ezclermont in silico Clermont Phylotyper (Clermont et al., 2013).

Assignment of sequence number to novel STs: Strains with novel STs have their raw sequences uploaded to the University of Warwick Enterobase platform (https://enterobase.warwick.ac.uk/species/ecoli/upload_reads), curated at https://enterobase.warwick.ac.uk/species/ecoli/my_strains

Table 1. Criteria used for classification of the Escherichia coli strains into pathotypes

Target marker(s)	Other diagnostic target(s)	Predicted Pathotypes	References
eilA/stx1B	*ehxA*, *stx1B*, *eilA*	Shiga toxin-Enteroaggregative *E. coli*	(Robins-Browne et al., 2016)
STA	*tibC*, *east-1*, *F17a*, *F17d*	Enterotoxigenic *E. coli*	(Robins-Browne et al., 2016)
eilA	*fyuA, irp2, chuA*, *traT*, *ompT*	Enteroaggregative *E. coli*	(Sheikh et al., 2006)
eae	*nleA-2*, *nleA-8*, *nleC-3*, *nleB-14*, *lifA*, *astA*	Atypical Enteropathogenic *E. coli*	(Mercado et al., 2016)
hlyF	*iss*, *iroN*, *ompT*, *cvaC*, *hlyF*, *papC*	Avian Pathogenic *E. coli*	(Ovi et al., 2023)

eilA, *Salmonella enterica hilA*-like gene; *fyuA*, yersiniabactin receptor; *chuA*, haem receptor; *ompT*, outer membrane protein; *ehxA*, enterohemolysin; *stx1B*, Shiga toxin 1B; *F17a, F17d, F17* fimbriae; *iss*, increased serum survival; *cvaC*, colicin V production; *hlyF*, hemolysin F; *eae*, intimin gene; *papC*, P fimbrial gene; nle genes, pro-inflammatory inhibitory proteins; *iroN*, enterobactin siderophore receptor protein; *astA/STA/east-1*, heat-stable enterotoxin; *tibC*, tib adhesin/invasion; *lifA*, lymphocyte inhibitory factor.

and STs were assigned based on the Achtman 7 gene multilocus sequence typing (MLST) scheme. Classification of strains into pathotypes: Genome sequences were inspected for the carriage of specific markers, and the pathotypes were determined using previously recommended protocols outlined in Table 1 (Sheikh et al., 2006; Mercado et al., 2016; Robins-Browne et al., 2016; Ovi et al., 2023).

Availability of whole genome sequence data: Raw sequence datasets generated during this study were deposited at the European Nucleotide Archive with bioproject number PRJEB8667. Accessions are available at the https://www.ebi.ac.uk/ena/browser/view/PRJEB8667.

Results

Detection of antibiotic resistance genes: Thirteen of the 32 *E. coli* isolates bore ARGs and the most prevalent was tetracycline (*tet*) found in 11 (34.36%) isolates followed by sulphonamide (*sul*) and aminoglycoside [*aac-6*(*Ib7)*/*ant-3(Iia)*/*aph-3(Ib)*/*aph-6* (*Id*)] resistance genes carried each by 6 (18.75%) isolates. Plasmid-mediated quinolone resistance (PMQR) genes *qnrs1* (n=3) and *qnrb7* (n=1) were borne by the isolates without co-occurrence. Simultaneous reading of the resistance determinants showed that the isolates carrying qnrs1 harboured *aph-6-Id, aac-6* (*Ib7*), and *blaTEM* while those carrying *aph-6-Id*, *aac-6* (*Ib7*), and *blaTEM-95* also possessed *dfrA14, mef-B, sul3*, and *tetA* genes. Other *ARGs – aadA5, dfrA17*, and *sul2* were found in one and two isolates accordingly (Table 2 and https://microreact.org/project/iEWL5GYVXBcvuje3kAyjkJ-escherichia-coli-antimicrobial-resistance-genes).

Carriage of virulence-associated genes by the isolates: The mean value of VAGs was nine and the range was from five to 28. The glutamate decarboxylase gad- and tellurite resistance terC genes were found in all the isolates while 30 (93.75%) isolates carried the long polar fimbriae *lpfA*. At least a gene responsible for bacterial iron metabolism (iron acquisition, yersiniabactin biosynthesis, and aerobactin) -*iroN, irp2, fyuA, chuA, iutA, iucC- and traT* - were detected in the strains, but *traT*- and *iroN* (n=7) occurred the most. The microcin (*mchC-, mchB-, mcmA-, mchF-, mchF-*) and colicin genes (*cba, cvaC, cma, cvaC*) were correspondingly detected in four and two isolates. A strain carried *iha*, an O157:H7 EHEC nonhemagglutinin, and *efa1* (*lifA*), a lymphocyte inhibitory factor: the toxin gene *astA* and *sitA* were accordingly found in nine and 17 isolates. Immunoglobulin repeat protein (*air*) and *eilA*, a *Salmonella enterica HilA-*like regulator seen in EAEC were present in two isolates. However, nine and 11 isolates carried outer membrane protein (*OmpT*-) and increased serum survival (*iss*) genes. Shiga-toxin-related gene (*stx1B*) was found in one *E. coli* containing extra 12 VAGs (Table 2 and https://microreact.org/project/mwV3AQPd59fJrXk8EetChZ-escherichia-coli-virulence-associated-genes).

Detection of plasmid replicons in *E. coli*: Seventeen strains (53.13%) carried various plasmid types while the rest of the isolates did not carry any plasmid replicons. The most occurring plasmid type was IncF found in 12 (37.5%) isolates. The IncF replicons were IncFI- (n=18), IncFII- (n=7), IncX (n=7), IncI-1-α (n=5), IncN (n=3), IncY (n=3), and Col-MG82 (n=1) (Table 2 and https://microreact.org/project/kgLorSjVnomL1Y5baXn3ga-escherichia-coli-plasmids).

Phylogroup and multilocus sequence type of *E. coli*: The phylogroup B1 (n= 22, 68.75%) was predominant as against phylogroup A (n=8, 25%) while one isolate belonged to phylogroup E and the cryptic clade. The most frequently occurring STs were ST2541 and ST7483 (n=5), and ST295 (n=3). The detected novel STs were ST13336 (n=2) and ST13327 (n=1). ST/phylogroup showed that 21 isolates belonged to group B1 while ST2541, ST206, and ST13336 belonged to phylogroup A. A novel ST (ST13327) belonged to group E (Table 2).

Table 2. WGS analysis of *E. coli* showing antimicrobial resistance, virulence, plasmid types, phylogroups, and sequence types

ST/ Phylogroup	Virulence-associated genes	Plasmids	AMR genes	Classifications
ST224/B1	*terC-5, terC-23, lpfA, gad-, hra-11, hra-6, F17a(6), F17a(7), mchC-4, afaA-2, mchB-2,* mcmA-3, afaC-7, mchF-8, mchF-2, f17G-5, hra-2, afaB-6		nil	ETEC
ST13336/E	*terC-5, terC-23, gad-, air-4, iss-3, air-3, chuA-50, eilA-3, air-2, ompT-8, ompT-, sitA, traT-*	IncFIC-FII, IncFIB	nil	EAEC
ST665/B1	*terC-5, terC-23, lpfA, gad-, efa1(7), esp(1), espA(11), tir(30), eae (5), eae(44), eae(7), eae-42, astA-8, iutA-20, tccP-15, iha-14, gad-, nleA-8, nleA-2, nleC-3, nleB-14, espB-7, espF-3, iucC-1, ompT-, traT*	nil	nil	aEPEC
ST7428/B1	*terC-5, terC-23, lpfA, gad-, ompT-8, fyuA-78, irp2, traT, iss-9*	IncFIA-HI1, IncFII-pCoo, IncFIB-pB171	nil	Commensal
ST13336/A	*terC-5, terC-23, lpfA, gad-, iss-4, cba-5, cvaC-10, papC-34, papC-29, iroN-6, mchF-11, ompT-240, cma-12, cvaC-6, hlyF-15, ompT-, sitA, traT-*	IncFII, IncFIB	nil	APEC
ST2467/ cryptic	*terC-5, terC-23, lpfA, gad-, air-4, ehxA(1), sta1-2, astA-4, air-3, stx1B-14, eilA-3, yfcV-42, sitA*	IncFII-pSE11, IncFIB	nil	ST-EAEC
ST2541/A	*terC-5, terC-23, lpfA, gad-, astA-4, sitA*	IncI1-I-α		Commensal
ST2541/A	*terC-5, terC-23, lpfA, gad-, sitA, tsh-3*	Col-MG828, IncFIC-FII, IncI1-I-α		Commensal
ST224/B1	*terC-5, terC-23, lpfA, gad-, hra-11, hra-6, F17a(7), mchC-4, afaA-2, mchB-2,mcmA-3,* afaC-7, mchF-8, f17G-5, hra-2, afaB-6			Commensal
ST155/B1	*terC-5, terC-23, lpfA, gad-, sitA*			Commensal
ST295/B1	*terC-5, terC-23, lpfA, gad- sitA*	IncN, IncX1.1, IncX1.2, IncY	*aac-6(Ib7), ant-3 (Iia), aph-6(Id), qnrS1, bla*$_{TEM-95}$*, dfrA14, mef-B, sul3, tetA*	Commensal
ST295/B1	*terC-5, terC-23, lpfA, gad-, sitA*	IncN, IncX1.1, IncX1.2, IncY	*aac-6(Ib7), aph-6 (Id), qnrS1, bla*$_{TEM-84}$*, dfrA14, mef-B, sul3, tetA*	Commensal
ST164/B1	*terC-5, terC-23, lpfA, gad-, hra-11, hra-6, iss-8, ompT-8, ompT-, sitA, traT-*	*IncFIC-FII, IncFIA, IncFIB*		Commensal

ST-EAEC, Shiga toxin/Enteroaggregative *E. coli*; ETEC, Enterotoxigenic *E. coli*; EAEC, Enteroaggregative *E. coli*; APEC, Avian Pathogenic *E. coli*; aEPEC, atypical Enteropathogenic *E. coli*

Classification of the Escherichia coli strains into pathotypes: One *E. coli* isolate each was grouped into five pathotypes, viz. highly contagious APEC, ETEC, EAEC, ST-EAEC, and aEPEC. The isolates possessed an average of nine VAGs, even though most of them (n=27/32) were unclassifiable into specific pathotypes based on our stringent criteria and were therefore regarded as commensals.

Continuous reading of plasmids, antimicrobial resistance, and virulence genes: The investigation of the co-occurrence of ARGs, VAGs, and plasmids using a Venn diagram revealed that none of the isolates carried only ARGs or plasmids (Fig. 1). However, 10 *E. coli* isolates contained VAGs only without harbouring ARGs and plasmids, while separate strains (n=10) carried both VAGs and plasmids. Five *E. coli* isolates carried both ARGs and VAGs as against seven isolates that carried ARGs, VAGs, and plasmid replicons altogether (Figure. 1).

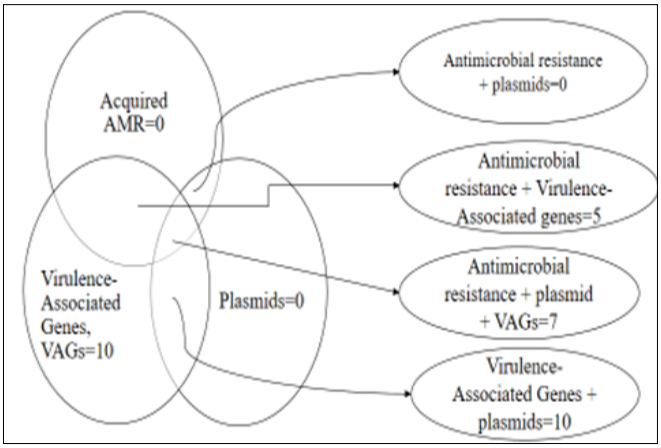

Figure 1. Representation of detected antimicrobial resistance genes (ARGs), virulence-associated genes (VAGs), and plasmids in *E. coli* (n=32) isolated from dog fecal samples. Additional information on accessions and other metadata are available in the Supplementary files.

Table 2 continuation. WGS analysis of *E. coli* showing antimicrobial resistance, virulence, plasmid types, phylogroups, and sequence types.

ST/Phylogroup	Virulence-associated genes	Plasmids	AMR genes	Classifications
ST211/B1 (n=2)	*terC-5, terC-23, lpfA, gad-, astA-4*	IncFII, IncFIB, IncI1-I-α	*aph-3(Ib), aph-6(Id), sul2, tetR, tetB*	Commensal
ST13327/A	*terC-5, terC-23, lpfA, gad-, iss-4, cba-5, cvaC-10, papC-34, iroN-6, mchF-11, ompT-240, cma-12, cvaC-6, hlyF-15, ompT -, sitA, traT-*	IncFII, IncFIB		Commensal
ST162/B1	*terC-5, terC-23, lpfA, gad-, iss-8, ompT-, sitA*			Commensal
ST297/B1	*terC-5, terC-23, lpfA, gad-, iss-8, ompT-8, sitA, traT-*	IncFIA-HI1, IncFII-pCoo, IncFIB-pB171		Commensal
ST2541/A (n=2)	*terC-5, terC-23, lpfA, gad-, hra-11, hra-6, F17a(5), f17G-6*			Commensal
ST13029/B1	*terC-5, terC-23, lpfA, gad-, astA-4, tsh-3, ompT-, sitA*	IncI1-I-α, IncX1.4	bla_{TEM-84}, *tetA*	Commensal
ST2541/A	*terC-5, terC-23, lpfA, gad-, hra-11, hra-6, F17a(5), f17G-6*			Commensal
ST295/B1	*terC-5, terC-23, lpfA, gad-, sitA*	IncN, IncX1.1, IncX1.2, IncY	*aac-6(Ib7), aph-6 (Id), qnrS1,* bla_{TEM-95}, *dfrA14, mef-B, sul3, tetA*	Commensal
ST2067/B1	*terC-5, terC-23, lpfA, gad-, hra-11, iss-3, ompT-147, ompT-, sitA*			Commensal
ST297/B1	*terC-5, terC-23, lpfA, gad-, iss-3, ompT-8, ompT-, sitA*	IncFIC_FII, IncFIB		Commensal
ST206/A	*terC-5, terC-23, gad-*	IncFIB-pHCM2	*aac-6(Ib7), qnrB7, aadA5, dfrA17, sul2*	Commensal
ST7483/B1(n=5)	*terC-5, terC-23, lpfA, gad-, astA-4, iroN-3*		*tetR, tetB*	Commensal

ST-EAEC, Shiga toxin/Enteroaggregative *E. coli*; ETEC, Enterotoxigenic *E. coli*; EAEC, Enteroaggregative *E. coli*; APEC, Avian Pathogenic *E. coli*; aEPEC, atypical Enteropathogenic *E. coli.*

Discussion

We recently reported multiple phenotypic antibiotic resistance for *E. coli* isolates retrieved from the fecal sample of healthy dogs in Ibadan (Falodun et al., 2022). In the current investigation, we leveraged the awareness that pets constitute channels for the spread of highly contagious diseases (Jacob et al., 2015) to sequence the antibiotic-resistant isolates and assess their genomes for resistance markers. We found *blaTEM (*n=4) as the sole ESBL gene that encoded resistance to β-lactam antimicrobials; no other ESBL genes were found in the strains. In 75% of the strains that contained *blaTEM*, there was a co-occurrence of *qnrS1, tetA, sul3, aac-6(Ib7), aph-6(Id)* genes with the highly versatile self-mobilizable IncN and host-specific IncX replicons. Our study is concordant with that of Sumrall et al. (2014) who reported that the occurrence of PMQR with IncX in *E. coli* attracted multiple and transmissible ARGs. The dosing of pets with antibiotics such as tetracycline, sulphonamide, and β-lactam antibiotics by veterinarians is a precursor for the different resistance genes found in the isolates. The presence of mobile antimicrobial resistance genes alongside self-conjugative plasmids in the isolates increases the risk of their contagion and this constitutes a major threat to public health.

The predominant STs of the strains isolated from freshly passed dog faecal samples were ST2541 and ST7483. However, most of the known epidemic *E. coli* strains such as STs-224, 162, 155, 297, and 2067 occurred rarely. It is thus clear that most isolates recovered in this study did not fall into widely circulated *E. coli* lineages of ST131, and ST10 complex-related isolates in Nigeria (Aibinu et al., 2012; Ogbolu et al., 2020; Afolayan et al., 2022). It is well known that ST2541 and others (STs-224, 162, 155, 297) belong to the global lineage associated with ESBL in *E. coli* isolated from dogs in Asia, Europe, and America

(Salgado-Caxito et al., 2020), although, none of the epidemic STs detected in this study carried ESBL genes.

Nevertheless, the *E. coli* phylogroup structure A (n=8,25.0%) and B1 (n=21,67.74%) agree with the previous report of Vega-Manriquez and colleagues who reported a similar pattern of phylogroup A (n=5) and B1 (n=21) in *E. coli* strains isolated from dogs (Vega-Manriquez et al., 2020). The clustering of diarrheagenic *E. coli* in phylogroups A, B1, and E indicates diarrhea association (Clermont et al., 2011) implying that these organisms can cause in the healthy pet dogs and even infect the pet owners.

The detection of highly infectious and pathogenic strains of ETEC, EAEC, ST-EAEC, aEPEC, and APEC strains was worrying because these strains, especially aEPEC have been attributed to diarrhea in dogs (Puño-Sarmiento et al., 2013). EPEC virulence markers (typical or atypical) lead to diarrhea complications as they are differential diagnostic markers in dogs with enteric infections (Kjaergaard et al., 2016). ST-EAEC, cryptic *E. coli* that harbored *stx1B*, enterohemolysin *ehxA*, *hilA*-homologue *eilA*, and toxigenic *sta1* genes, are predictors for bloody diarrhea and hemolytic uremic syndrome in STEC infections (hua et al., 2021). Even though in Nigeria, there is a rarity of information on the incidence of STEC in dogs; PCR characterization of STEC genes retrieved from human clinical samples (Olowe et al., 2015) showed a moderate prevalence of *stx1* (13.9%), *stx2* (6.9%) and *hlyA* (2.0%). Therefore, given the opportunity of exposure, ST-EAEC acquisition by humans from dogs is possible.

The pathogenicity island, PAI O-122 (*lifA/efa-1 and nleA-E*), present in some strains showed that the strains can cause severe diarrhea in children (Mercado et al., 2016). These strains possibly progressed through enforcing maintenance in their host (Doumith et al., 2012) and then acquired virulence factors to emerge as new *E. coli* subtypes. Feng et al. (1988) already showed evidence of genetic re-arrangements of a novel assortment of *E. coli* virulence genes in EHEC. A key study limitation is that the number of *E. coli* isolates obtained and sequenced was small to generalize the evolution of novel *E. coli* strains in apparently healthy pet dogs. Additional studies are needed to examine the specific mechanism(s) that could be responsible for the evolution of new pathogenic subtypes especially in healthy pet animals.

Conclusions

The possible exposure to distinct *E. coli* pathogenic lineages in fecal materials of healthy dog by humans poses a risk of disease spread from pets to humans. The delineation of the strains into specific lineages afforded by the whole genome sequencing further revealed the co-incidence of antimicrobial resistance genes and mobile genetic elements such as plasmids. Therefore, pet owners are strongly advised to handle fecal samples from their household dogs with care to minimize the risk of the environmental spread of infections and diseases. Antimicrobial surveillance systems should be expanded to incorporate healthy pet dogs to reduce the chance of disease spread between pets and their owners.

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Conflict of Interest

The authors declared that there is no conflict of interest

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Factors influencing ovum pick-up technique results in cattle

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ABSTRACT

The Ovum Pick-Up (OPU) technique, which is used in in vitro embryo production (IVP) to retrieve immature oocytes from live donor animals, is one of the most important biotechnological procedures used in cattle breeding. The most important advantage of this technology is that it allows for the reproducible retrieval of immature oocytes from living donor animals. It is particularly useful in dairy cattle breeding to address infertility issues and boost the production of superior animals with high genetic value. The OPU technique offers several advantages, including its applicability to cows ranging from six-month-old calves to the first three months of pregnancy, its effectiveness in animals with genital tract infection or acyclic cattle, and its ability to yield a higher number of embryos within the same period compared to the Multiple Ovulation and Embryo Transfer (MOET) technique. Understanding and improving the technical and biological factors influencing the OPU procedure is necessary to increase and optimize donor animal use in IVP and the number of quality oocytes obtained. This review aims to examine the specifics of the OPU approach and the factors influencing its performance in light of contemporary literature, as well as to propose fresh ideas to researchers.

Keywords: bovine, in vitro embryo production, ovum pick-up, oocyte retrieval, follicle aspiration

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Introduction

Pieterse et al. (1988) pioneered the technique of transvaginal ultrasonography-guided follicular aspiration from live donor bovine ovaries in the late 1980s. This technique was initially used in cows who did not respond to superovulation (Kruip et al., 1994; Looney et al., 1994). The OPU technique aims to produce a large number of high-yielding calves while shortening the generation gap by using oocytes acquired repeatedly from the ovaries of heifers with high genetic value (Pieterse et al., 1991).

In 2020, in vitro embryo production (IVP) produced 79.7% of all embryos transplanted worldwide. Additionally, 71.2% of the embryos obtained from IVP were obtained with OPU. This increase in IVP is attributed to OPU (Viana, 2021 and 2022).

Two methods are primarily used as oocyte sources in in vitro embryo production. Transvaginal ultrasonography-guided follicular aspiration technique in live donor animals is one of these approaches, while postmortem follicular aspiration from slaughterhouse material is another (Galli et al., 2014; Ferré et al., 2019). OPU is a non-invasive and reproducible approach for retrieving large numbers of immature oocytes from the antral follicles of live donor animals (Choudhary et al., 2016; Watanabe et al., 2017; Çizmeci, 2022). OPU/IVP devices can extract 3-8 mm diameter follicles from the ovaries of calves, heifers, and even pregnant animals. The OPU procedure offers

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the only alternative for obtaining cumulus-oocyte complex (COC) from pregnant animals (Aller et al., 2012; Ferré et al., 2019). OPU does not disrupt the reproductive cycle of the donor. It can be performed successfully at any time, regardless of the donor's reproductive state. It can be repeated at regular intervals (every 1-2 weeks). It causes the least amount of stress for the donor animal. Additionally, each application has a reduced cost. An immense amount of oocytes can be fertilized in vitro (IVF) with a single straw spermatozoa. It allows the use of several premium bull semen and sexed spermatozoa. It enables dominant follicle ablation (DFR). With OPU, more transplantable embryos can be obtained from each donor monthly (Qi et al., 2013; Boni, 2012; Choudhary, 2016; Kimble, 2020). Unlike oocytes taken from ovaries collected at a slaughterhouse, the genetic characteristics and health state of the donor are known in OPU oocytes. Furthermore, the OPU procedure is a promising technique that can be included in programs for animal breeding that use endangered breeds (Borş et al., 2018).

Retrieving oocytes from the ovaries of donor animals by OPU technique has brought a new perspective to assisted reproductive techniques and made it possible to use donor animals with greater genetic potential for IVP for many years (Bols and Stout, 2018).

Factors influencing ovum pick-up technique results

The final outcome of the OPU technique is affected by two main factors: technical and biological factors. Technical factors influencing the success of the OPU technique include aspiration pressure, the type and frequency of the ultrasound device, aspiration needle characteristics, and operator experience. Biological factors influencing the success of OPU include individual differences among donors, breed, age, health status, body condition score (BCS) and nutrition, as well as aspiration frequency and superstimulation. In OPU programs, the generation of embryos also requires specialized laboratory support (Galli et al., 2014; Da Silva et al., 2016; López, 2020).

Aspiration pressure value

The aspiration pressure in the OPU mechanism depends on the aspiration pump, the length and diameter of the tube used, the size and type of the collection tube, and the diameter of the aspiration needle. By adjusting the aspiration needle's width, the rate of liquid aspiration can be increased up to three times while maintaining the same aspiration pump pressure. Although high aspiration pressure increases the number of oocytes collected, it is stated that high aspiration pressure (70-130 mm/Hg) reduces the rate of oocytes with cumulus cells. It is suggested that low aspiration pressure increases the rate of reaching the blastocyst by increasing the rate of cumulus cell oocytes (Bols et al., 1996; Fry et al., 1996; Ward et al., 2000; López, 2020). The value of the aspiration pressure may damage the cumulus-oocyte complex. Following follicle aspiration, the morphology of the COCs should be assessed when experimenting with various aspiration pressures and needle sizes. As a result, it is possible to identify the aspiration pressure range or threshold value that will limit harm to the aspirated COCs. This technique can be used in systems using disposable injection needles to calibrate in vitro (Bols et al., 1996).

Resolution of the ultrasound devices and features of the probes

Using ultrasonography, the follicles must be readily visible during the OPU procedure. The follicles are aspirated with the least harm to the donor and the COCs when clearly visible in the ovary. Research has shown that the ultrasonic screen's resolution and probe characteristics significantly impact the number of oocytes retrieved and minimize needless ovarian tissue damage (Bols et al., 2004; Çizmeci, 2022). For the operator to see the follicles and other ovarian structures in the ovary as clearly as possible, it is advised that high frequencies (6-8 MHz) be used (Da Silva et al. 2016; López 2020). New ultrasound devices even show follicles with tiny diameters (2-3 mm), which are not suitable for oocyte retrieval. Although sector probes are the most commonly used in OPU systems, linear probes can also be used. There is no statistical difference between sector and linear probes in terms of oocyte number and quality, although the sector probe visualizes tiny follicles better than the linear probe. The linear probe, on the other hand, restricts ovary movement to a rotation along its longitudinal axis and hence cannot observe the ovary's outside margins. (Bols et al., 2004; López, 2020).

Aspiration needle diameter and type

The sharp aspiration needle is the most essential technical aspect influencing a successful OPU application (Seneda et al., 2001; Singh et al., 2003; Bols et al., 2004). Previously, operators used needles that were 50-60 cm long and had an outside diameter of 1-1.5 mm (Looney et al., 1994). The main downside of these needles is that they lose their sharpness rapidly and cannot be re-sharpened to their previous sharpness. Furthermore, these long, non-disposable needles are rather costly. Alternative OPU systems that use disposable 18-gauge (1.02 mm) epidural needles or subcutaneous injection needles that are somewhat less expensive have been developed (Rath, 1993; Bols et al., 1995). These needles have the

advantage of being sterile and available in various sizes and lengths (Bols and Stout, 2018).

It has been reported that using needles with a diameter of less than 18 g in OPU systems results in a higher number and quality of oocytes. The oocytes are separated from the surrounding cumulus cells because they move quicker than the cumulus cells due to the use of large-diameter needles. Furthermore, the use of large diameter needles causes more damage to the ovary. Damage causes an increase in blood aspiration. The use of needles with tiny diameters (more than 21 g) decreases the aspiration rate, increasing the amount of oocytes lost. As a result, 18-20 g aspiration needle sizes are recommended (Bols et al., 1996; Da Silva et al., 2016; López, 2020).

The optimal needle length is thought to be between 40 and 75 mm. Aspiration needles 40 mm long require extensive manipulation during follicular aspiration because they cannot reach all follicles from the perforated area of the ovary. When aspiration needles longer than 75 mm are used for follicular aspiration, they are flexible and easily bend (López, 2020).

The curve of the needle tip, on the other hand, influences the fraction of oocytes with compact cumulus cells. There are two types of needle tips available on the market: short and long conical, and it has been reported that using a long conical needle enhances oocyte yield (Bols, 1997).

OPU session application frequency

The OPU technique is highly reproducible (Pieterse et al., 1991; Watanabe et al., 2017). The frequency and duration of the OPU session used on the donor animal, on the other hand, influences the quality and number of oocytes retrieved (Nolan et al., 1998; Merton et al., 2003; McEvoy et al., 2006). Although a 3- or 4-day delay between two OPU treatments has been shown to give fewer COCs than a 7-day interval, a 3-day interval has also been shown to yield better quality COCs and a higher blastocyst rate. This is because the dominant follicle (DF), which arises roughly three days after the OPU treatment, suppresses the development of other follicles. The number of oocytes collected per session does not differ between the OPU program performed at 3 and 4-day intervals, which is often used, and the OPU program performed at 2 and 5-day intervals (Merton et al., 2003; Sirard, 2012; López, 2020).

Donor animal's diet

The donor's diet influences follicular development and ovulation through its role in the hypothalamus-pituitary-gonadal axis (Armstrong et al., 2003). Negative energy balance in cattle negatively affects pre-ovulatory follicle diameters and follicular development (Armstrong et al., 2001). Malnutrition has negative effects on the in vitro developmental competence and blastocyst rate of oocytes retrieved from the donor animal (Dominguez, 1995, Ruiz et al., 1996). Furthermore, a correlation has been noted between an increase in BCS and an increase in the developmental capacity of the oocyte (Dominguez, 1995). Conversely, low BCS in the donor animal due to malnutrition has a negative impact on the blastocyst rate and the ability of the resulting oocytes to grow in vitro (Bols and Stout, 2018; López, 2020). It has been noted that moderate to high BCS is linked to the negative effects of excessive calorie intake (Sartori et al., 2017). Furthermore, prolonged hyperinsulinemia decreases the ovaries' sensitivity to gonadotropins and oocyte quality, even though high energy intake raises blood sugar, insulin, and insulin-like growth factor-1 (IGF-1) concentrations (Diskin et al., 2003; Bender et al., 2014; Sales et al., 2015).

Donor animal species

Depending on the breed of the animal, the AFC in the ovaries of cattle receiving repeated OPU treatments differs (Goodhand et al., 1999; Viana et al., 2010). Bos taurus and Bos indicus cow breeds vary considerably in the number of follicular waves, the diameter of the DF, and the rate of follicle development (Figueiredo et al. 1997; Bó et al. 2003). The quantity of oocytes extracted from each OPU varies significantly between breeds, according to several research groups (Pontes et al., 2010; Gimenes et al., 2015). It has been noted that compared to cattle of the Bos taurus breed, the number of follicular waves and the AFC that occur during the follicular wave in Bos indicus breed cattle are higher. Oocytes from Bos indicus cow breeds are obtained in greater quantities as a result of this circumstance (Watanabe et al. 2017; Bó et al. 2019).

In cattle of the Bos taurus species, the average number of oocytes recovered per OPU treatment is 4–14, whereas in the cattle of the Bos indicus species, it is 18 –25 (Thibier, 2004; Rubin et al., 2005; Martins et al., 2007; Pontes et al., 2011). According to Sartori et al. (2010a), the average AFC in the ovaries of Bos taurus cattle is twice that of Bos indicus cattle. Breed-related variations in circulating insulin, IGF-1, and cholesterol are thought to be connected to this (Alvarez et al., 2000; Sartori et al., 2010b and 2018a).

Because of their larger AFC, superior oocyte quality, and greater sensitivity to gonadotropic hormones, Bos indicus breed cattle have substantially more economical IVP than Bos taurus breed cattle (Sartori et al., 2018b).

Donor animal age

Many eligible donor animals, ranging from 6-month-

old calves to 3-month-old pregnant cows, can be used with the OPU approach (Aller et al., 2012; Ferré et al., 2019). Because the follicular reserve in the ovaries of five-year-old cows declines, the age of the cattle must be considered in IVP (Cushman et al., 2009). It is reported that the blastocyst rate of oocytes collected from donors aged one to three years is higher than that of older donors (Ali et al., 2021).

This is significant because genetic development can be expedited by retrieving oocytes from prepubertal calves and decreasing the intergenerational period (Landry et al., 2016; Çiftçi, 2022; Çizmeci, 2022). The developmental competence of oocytes retrieved from calves less than six months old, on the other hand, is poorer (Duby et al., 1996; Ax et al., 2005). It has been reported that the number of small to medium-sized follicles and the total number of follicles aspirated after ovarian stimulation are higher in young Bos taurus breed donors compared to cyclic donor cattle. Although in vitro maturation produces more COCs, young animals produce less blastocysts than cyclic cows (Landry et al., 2016; Baldassarre et al., 2018; Zacarias et al. 2018; Seneda et al., 2020). It has also been observed that IVP efficiency is higher in heifers of Holstein breed donors than in lactating cows (Ferreira et al. 2011; Ali et al., 2021).

The OPU procedure in calves is dependent on the pelvic size to accommodate the vaginal probe. Oocytes can be acquired with the OPU technique in Holstein breed heifers at the age of 6-9 months, depending on the size of the probe used (Bols, 1999; Bols and Stout, 2018).

Heat stress and season

Heat stress during follicle development in cattle has been shown to affect oocyte quality and embryo development by preventing follicular dominance (Qi et al., 2013). It is suggested that the negative effects of heat stress on fertility lower oocyte quality in a short period of time by causing problems such as the small diameter of the DF of follicular waves, inadequate dominance formation, and an increase in the number of large-diameter follicles (Çizmeci, 2022). Heat stress has been shown to reduce the developmental ability of oocytes retrieved from both OPU and slaughterhouse material. During the summer months, the quality of bovine oocytes decreases, and the lipid composition of oocyte membranes varies (Al-Katanani et al., 2002; Takuma et al., 2010; Boni, 2012; López, 2020). According to Roth et al. (2001), the quality of oocytes acquired at the beginning of autumn is low and gradually improves as winter approaches. Heat stress has been shown to disrupt the GnRH release mechanism from the hypothalamus, resulting in lower levels of FSH and LH in the bloodstream, which negatively affects follicle selection and development and reduces oocyte quality (De Rensis and Scaramuzzi, 2003; Camargo et al., 2006; De Rensis et al., 2021; Çizmeci et al., 2022).

Follicular wave synchronization

It has been shown that follicular development in the bovine ovary occurs in waves and that two or three follicular waves are often seen in a single estrous cycle. (Adams and Pierson, 1995; Adams, 1999; Sirard, 2018). On random days of the estrous cycle, follicles with a diameter of at least 2 mm are aspirated in several OPU/IVP protocols (Pontes et al., 2011; Dos Santos et al., 2016). Oocyte quality, oocyte growth rate, and IVP are all affected by the time of the estrous cycle during follicle aspiration (Seneda et al., 2001; Hendriksen et al., 2004; Camargo et al., 2006). It has been discovered that oocytes aspirated on the fourth, fourteenth, and eighteenth days after ovulation of the estrous cycle yield a greater rate of blastocysts (Gonjalves et al., 2022). It has been observed that more and higher quality oocytes are retrieved during the recruitment phase when the DF does not develop due to follicular atresia. It has also been reported that oocytes retrieved at this stage are more likely to reach the blastocyst (Bacelar et al., 2010; Gimenes et al., 2015; Ongaratto et al., 2015). The synchronization of the follicular wave in donor animals makes the follicles more homogenous in terms of diameter and developmental stage, allowing for the retrieval of higher-quality oocytes. Synchronizing the follicular wave before OPU has been shown to boost the number of embryos generated and pregnancy rates after the transfer. The estrous cycle of cattle and the physiology of the follicular wave ensure that follicular waves are synchronized before the OPU. As a result, follicular wave synchronization can be used to improve IVP findings prior to OPU (Cavalieri et al., 2018; Seneda et al., 2020). Follicular dynamics in the estrous cycle in cattle can be manipulated with exogenous gonadotropin applications (Aerts and Bols, 2010). A follicular wave has been observed in cattle approximately 36 hours following gonadotropin application (Bergfelt et al., 1994 and 1997; Ongaratto et al., 2015).

The goal of administering PGF2α four days before OPU is to ensure CL regression. In the absence of CL, follicular visibility and puncturing are easier, vascular perfusion reduces, and thus, the number of COCs collected increases (Bacelar et al., 2010; Ongaratto et al., 2015; da Silva et al., 2017). However, oocyte quality has been found to decline as a result of a decrease in plasma P4 concentration by inducing

luteolysis prior to OPU (Nasser et al., 2011). It has been shown that adding P4 to the treatment protocol in dairy cows that receive superstimulation during the first follicular wave improves embryo quality (Rivera et al., 2011).

GnRH stimulation 48 hours before OPU in the early lactation phase improves embryo production efficiency in Holstein breed donors. Furthermore, it has been found that EB stimulation of donor cattle enhances the effectiveness of embryo formation in OPU-IVP technology. In cow studies, it has been shown that those who received EB application had higher levels of AFC and COCs than those who had GnRH stimulation (Ogata et al., 2015; Cavalieri et al., 2018; Hidaka et al., 2018).

Nonhormonal application and superstimulation

The OPU approach is a non-invasive, reproducible procedure that can be performed with or without hormonal stimulation (Watanabe et al., 2017; Wrenzycki, 2018). Once or twice a week, two distinct OPU procedures, with or without hormonal stimulation before OPU, might be applied (Chaubal et al., 2007). There is no stimulation in the standard OPU procedure, and OPU can be applied to each donor animal twice a week. It has been found that OPU application twice a week delivers the largest amount of oocytes of adequate quality when compared to OPU application once a week. This is because all observable follicles are aspirated, preventing the DF from growing and inhibiting the growth of other follicles. Thus, there is no need for ablation (DFR) of the DF. (Qi et al., 2013; Çizmeci 2022). OPU application twice a week can achieve 130 embryos every year, resulting in the delivery of 70 calves (Merton et al., 2003).

The second method is the application of hormones called "Superstimulation". The purpose of superstimulation is to increase the number of oocytes available for aspiration per OPU administration. The number of oocytes retrieved from superior cows can be enhanced by using exogenous gonadotropins and managing follicular dynamics. Equine chorionic gonadotropin (eCG), pituitary extracts from pigs and sheep, and human menopausal gonadotropin (hMG) are all used for superstimulation in cattle (Mapletoft et al., 2002; Aerts and Bols, 2010). Due to variations in properties such as half-lives and LH secretion, a variety of protocols have been developed for superstimulation with these hormones. Changes in the dosage and timing of gonadotropins are necessary because the primary goal of pre-OPU superstimulation is to generate more follicles rather than multiple ovulation (Bols and Stout, 2018). Numerous techniques have been tested for this aim, including lowering the amount of FSH and dissolving it in various polymers, using a single dosage of FSH in pre-OPU superstimulation protocols, and using different application routes (Chaubal et al., 2007; Ongaratto et al., 2010; Vieira et al., 2016). Applying exogenous FSH to donor cattle can enhance the quantity and quality of oocytes retrieved with OPU in cattle. This temporary increase in serum FSH concentration can delay the development of the DF, atresia of the subordinate follicles, and the increase in the size of the ovarian follicles, making them suitable for OPU (Ongaratto et al., 2015 and 2020; Fernandes et al., 2020; Çiftçi and Dinç, 2023). One of the major parameters for increasing the effectiveness of IVP systems is the diameter of the follicles in the ovary during OPU (Vassena et al., 2003). FSH treatment before OPU can improve the number of medium and big follicles as well as the effectiveness of OPU-IVP (Bó et al., 2019; de Carvalho et al., 2019; Ongaratto et al., 2020; Çiftçi and Dinç, 2023). However, the response to exogenous FSH can be affected by factors such as the change in the applied FSH concentration, breed, application method, application frequency, and the interaction of FSH with LH (Sartori et al., 2010a; Zacarias et al., 2018; Kaya et al., 2018; Fernandes et al., 2020). It is reported that FSH treatment and regimen, although it increases the number of medium-sized follicles (4-6 mm), has no effect on the quality and number of oocytes obtained. It has been shown that dividing the FSH regimen into 7 days rather than 4 reduces the number of small antral follicles (1-5 mm) while increasing the number of large antral follicles (>9 mm) (Mapletoft et al., 2015).

More AFC, COCs, and embryos can be obtained with superstimulation. Long-term exogenous hormone use, on the other hand, may alter the donor's hormonal condition and result in infertility. Additionally, the response of other donors to hormone stimulation may differ. As a result, it has been observed that short-term hormonal stimulation is beneficial (Qi et al., 2013; Vieira et al., 2014). In donors with low AFC, AFC can often be raised using FSH-LH combinations or eCG. These hormones are usually used in ET programs. Because the primary goal of pre-OPU superstimulation is to produce more follicles rather than to ensure multiple ovulation, the dose and timing of the treatment are critical (De Rover et al., 2008; Bols and Stout, 2018).

Because of the low number of oocytes retrieved each OPU session from Holstein cattle, follicular wave synchronization becomes necessary. As a result, it has been reported that FSH superstimulation is essential to increase embryo production (Demetrio et al., 2020). In Holstein breed donors without hormonal stimulation, 4-5 quality oocytes are retrieved each

OPU session, but up to 20 are obtained in those with hormonal stimulation (de Loos et al., 1989; Hasler, 1998; Bols et al., 2005; Vieira et al., 2014 and 2016). It has also been shown that the average number of aspirated follicles, COCs, and embryos is higher in superstimulated animals than in non-stimulated animals (Chaubal et al., 2007; De Roover et al., 2008).

Prolonged stimulation of the ovaries following a single eCG injection causes problems for practitioners all around the world (Bo and Mapletoft, 2014). The half-life of eCG in the cow is 40 hours, and it can stay in the bloodstream for up to 10 days (Murphy and Martinuk, 1991). Consequently, eCG's extended half-life results in persistent ovarian stimulation, non-ovulated follicles, abnormal endocrine profiles, and poor embryo quality (Kruip et al., 1984; Aller et al., 2012). However, Ribas et al. (2018) found that using 800 IU eCG before OPU increased the rate of follicles >6 mm in diameter and that the oocytes obtained from these follicles had a higher fertilization rate that supported the first embryonic development, and that this was an alternative protocol for ovarian superstimulation before OPU.

Coasting, or ovarian stimulation followed by a gonadotropin-free rest interval, has been proven to be a successful regimen for producing higher quality oocytes and improving blastocyst output in cattle. The coasting process is described as depriving cows of FSH for 36-48 hours prior to OPU. In other words, the time between the last FSH application and OPU is referred to as partial in vivo prematurity. Coasting is achievable in FSH-stimulated cows by ceasing FSH application 36-48 hours before OPU, but it is not possible in eCG-stimulated cows due to the extended half-life. Coasting in ovarian stimulation regimens has been shown to increase blastocyst output in cyclic cows by up to 80% (Blondin, 2002; Nivet et al., 2012; Landry et al., 2016; da Silva et al., 2017).

Dominant follicle ablation (DFR)

When a DF is absent or eliminated at the start of superstimulation, the overall number of oocytes and live embryos increases significantly when compared to the outcomes of superstimulation in the presence of a DF (Merton et al., 2003). The sensitivity of the ovaries to superstimulation differs greatly between donors (Looney, 1986). Ablation of the DF or hormonal treatments can be used to optimize the superstimulation response and increase the number of antral follicles. The DF in the ovary suppresses follicular development by stimulating the release of inhibin and estradiol (Aerts and Bols, 2010). It has been observed that the presence of a DF lowers the in vitro developmental competency of oocytes produced from subordinate follicles (Hendriksen et al., 2004). DFR is typically applied 48 hours before the OPU session. DFR causes an increase in FSH to begin within 12 hours and a new follicular wave to start within 24 hours. It is also claimed that DFR is as effective as protocols combining progesterone and estradiol in follicular wave synchronization for superstimulation in cattle (Bó and Mapletoft, 2014; Adams and Singh, 2021; Çizmeci 2022).

Reproductive health of the donor animal

OPU induces both short- and long-term alterations in donors' ovaries and vagina. The vaginal fornix can be perforated up to 48-72 hours after puncture (Da Silva et al., 2016). According to one study, although bruising was found in the perivaginal area, this did not cause significant harm to the donor (Viana et al., 2003). It has also been noted that needle puncture can cause vaginal tears or pathogen contamination (Younis et al., 1997; Cho et al., 2004). Adhesions and fibrosis can be seen in cow ovaries, particularly those subjected to conventional OPU treatments over an extended period (Da Silva et al., 2016). The frequency and high number of repeated OPU sessions result in the formation of an abnormally large amount of fibrous connective tissue in the tunica albuginea and ovarian stroma (Viana et al., 2003; McEvoy et al., 2006; Çizmeci, 2022). Many researchers, however, have reported that ovarian tissue is resistant to sclerosis. It has been reported that cattle can tolerate the follicular puncture process, which disrupts ovarian surfaces and significantly changes tissue dynamics (McEvoy et al., 2002; Bogh et al., 2003). The OPU technique can be applied to the same donor animals for 4-5 months without significant complications (Kruip et al., 1994; Petyim et al., 2007; López, 2020). In the ovaries of donor animals treated with OPU, connective tissue formation, inflammatory cell infiltration, and the presence of luteal tissue scattered in the stroma may occur (Viana et al. 2003; López 2020).

The use of epidural anesthetic on donor animals is one of the concerns that must be considered in OPU operations to ensure that the follicular aspiration process is carried out in a healthy manner and to prevent the entry of pathogens. Epidural anesthesia is the treatment that causes the most discomfort to donor animals during OPU application (Petyim et al., 2007). Epidural anesthesia reduces the donor animal's disturbing movements and abdominal tension. Repeated OPU applications show that lasting changes may develop in the epidural anesthesia area in cattle. Animal welfare, problems of epidural anesthesia, disruption of the integrity of the ovarian stroma, and adhesions should all be considered in aggressive OPU administration twice a week (McEvoy et al., 2006; Chaubal et al., 2007; Çizmeci, 2022).

Conclusion

As a result, the OPU technique is frequently used to retrieve oocytes in IVP, both in commercial enterprises and embryo transfer research. In comparison to MOET, IVP has become a better commercial choice because it produces more embryos in the same time frame as OPU, permits the use of different elite bull sperm in IVF, and does not require the use of medications. For this reason, veterinarians and researchers intending to use the OPU option should familiarize themselves with the intricacies of this technique and the factors influencing its success. This knowledge ensures a more extensive use of donor animals and the acquisition of higher quality oocytes.

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The effect of adding cod liver oil to the diet on the productivity and blood parameters of broiler chickens

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ABSTRACT

The experiment was performed to evaluate the production performance and blood parameters of broiler chicken fed on cod liver oil enriched feed. A cohort of 96 day-old chickens was allocated at random into four treatment groups, denoted as T0, T1, T2, and T3. Each treatment group consisted of three replicates. In contrast to each replication consisting of eight birds, each treatment group comprises 24 birds. Experimental birds in T1, T2, and T3 were fed rations enriched with cod liver oil at 0.5%, 1%, and 2% by weight, respectively, whereas T0 received only standard feed and was designated as the control group. The findings of this research demonstrated that the final live weight and live weight gain of the birds in group T3 which was provided with feed enriched with 2% cod liver oil was significantly greater ($p<0.05$) than the other treatment groups. According to the findings of this research, the best feed conversion ratio was found in T3 group. With regard to blood parameters, treatment groups differed significantly and it was within the normal range. The highest RBC, WBC, PCV and Hb were found in T3 and lowest in T0 group. The optimum net profit was found in T3. On the basis of the findings, it is possible to conclude that the addition of 2% cod liver oil to broiler chicken feed has the potential to stimulate growth.

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Introduction

The poultry industry in Bangladesh is one of the most prospective and vital industrial sectors for the country's economic growth. Poultry assumes a critical function within the subsistence economy of Bangladesh, contributing 1.6% to the country's Gross Domestic Product (SAEDF, 2008). Rural women and unemployed adolescents can generate income rapidly through broiler farming, according to Hossain et al. (2010).

Despite the abundance of protein sources, meeting the growing demands of the population without broilers is problematic. Protein is derived from the meats of broilers. Farm proprietors also receive a rapid return on their investments. The detrimental impact on health is minimal, and there are no religious prohibitions regarding the consumption of grill meat. As a result, individuals of all ages, genders, and castes enjoy it, which contributes to the proliferation of poultry farms in Bangladesh. The majority of producers in our nation lack formal education and

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inadequate training in grill production. Pharmaceutical companies are taking advantage of this circumstance. They are attempting to persuade the producers to administer medication to the chickens. Huge quantities of chemical agents are consequently found in broiler meat. Antibiotic residues that penetrate the human body through the consumption of these broilers may cause severe health complications in humans (Kibria et al., 2009). Exploiting the potential of particular dietary supplements to improve poultry performance and feed conversion represents a novel challenge in the poultry industry. Presently, there is an increased consumer inclination towards functional foods that are fortified with advantageous natural constituents, to enhance their long-term health goals (Sloan, 2004). Polyunsaturated fatty acids (PUFA), specifically Omega-3 (n-3) fatty acids (FA), are among the numerous vital nutrients that must be included in the daily diet of humans. Among these, PUFA significantly contribute to the prevention of diseases such as cardiovascular, hypertension, diabetes, arthritis, and autoimmune disorders (Adkins & Kelley, 2010). In human nutrition, alpha-linolenic acid and its long-chain metabolites docosahexaenoic and eicosapentaenoic acids are the most essential n-3 fatty acids. Although ALA (α-linolenic acid)) can be used as a precursor in the synthesis of EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), research has shown that this conversion is limited in the human body (Keten, 2019). Therefore, it can be hypothesised that omega-3 fatty acids, which are essential for health protection, can be deposited in the human body through the consumption of chicken meat (Bharath et al., 2017). A commercial diet, both supplemented and unsupplemented, was provided for the animals' 21-day diet, and water was available for their adlibitum consumption. To the commercial diet for supplemented animals, 0.5–0.8 g of omega-3 derived from the cod population was added daily.

As a result, omega-3-enriched poultry products may represent a feasible alternative within the production system. ALA can be converted by grill chickens into docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which are subsequently deposited in the meat. Nevertheless, the conversion efficiency is restricted (Kralik et al., 2008; Zuidhof et al., 2009). Particularly abundant in n-3 long-chain PUFA are EPA and DHA in fish oil (Koreleski and Swiatkiewicz, 2006). The lipid profile of grill meat is enhanced by dietary omega-3 PUFA (Schreiner et al., 2005). Peroxidation is initiated when free radicals attack polyunsaturated fatty acids, which makes them susceptible to oxidation (Scislowski et al., 2005; Estevez, 2015). The byproducts of lipid oxidation contribute to degradation. At present, there is a surge in the attention given to natural antioxidants due to their perceived safety in comparison to synthetic antioxidants and their potential for enhancing the palatability, stability, acceptability, and shelf life of meat products (Park and Kim 2008; Laila et al. 2019).

The utilization of oils in poultry diets offers several benefits, including enhanced hydrolysis and absorption of the lipoproteins that provide fatty acids, as well as a decrease in feed particles (Nobakht et al., 2011). With the maximum caloric content of all dietary nutrients, oils also serve as the primary energy source for the birds. Additionally, they can optimize the utilization of ingested energy, enhance the palatability of diets, and facilitate the absorption of fat-soluble vitamins. Furthermore, by decreasing the rate at which food traverses the gastrointestinal tract, the absorption of all dietary nutrients can be enhanced (Poorghasemi et al., 2013). Cod liver oil is considered a source of significant energy. It has been demonstrated that feed efficacy and growth are enhanced by high-energy diets (Hosseini-Vashan et al., 2010; Sahito et al., 2012).

Hence, the objective of this research endeavour was to ascertain the optimal concentration of cod liver oil-enriched feed meal that would impact growth performance, calculate the cost-benefit analysis of broiler poultry production, and estimate blood parameters.

Material and Methods

Ethical approval: Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur-5200,Bangladesh[Approval code: HSTU/VAS/ASN/ EA/011], gave their approval for the trial.

Location and Period: The trial was carried out from July 2019 to December 2019 at Malek's Poultry Farm, located in Karnai, Dinajpur.

Table 1. The layout of the experiment

Dietary Groups		Number of Broilers in each replication			Total
		R_1	R_2	R_3	
Control (without liver oil)	T_0	8	8	8	24
0.5% Cod liver oil	T_1	8	8	8	24
1% Cod liver oil	T_2	8	8	8	24
2% Cod liver oil	T_3	8	8	8	24
Total No. of broilers		32	32	32	96

Experimental birds: A total of 96 Cobb 500 day-old broiler chicks were bought from CP Limited in Sadar, Dinajpur, Bangladesh.

Layout of the experiment: For seven days, the day-old chicks were raised in a brooder house to acclimate to their environment. Following a period of seven days, the chicks were assigned at random to one control group and three (3) dietary treatment groups, with eight birds per replication (Table 1).

Procurement of feed ingredients: The experimental regimens will be prepared using feed ingredients procured in the necessary quantities from the local market in Dinajpur town (Table 2).

Table 2. Composition of basal diets

Ingredients (%)	Starter (8-16 d)	Grower (17-35 d)
Corn	54.87	61.78
Soybean meal	36.72	26.36
Fishmeal	1.31	4.50
Vegetable oil	3.00	4.00
Limestone	1.15	1.05
Dicalcium phos-phate	1.94	1.49
Vit. and min.premix1	0.50	0.50
Salt	0.30	0.30
DL-methionine	0.21	0.02
Total	100.00	100.00
Calculated chemical composition		
ME(kcal/kg)	2900	3100
CP (%)	21.44	19.37
Calcium (%)	1.05	1.00
Phosphorus (%)	0.16	0.50
Sodium (%)	1.41	0.14
Arginine (%)	1.41	1.23
Methionine+Cystine (%)	0.91	0.69
Lysine (%)	1.20	1.10
Tryptophan (%)	0.31	0.26

Source: Isalm et al., 2017

Collection, processing and storage of Cod liver oil: Dinajpur, Bangladesh, is the location where cod liver oil is purchased locally. The purchased cod liver oil was preserved in a plastic container.

Preparation of the experimental diet: In this investigation, the formulated feed was utilized. Initially, the prescribed quantity of the formulated feed ingredients was determined using a digital weighing balance. Two phases comprised the experimental period: broiler-starter and broiler-grower. The broiler chickens were provided with a broiler starter for a duration of 0 to 10 days, followed by a broiler grower from 11 to 35 days.

Immunization: On the first day, the company vaccinated all birds against Ranikhet Disease and Infectious Bronchitis, which affect newborn chicks. The birds were administered the Ranikhet and Infectious Bursal (Gumboro) vaccines following the subsequent evening schedule (Table 3).

Management of the experimental birds: Individual chick weights were recorded as initial body weights on the first day of the experiment. In the current study, cages measuring 120 cm by 76 cm on each floor of the experimental home were considered. The enclosures underwent thorough cleaning, washing, and bleaching powder disinfection. The room was disinfected with Virkon solution (50 ppm) after 15 days. Concurrently, every essential piece of equipment was meticulously cleansed and laundered. Garbage was encased in fresh newspaper for a duration of seven days, after which the newspaper was removed as it became soiled. Following that time, the birds were confined to a floor strewn with rice husks, which reached a depth of 4 cm. Before the application of refuse, calcium carbonate was dispersed across the floor. The upper portion of the litter containing the droppings was routinely removed after the first week and agitated three times per week until the conclusion of the experiment. Every other day, the refuse was disinfected with Virocid® solution. The litter was agitated at the conclusion of each week in order to disrupt its compaction and supply it with adequate moisture. After the second and third weeks of life, the litter was cleared of droppings. From the initial week of age, until the chickens acclimated to the ambient temperature of the house, the incubation temperature was maintained at 34°C. Subsequently, it was progressively reduced until the final temperature of 23°C was reached at the conclusion of the experiment. An additional source of heat was provided by placing a 100-watt incandescent bulb in the centre of pen, 12 inches above the ground level, away from the 7-day-old chicks. The daily room temperature (°C) was monitored using a thermometer every six hours. Throughout the experiment, each bird was subjected to a daily cycle of nineteenth hours of continuous illumination followed by an hour of darkness. During the initial week, the birds were fed on clean newspapers every three hours for the first three days. Round plastic pitchers and linear feeders were utilized during the brooding phase. The linear feeder was subsequently substituted with the circular plastic

Table 3. Vaccination program

Diseases	Day	Vaccine	Route	Time
Ranikhet	4	BCRDV	Eye	Evening
Gumboro	10	Gumborovac	Eye	Evening
Gumboro	16	Gumborovac	Eye	Evening
Ranikhet	21	ND Lasota	Eye	Evening

tumbler. In the treatment groups, feeds were administered thrice daily (in the morning, at midday, and again at night) and were weighed using a measuring balance.

Mortality, live weight, feed intake, and feed conversion ratio were all monitored for the duration of the 35-day rearing period. In order to estimate the dressing yield, one bird was removed from each replication and blood samples were obtained at the moment of slaughter for hematological parameter analysis.Blood samples from two broilers in each replicate randomly were collected from the wing vein in a test tube with EDTA anticoagulant. Hematological parameters such as RBC, WBC, PCV and Hb were determined by Complete blood count (CBC) test at Update Laboratory in Rangpur, Bangladesh.

Statistical analysis: Analysis of variance (ANOVA) was performed on the data pertaining to various variables within a Completely Randomised Design (CRD) as described by Steel and Torrie (1980). The analysis of variance (ANOVA) table was utilized to determine the significance of the differences among the treatment means. Every analysis was conducted using the "IBM SPSS Statistics 22" software.

Results and Discussion

Effect of cod liver oil on live weight (g) of broilers : According to the data presented in Table 4, cod liver oil influences the live weight of broilers. There was no statistically significant (P>0.05) difference in live weight between the treatment groups on the first and seventh day of the experiment, according to the findings of the present study. By the 7th day of the trial, the body weight of the various dietary treatment groups was nearly identical. The treatment groups exhibited significant differences in live weight (P<0.05) on the 14^{th}, 21^{st}, 28^{th}, and 35^{th} days of age. Upon the conclusion of the experiment, it was observed that T3 (1692.9 ± 7.940) containing 2% cod liver oil mixed with feed had the maximum body weight compared to T2 (1602.9 ± 4.588), T1 (1550.4 ± 22.331), and T0 (1494.9 ± 28.283).

Birds in diet group T0 had the lowest live weight (P<0.05), while birds in diet group T3 had the highest live weight (P<0.05). The growth of dietary group T3 was enhanced through the administration of a 2% mixture of cod liver oil and feed.

A study conducted by Elzobier et al. (2016) observed a significant (P<0.05) rise in the live weight of chickens that were administered a diet containing up to 3% Fish Oil (FO), in comparison to the control group.

Table 4. Effect of cod liver oil on live weight (g) in different dietary treatment groups of broiler

Age (Day)	Live weight (g)				Level of significance
	T_0	T_1	T_2	T_3	
Initial BW	39.2 ± 2.10	38.7 ± 1.10	38.2 ± 1.80	38.9 ± 2.90	NS
7^{th}	187.9 ± 7.978	182.5 ± 1.847	185.3 ± 2.810	196.1 ± 2.862	NS
14^{th}	347.5 ± 17.859^{a}	384.7 ± 19.637^{ab}	437.8 ± 24.260^{b}	453.5 ± 29.266^{b}	*
21^{th}	742.0 ± 9.246^{a}	761.9 ± 10.664^{ab}	771.0 ± 7.725^{b}	798.4 ± 4.101^{c}	*
28^{th}	1117.2 ± 16.803^{a}	1136.6 ± 16.208^{ab}	1187.3 ± 9.429^{b}	1249.7 ± 22.056^{c}	*
35^{th}	1494.9 ± 28.283^{a}	1550.4 ± 22.331^{ab}	1602.9 ± 4.588^{b}	1692.9 ± 7.940^{c}	*

Legends: BW =Body weight, T0= Control diet,T1= Control diet + 0.5% cod liver oil,T2= Control diet + 1% cod liver oil ,T3= Control diet + 2% cod liver oil, ±= Standard error, abc means having different superscript in the same row differed significantly (P<0.05), *= P ≤ 0.05, NS= Non significant

Table 5. Effect of cod liver oil on body weight gain (g) and mortality in different dietary treatment groups of broiler

Age (Day)	Body weight gain (g/day)				Level of significance
	T_0	T_1	T_2	T_3	
Initial BWG	39.2 ± 2.10	38.7 ± 1.10	38.2 ± 1.80	38.9 ± 2.90	NS
7th	148.7 ± 7.97	143.8 ± 1.84	147.1 ± 2.81	157.2 ± 2.86	NS
14th	159.4 ± 10.65^{a}	202.2 ± 20.34^{ab}	252.1 ± 25.98^{b}	257.3 ± 30.59^{b}	*
21st	394.5 ± 20.61	377.2 ± 11.01	333.5 ± 16.78	344.9 ± 32.83	NS
28th	375.2 ± 17.11	374.6 ± 17.83	416.3 ± 17.14	451.3 ± 26.09	NS
35th	377.7 ± 12.49^{a}	413.8 ± 35.17^{b}	415.9 ± 4.80^{b}	443.1 ± 21.77^{c}	*
Final BWG	1455.5 ± 15.40^{a}	1511.7 ± 17.83^{b}	1565.0 ± 18.40^{c}	1653.9 ± 16.92^{d}	*
Mortality (%)	00.0	00.0	00.0	00.0	NS

Legends: BWG =Body weight gain, T0= Control diet, T1= Control diet + 0.5% cod liver oil, T2= Control diet + 1% cod liver oil , T3= Control diet + 2% cod liver oil, ±= Standard error, abcd means having different superscript in the same row differed significantly (P<0.05), *= P ≤ 0.05, NS= Non significant

Table 6. Feed intakes (g) in different dietary treatment groups at different ages of birds

Age (Day)	Feed intakes (g)				Level of significance
	T_0	T_1	T_2	T_3	
7th	167.9 ± 7.96	158.1 ± 2.56	163.3 ± 3.01	178.7 ± 5.28	NS
14th	222.0 ± 14.35^{a}	267.0 ± 24.19^{b}	289.0 ± 29.43^{c}	306.0 ± 43.90^{d}	*
21st	593.3 ± 35.10	523.1 ± 15.41	462.7 ± 25.55	481.8 ± 46.50	NS
28th	610.1 ± 15.80	551.1 ± 30.43	613.6 ± 29.53	607.2 ± 38.88	NS
35th	725.4 ± 54.57b	736.1 ± 60.80c	730.4 ± 2.492bc	635.2 ± 25.45a	*
Total FI	2318.8 ± 127.78^{d}	2235.6 ± 133.39^{b}	2259.1 ± 90.012^{c}	2209.1 ± 160.01^{a}	*

Legends: FI= Feed intake, T0= Control diet, T1= Control diet + 0.5% cod liver oil, T2= Control diet + 1% cod liver oil , T3= Control diet + 2% cod liver oil, ±= Standard error, abcd means having different superscript in the same row differed significantly (P<0.05), *= P ≤ 0.05, NS= Non significant

Effect of cod liver oil on body weight gain: The impact of cod liver oil on broiler body weight gain is illustrated in Table 5. Based on the findings of the current study, it was determined that at seven days of age, the body weight gain of the various dietary treatment groups were nearly identical. At the end of the trial, the body weight gain was significantly (P<0.05) higher in T3 comparison to the other groups. The body weight gain of group T3 was increased by supplementing 2% mixture of cod liver oil in a basal diet.

In line with the results obtained in the current investigation, Lopez-Ferrer et al. (2001) documented a statistically significant (P<0.05) elevation in weight gain among chickens that were administered a 4% Linseed Oil(LO) diet as opposed to the control diet. The authors also documented comparable outcomes when using a diet with the maximum Fish Oil (FO) content (4%) in comparison to the control diet. That is to corroborate the current research findings. In regards to body weight gain, the current research result was further corroborated by Bharath et al. (2017).

Effect of cod liver oil on feed intake: Table 6 demonstrates the impact of cod liver oil on the feed consumption of broiler chickens. At the 35th day of age, the dietary treatment group T3 had the lowest feed intake, whereas the dietary group T0 had the maximum feed intake. Farrell (1995) found that the feed intake of feed mixed with cod liver oil might vary, sometimes being lower and other times higher. In addition, Alparslan and Özdogan (2006) conducted a study which found that the consumption of feed combined with 2% fish oil is greater. The findings of the present study have been validated by Crespo and Esteve-Garcia (2001) as well as Alparslan and Özdogan (2006).

Effect of cod liver oil on feed conversion ratio: Table 7 shows the Feed Conversion Ratio (FCR) of the birds that were tested during the trial. The FCR did not show any significant differences (P>0.05) across the various treatment groups throughout the first 7 days of age. A statistically significant difference (P<0.05) in FCR was seen in the rest of the experimental period. The dietary treatment group T3 exhibited the lowest but best FCR in comparison to other treatment groups. The administration of cod liver oil demonstrated superior FCR when compared to the control group.

The results of this study align with previous research conducted by Crespo and Esteve-Garcia (2001), Farhoomand and Checkaniazer (2009), Newman et al.

Table 7. Feed Conversion Ratio (wt gain/feed intake) of different birds of different dietary treatment groups

Age (Day)	Feed Conversion Ratio (weight gain/feed intake)				Level of significance
	T_0	T_1	T_2	T_3	
7^{th}	1.13 ± 0.01	1.10 ± 0.01	1.11 ± 0.01	1.13 ± 0.02	NS
14^{th}	1.39 ± 0.01^{c}	1.32 ± 0.02^{b}	1.14 ± 0.01^{a}	1.19 ± 0.02^{b}	*
21^{st}	1.49 ± 0.03^{b}	1.38 ± 0.01^{a}	1.38 ± 0.01^{a}	1.39 ± 0.01^{a}	*
28^{th}	1.62 ± 0.01^{b}	1.47 ± 0.01^{a}	1.47 ± 0.01^{a}	1.44 ± 0.00^{a}	*
35^{th}	1.91 ± 0.09^{b}	1.78 ± 0.02^{b}	1.75 ± 0.02^{b}	1.44 ± 0.03^{a}	*
Final FCR	1.59 ± 0.03^{c}	1.47 ± 0.02^{b}	1.44 ± 0.02^{b}	1.34 ± 0.02^{a}	*

Legends: FCR= Feed conversion ratio, T0= Control diet, T1= Control diet + 0.5% cod liver oil, T2= Control diet + 1% cod liver oil , T3= Control diet + 2% cod liver oil, ±= Standard error, abc means having different superscript in the same row differed significantly (P<0.05), *= P ≤ 0.05, NS= Non significant

Table 8. Live weight and carcass weight of broilers of different dietary treatment groups

Parameters	Dietary groups				Level of significance
	T_0	T_1	T_2	T_3	
Live weight (g)	1494.9 ± 28.28^{a}	1550.4 ± 22.33^{ab}	1602.9 ± 4.58^{b}	1692.9 ± 7.94^{c}	*
Carcass weight (g)	1052.6 ± 8.10^{a}	1158.8 ± 18.41^{a}	1150.7 ± 52.58^{a}	1288.6 ± 34.81^{b}	*

Legends: T0= Control diet,T1= Control diet + 0.5% cod liver oil,T2= Control diet + 1% cod liver oil ,T3= Control diet + 2% cod liver oil, ±= Standard error, abc means having different superscript in the same row differed significantly (P<0.05), *= P ≤ 0.05

Table 9. Hematological parameters of broiler

Parameters	Dietary groups				Level of significance
	T_0	T_1	T_2	T_3	
Hb (mg/dl)	9.51 ± 0.176^{a}	10.1 ± 0.278^{ab}	11.1 ± 0.379^{bc}	12.1 ± 0.581^{c}	*
PCV (%)	27.3 ± 0.888^{a}	32.2 ± 0.318^{b}	32.6 ± 0.370^{b}	35.2 ± 0.160^{c}	*
WBC ($1x10^3/mm^3$)	18.5 ± 0.405^{a}	19.9 ± 0.081^{b}	20.6 ± 0.356^{b}	23.9 ± 0.071^{c}	*
RBC ($1x10^6/mm^3$)	4.5 ± 0.289^{a}	5.08 ± 0.068^{ab}	5.8 ± 0.306^{b}	7.3 ± 0.584^{c}	*

Legends: T0= Control diet,T1= Control diet + 0.5% cod liver oil,T2= Control diet + 1% cod liver oil ,T3= Control diet + 2% cod liver oil, ±= Standard error, abc means having different superscript in the same row differed significantly (P<0.05*= P ≤ 0.05

(2002), Alparslan and Ozdogan (2006), and Bharath et al. (2017). These studies also found that adding LO and FO to the diet of broilers improves feed conversion efficiency.

Effect of cod liver oil on carcass weight: The impact of cod liver oil on the weight of the carcass is shown in Table 8. The results indicate that there were significant differences (P<0.05) in live weight (g) and carcass weight (g) among the different nutrition treatment groups. The treatment group T3 had the highest carcass weights than the other treatment groups. Consistent with the results of the current study, Lopez-Ferrer et al. (2001), Chekani-Azar et al. (2007), and Bharath et al. (2017) also found that the inclusion of FO (3-4%) in the diet has a significant (P<0.05) impact on relative carcass metrics.

Effect of cod liver oil on blood profile: The hematological parameters of cod liver oil-treated broilers are detailed in Table 9. In comparison to the control group, all groups supplemented with cod liver oil exhibited significantly (P<0.05) higher levels of hematological parameters (Hemoglobin, Packed Cell Volume, White Blood Cell, and Red Blood Cell). The normal ranges of the haematological parameters in chickens are RBC: 2.5-3.5 $x10^6$ µl, PCV: 22-35 %, Hb: 7-13 g/dl and WBC: 12-30 x 10^3 µl (Bounous & Stedman, 2000). The findings suggest that 2% cod liver oil significantly increases RBCs, WBCs, PCV, and Hb (P<0.05). We concur with the findings of several studies (Bond et al., 1997; Kadhim, 2010; Radwan et al., 2012; Jameel, 2013; Al-Zuhairy and Alasadi, 2013; Jameel and Sahib, 2014) that omega-3 fatty acids significantly increased Hb, WBCs, and RBCs.

Table 10. Data showing the economics of broiler production per bird kept under different treatment groups from day old chick to 35 days of age

Parameters (Tk.)	T_0 control	T_1 0.5% cod liver oil	T_2 1% cod liver oil	T_3 2% cod liver oil
Chick cost (Taka)	17	17	17	17
Average feed consumed (Kg)/chicks/35 days	2.31	2.23	2.25	2.20
Cost of medicine, Vaccine and cod liver oil (Taka)	11	16	22	28
Feed (price/kg)	35	35	35	35
Miscellaneous cost (Taka)	14	14	14	14
Total feed cost/broiler (Taka)	80.8	78.0	78.7	77
Total cost /broiler (Taka)	122.8	125.0	131.7	136
Average live weight (kg/broiler)	1.49	1.55	1.60	1.69
Sale price Tk./kg live wt. (Taka)	120	120	120	120
Sale price /broiler (Taka)	179	186	192	203
Net profit /broiler (Taka)	56.1	60.9	60.2	67

Legends: T0= Control diet, T1= Control diet + 0.5% cod liver oil, T2= Control diet + 1% cod liver oil , T3= Control diet + 2% cod liver oil

Cost benefit analysis: The prices of several bird groups are shown in Table 10. According to the data provided in Table 10, the mean expenses associated with broiler chicken rearing in treatment groups T0, T1, T2, and T3 were 122.85, 125.05 taka, 131.75 taka, and 136 taka, respectively. Based on the results of cost-benefit production the highest net profit was found in the T3 group and the lowest in T0.

Conclusion

The findings of the research indicate that when broilers were supplemented with 2% cod liver oil per kilogram of feed, their body weight, body weight gain, feed intake, feed conversion ratio (FCR), and blood parameters (RBC, WBC, PCV, and Hb) all improved. Additionally, the carcass weight and performance parameters exhibited no detrimental effects. As a result, 2% cod liver oil can be utilized as a growth promoter in the diet of broilers.

Authors' contributions

MAH, MNA, US, MB, and SHS conceived and designed the experiment. MB, MAH, and MNA performed the study, MB conducted lab analyss. MAH and MNA supervised and coordinated the experiments. MB performed statistical analyses of the experiental data. MB and MAH prepared the draft of the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript and also approved the final version.

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Production techniques and product characteristics of “kaymak” produced in Türkiye

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ABSTRACT

With its unique creamy structure, delicious consistency, slightly acidic taste and aroma, “kaymak” is a traditional dairy product that is consumed with honey and jam for breakfast, in the production of Turkish delight with cream and cream candy, or in Turkish desserts such as kadayif and baklava because of its many features that increase the ornamentation, flavor and nutritional value. In Türkiye, two types of “kaymak” are produced as “Afyon Kaymağı” and “Lüle Kaymağı”, especially in the Aegean region (Afyonkarahisar and its surroundings) and Central Anatolia, as well as in Ankara, Bursa, Edirne, Erzurum, Istanbul, Izmir, Kilis and Kocaeli provinces. It is also produced from buffalo milk in countries such as the Balkans, Middle East, Central Asia, Iran, Afghanistan and India. It is described by names such as “kajmak”, “kaimak”, “gemagh” or “geymar”. The fact that the “kaymak” produced by the traditional method has a very open production process to microbial contamination, is a product that does not undergo fermentation, has a high water content and is rich in usable nutrients, increases the importance of the risk of contamination after the pasteurization process. It is evaluated that “kaymak” can provide an extremely favorable environment for the development of pathogenic microorganisms that *Escherichia coli*, *E. coli* O157:H7, coliform bacteria, *Listeria* spp., *Listeria monocytogenes*, *Pseudomonas* spp., *Salmonella* spp., *Salmonella-Shigella, Staphylococcus, Staphylococcus aureus*, total aerobic mesophyll bacteria, total aerobic psychrophilic bacteria, yeast and mould, which cause foodborne infections and food intoxications in humans and this situation may pose a risk for public health.

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Introduction

Kaymak is an important dairy product in Türkiye, which is generally produced in small family businesses as well as in many enterprises, from small dairies to large modern facilities (Kurt and Özdemir, 1988; Hasdoğan, 2004). Cream-like dairy products are also produced in some European countries. There is a local type of cream called "clotted cream", produced especially in England. This traditional product is yellowish in color and has a granular structure, and is generally consumed by spreading it on products such as pastries, buns, or on fruits (Early, 1991).

According to the Turkish Food Codex Communique on Cream and Turkish Cream, Kaymak is defined as cream containing at least 60% milk fat by weight and also "Afyon Kaymağı" is described as the product obtained by boiling buffalo milk in accordance with the technique, keeping it at 92°C for at least 2 minutes and cooling it in accordance with the technique (TFC,

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2003). However, in recent years, since the number of buffaloes has been far below the desired level and sufficient amounts of buffalo milk cannot be produced, Kaymak produced from cow's milk or its mixtures is also produced and sold under the same name (Şenel, 2011). It is stated that the Kaymak must have a unique taste, smell and structure and must not contain any visible microorganism colonies. Kaymak to be offered for direct consumption must be put on the market after undergoing at least pasteurization or a heat treatment equivalent to pasteurization (TFC, 2003). In the Turkish Standards Institution (TSI) Cream and Kaymak Standard (TS 1864); it is stated that these oil-based products must have their own unique colour, taste, odor and consistency, and if they are to be offered for direct consumption, they must be put on sale after heat treatment at the lowest pasteurization norm (15-20 seconds at 72°C) (TSI, 2008).

Kaymak; being a dairy product rich in fat, it can easily spoil due to reasons such as not following adequate hygiene and sanitation rules in its production, not being able to prevent cross-contamination, and not paying enough attention to packaging and storage conditions (Bilir-Ormancı, 2020). For this reason, Kaymak and similar ready-to-eat dairy products; if it is to be consumed later, it must be cooled quickly and in a short time using other appropriate processes up to 5°C to kill bacterial cells or stop proliferation, and it should be known that heat treatments to be carried out after toxin formation will not be effective in preventing food-borne intoxications (Cretenet et al., 2011; Schelin et al., 2011; Ray and Bhunia, 2016). As a matter of fact, staphylococcal intoxications and related cases of staphylococcal gastroenteritis are frequently observed due to not paying due attention to Kaymak and similar protein-rich and ready-to-consume foods, especially during the production, preservation and marketing stages (Özcan-Yılsay and Akpınar-Bayizit, 2002; Erol and İşeri, 2004; Erol, 2007; Schelin et al., 2011; Ray and Bhunia, 2016).
In this study; it is aimed to reveal the importance of Kaymak, which has the potential to pose risks in terms of both consumer health and product quality, in terms of public health, with its microbiological, chemical and sensory properties that determine the quality of Kaymak, which is generally produced, sold and marketed as a traditional dairy product in small family businesses in our country.

The importance of kaymak in nutrition

Milk and dairy products are rich in phosphorus and calcium, important amino acids (lysine) that cannot be synthesized by the body and that the body needs to perform its normal functions, high quality protein, fat, carbohydrates, sugar, minerals, riboflavin and vitamin B12 (Galvano et al., 1998; Tekinşen, 2000; Tekinşen and Nizamlıoğlu, 2004; Fagundes et al., 2011; Üçüncü, 2018).

Kaymak has a very important place in nutritional physiology thanks to its unique sensory properties derived from milk fat (Metin, 2005; Tekinşen, 2005). Because milk fat contains very high levels of essential unsaturated fatty acids such as medium-chain fatty acids linoleic, linolenic and arachidonic acids, and fat-soluble vitamins (vitamins A, D, E and K) (Tekinşen and Nizamlıoğlu, 2004; Bilir-Ormancı, 2020). Milk fat plays a major role in the physical properties, taste, aroma and nutritional value of Kaymak, as in milk and other dairy products (Tekinşen, 2005; Bilir-Ormancı, 2020). Kaymak is also a good source of energy and provides 5.90-6.13 g-1 calories of energy (Tekinşen, 2005).

Recent studies have reported that conjugated linoleic acid (CLA) contained in milk fat has beneficial qualities for human health (Seçkin et al., 2005). In addition, milk fat is easily digested due to the short and medium chain fatty acids in its structure (Tekinşen, 2000). In a study conducted by Akalın et al. (2005), the amount of CLA was found to be higher in animal products compared to plant products, and it was reported that the level of CLA in the tissues of ruminant animals was found to be higher among animal products. Therefore, dairy products such as milk and milk fat-based Kaymak, butter and cheese are considered the most basic source of CLA. Kaymak, along with foods such as butter and cheese with high milk fat content, are among the preferred dairy products because they are rich dietary sources of CLA intake (Akalın et al., 2005).

Formation of the kaymak layer

The Kaymak binding power of milk depends on the formation of a layer consisting mostly of fat on the surface of the milk left alone after a certain period of time (Bilir-Ormancı, 2020). Although the specific gravity of milk fat in the form of globules is 0.931 g/ml, the specific gravity of the plasma part of milk is 1.034 g/ml. The fat part with lower specific gravity gradually accumulates on the surface of the milk as the fat globules rise. Fat globules partially combine to form large masses. The layer collected on the surface of the milk gradually becomes richer in fat, thus forming a Kaymak layer (İnal, 1990; Bilir-Ormancı, 2020).

The quality of the milk used in the formation of the Kaymak layer is one of the most important factors. The high fat content of milk also increases the amount of Kaymak obtained. In addition, the taste, aroma and color of the resulting product vary depending on the type of animal from which the milk is obtained. Buffalo

milk is richer in fat-soluble vitamins than cow's milk and also contains high amounts of the glycoprotein lactoferrin. However, since buffaloes convert all of the β-carotene they take in feed into vitamin A, the color of the Kaymak obtained from buffalo milk is white (Bilir-Ormancı, 2020). Due to its high Kaymak binding ability, the Kaymak produced from buffalo milk is thick, viscous and white in color, while the Kaymak produced from cow milk is thin and yellowish in color (Çon et al.,2000; Bilir-Ormancı, 2020).

Kaymak production technology

When the historical background of Kaymak making is examined, the first technological initiative took place in 1864 and the milk was separated from the Kaymak by rotating the milk placed in the containers. However, due to the low capacity of the first technological machines, it was reported that the Kaymak obtained was not of the desired quality. It is noted that Ledfeld, who discovered the hood centrifuge in 1877, managed to separate the milk Kaymak from the milk in a short time and completely, and later, with the advancement of technology, today's machines were developed by making some changes to this first discovered device (such as adding a disc device) (İnal, 1990).

"**Afyon Kaymağı" production with traditional method**

Kaymak production with the traditional method is generally carried out in small family businesses in Afyon, where modern tools and equipment are not available, with production lasting approximately 36 hours (Eralp, 1969; Hamzaçebi, 1973; Korkmaz, 1990; Yılmaz, 1998). "Afyon Kaymağı" is a geographically indicated dairy product (TURKISH PATENT, 2009). In the production of this Kaymak, respectively; preparation of milk, filtering of milk, thermal treatments applied to milk (first heating process, cooling and resting, second heating process, cooling and resting), cutting and packaging of Kaymak and final product storage processes are applied (Tekinşen, 2005).

"**Lüle Kaymağı" production with traditional method**

Although there are processes similar to the production of "Afyon Kaymağı" in the production of "Lüle Kaymağı" with the traditional method, there are some differences. In our country, "Lüle Kaymağı" production is mostly done in large cities such as Istanbul, Ankara, Izmir and Bursa (Erkmen and İzmen, 1942; İzmen and Eralp, 1967; Yöney, 1970). In the production of this Kaymak, respectively; preparation of milk, thermal treatments applied to milk (first heating process, cooling and resting, second heating process, cooling and resting), binding and packaging of Kaymak and storage of Kaymak are applied (Tekinşen, 2005).

Kaymak production with industrial method

It is a method applied using separators, based on the principle of separating milk fat by centrifugal force. In this method, the milk is heated to 60°C and the cream part is separated with a separator. After the cream obtained following the separation process is standardized to contain 60% fat, heat treatment is applied at 90-95°C for 3-5 minutes. After the heat treatment, it is cooled to 25-30°C. After the cream is filled into suitable containers for sale, it is kept at 4-6° C for 12 hours and rested (Bilir-Ormancı, 2020).

Features that determine quality in kaymak

Microbiological properties of kaymak: Kaymak differs from milk fat-based butter and other local products known as ghee, sanma, meshho, samin and samuli in terms of its composition and shelf life. Kaymak has a higher moisture content and lower milk fat content (Eralp, 1969; Bilir-Ormancı, 2020). For this reason, Kaymak creates an extremely favorable environment for the development of pathogenic microorganisms due to its high water content and rich content of usable nutrients. Additionally, the fermentation process is not used in Kaymak production. These differences affect the shelf life of Kaymak (Bilir-Ormancı, 2020). Although the shelf life of other products is 6-8 months, the shelf life of Kaymak is at least 3-4 days in summer and 6-7 days in winter at 4°C, since it is a product that does not undergo fermentation (Tekinşen, 2005; Bilir-Ormancı, 2020).

According to the Turkish Food Codex Communique on Cream and Turkish Cream; Kaymak that will be offered for direct consumption must be put on the market after undergoing at least pasteurization or a heat treatment equivalent to pasteurization (TFC, 2003). The possibility of Kaymak being exposed to microbial contamination after pasteurization is considered to be one of the factors that shorten its shelf life (Bilir-Ormancı, 2020).

One of the effective factors in controlling microbial growth and extending shelf life is storage temperature. In order to preserve its quality, it is recommended to store Kaymak at temperatures close to 0°C. If the storage temperature exceeds 6°C, bacteria develop faster, quality values in the product decrease and the rate of deterioration increases (Bilir-Ormancı, 2020). The microbiological criteria specified for Kaymak according to the Turkish Food Codex Regulation on Microbiological Criteria are shown in Table 1 (TFC, 2011).

Although the most important factor determining the quality and storage time of Kaymak is its microbiological properties, there are very few studies

Table 1. Microbiological criteria specified for Kaymak (TFC, 2011).

Microorganisms	Sampling plan (1)		Limits (2)	
	n	c	m	M
Coagulase positive *Staphylococci*	5	2	10^2	10^3
Salmonella spp.	5	0	0/25 g-mL	
L. monocytogenes	5	0	0/25 g-mL	

(1) n: Number of samples, c: Number of samples allowed to have values between m and M limit, (2) Unless stated otherwise, the limit is evaluated as cfu/g-mL. cfu: Colony forming unit (in solid medium)

Table 2. Microbiological properties of Kaymak determined in some studies (Pamuk, 2017).

Microorganisms	Hamzaçebi, 1973	Kurt and Özdemir, 1988	Çon et al., 2000	Öksüz et al., 2000	Özcan-Yılsay and Akpınar-Bayizit, 2002	Akalın et al., 2006
Coliform (log_{10} cfu/g^{-1})	0.70-7.97	1.48-3.34	1.30-5.90	2.69-3.90	-	-
Yeast-Mould (log_{10} cfu/g^{-1})	0-5.85	2.23-4.26	2.30-4.98	2.77-4.40	2.11-6.20	3.88-7.53
Total Aerobic Mesophilic Bacteria (log_{10} cfu/g^{-1})	3.78-10.48	3.68-6.52	3.51-7.77	3.23-4.74	2.71-6.35	-
Staphylococcus aureus (log_{10} cfu/g^{-1})	-	0-3.20	0.60-4.20	1.00-2.92	0.00-5.44	0-6.86
Coliform bacteria (log_{10} cfu/g^{-1})	-	-	-	2.69-3.90	-	0-3.38
Salmonella-Shigella (log_{10} cfu/g^{-1})	-	-	-	-	0.00-4.25	-

on this product (Özcan-Yılsay and Akpınar-Bayizit, 2002). The results obtained from the studies conducted to determine the microbiological properties of Kaymak are summarized in Table 2 (Pamuk, 2017).

In addition, studies have shown that Kaymak contains or has the potential to develop pathogenic microorganisms such as *Clostridium perfringens*, *Escherichia coli*, *E. coli* O157:H7, lactic acid bacteria, *Listeria* spp., *Listeria monocytogenes*, *Pseudomonas* spp., *Salmonella* spp., *Staphylococcus* and total aerobic psychrophilic bacteria (TAPB) that cause foodborne infections and food intoxications in humans (Akalın et al., 2006; Öncü, 2012; İpekçioğlu and Gürler, 2017; Tomar and Akarca, 2018).

Chemical properties of kaymak

Kaymak is very rich in milk fat and contains 56-69% fat in its composition. It also contains some of the protein, lactose and mineral substances in milk (Yılmaz, 1998; Öksüz et al., 2000). The chemical composition of milk used in Kaymak production may vary depending on animal species (Tekinşen, 2005). The chemical composition of milk according to animal species is shown in Table 3 (Tekinşen et al., 1997).

The results obtained from the studies carried out in Türkiye to determine the chemical composition of Kaymak are summarized in Table 4 (Öncü, 2012).

Table 3. Chemical composition of milk according to animal species (%) (Tekinşen et al., 1997).

Animal Species	Water	Protein	Fat	Mineral Substance
Buffalo	82.2	4.2	7.90	0.8
Cow	87.5	3.3	3.60	0.9
Sheep	81.6	5.2	7.50	0.9
Goat	87.0	3.6	4.2	0.9

As a result of the studies, the low dry matter and fat rates detected are attributed to the use of cow's milk during the Kaymak making process. In some of the studies, the amount of protein contained in Kaymak was also examined and it was reported that the differences in protein values depended on the dry matter. The detection of different acidity values is associated with the fact that the studies were carried out in different seasons (İzmen and Eralp, 1967; Hamzaçebi, 1973).

In recent years, there have been different studies conducted to determine the chemical properties of Kaymak samples (Kocaoğlu, 2009; Anlı and Gürsel, 2013; Albay and Şimşek, 2019; Kocatürk et al., 2019; Özbek et al., 2021).

Table 4. Average chemical composition of various types of Kaymak in Türkiye (%) (Öncü, 2012).

Sample		Dry Matter	Fat	Protein	Ash	Acidity (l.a.)	Reference
Type	Number						
Lüle Kaymağı	23	68.61	65.0	2.40	0.29	0.29	Adam, 1955
Lüle Kaymağı	42	68.57	63.4	3.58	0.45	0.20	İzmen and Eralp, 1967
Lüle Kaymağı	10	68.32	62.6	4.03	0.44	0.18	Eralp,1967
Lüle Kaymağı	25	-	65.0	2.40	-	0.29	Eralp,1969
Afyon Kaymağı	250	69.21	62.7	-	-	0.17	Hamzaçebi, 1973
Kaymak	10	55.02	29.1	8.40	2.13	0.44	Kurt and Özdemir, 1988
Afyon Kaymağı	4	66.19	60.0	-	-	0.13	Yılmaz, 1998
Kaymak	21	67.40	62.7	3.36	-	0.34	Öksüz et al., 2000
Afyon Kaymağı*	1	63.04	58.0	-	-	0.14	Çon et al., 2000

* : First day on sale, l.a.: Titratable acidity, as lactic acid.

Sensory properties of kaymak

The composition and qualities of the milk used in Kaymak production and the processes applied directly affect the sensory qualities of the final product by causing differences in its color and structure. For example; the flavor (taste and smell) and aroma of the Kaymak made from buffalo milk in accordance with the technique is unique, distinct and pleasant. However, its consistency is medium dark and its structure is granular (grained), white in color and thick, while the Kaymak produced by adding cow's milk has a yellowish color and non-homogeneous appearance, thin and brittle structure (Tekinşen, 2005; Bilir-Ormancı, 2020).

Exposing milk to high heat treatment during production also causes the product to develop a cooked taste. In addition, cooling the Kaymak quickly causes it to have a fine structure, and keeping it in the cold for a long time causes it to have a granular structure (Bilir-Ormancı, 2020). It is reported that the sticky, sandy and fluid structure, which are among the defects of Kaymak, may be related to the fatty acid composition of the cream (deterioration of the emulsion stability of the cream) and the feed fed to the dairy animal (for example; the relationship with alfalfa) (Bodyfelt, 1988). There should be no loose cream residue in the Kaymak package (Tekinşen, 2005).

As a result of the studies, it was found that in the Lüle Kaymağı samples stored at low temperatures (3-5°C), the acidity level increased from the third day onwards, the increase continued at temperatures above 0°C, and the acidity remained constant at temperatures between -5°C and 0°C. It was determined that in the Kaymak samples, a hard structure was formed at the degrees where the acidity remained constant, and fractures and cracks occurred on the surface. As a result, considering that the sensory and visual qualities of Lüle Kaymağı do not change, it was stated that a storage temperature of 3-5°C and consumption within 1-2 days after its production would be appropriate (Eralp, 1969).

Studies on kaymak

The diversity among the findings detected in some studies where Kaymak samples were examined; it is thought that this may be due to differences in the technology used in the production of Kaymak, the number of samples, the analyzes applied, the isolation methods, the isolate obtained, the time of collection of the samples and the geographical features (Muratoğlu, 2010; Tomar and Akarca, 2018).

Conclusion

Kaymak produced with traditional and unhygienic methods is exposed to microbial contamination during production, packaging and storage because the milk boilers, cream pans, cheesecloth, plastic containers and other equipment used in production are not cleaned or disinfected at the desired level. This situation causes microorganisms to multiply on the

equipment surfaces and causes the Kaymak to deteriorate or undesirable defects to appear during processing. Similarly, process tanks and all equipment within the facility to be used in Kaymak processes carried out in large capacity facilities should be placed in a way that ensures sanitation, maintenance and control in accordance with hygienic design principles, should be made of stainless steel and should be capable of preventing the development of microorganisms. Effective hygiene, sanitation and disinfection should be applied to the equipment used for Kaymak production after use. The shelf life of Kaymak is also shortened due to negative effects such as rancidity, mould, bitterness and putridity, which occur as a result of Kaymak being exposed to microbial contamination during production, packaging and storage. In addition, economic losses occur in milk and dairy products processing facilities that produce Kaymak. Studies have shown that Kaymak can provide an extremely favorable environment for the development of pathogenic microorganisms such as; "*Clostridium perfringens*, *Escherichia coli*, *E. coli* O157:H7, coliform bacteria, lactic acid bacteria, Listeria spp., *Listeria monocytogenes*, *Pseudomonas* spp., Salmonella spp., Salmonella-Shigella, *Staphylococcus*, *Staphylococcus aureus*, total aerobic mesophyll bacteria (TAMB), total aerobic psychrophilic bacteria (TAPB), yeast and mould" that cause foodborne infections and food intoxications in humans.

In order to protect public health; production and sales places must be inspected at appropriate intervals by official institutions, and manufacturing enterprises must take all measures to reduce the microbial load on products and fulfill the legal procedures and requirements. All personnel working in businesses that produce and sell food must receive training on hygiene and sanitation, apply hygiene rules, and effectively apply the HACCP (Hazard Analysis Critical Control Points) system with a proactive approach, especially in production activities.

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The relationship between dietary fiber, microbiota and kidney diseases in cats and dogs

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ABSTRACT

Chronic kidney disease (CKD), which is an increasingly common disease in humans and a global health problem, is also a very common disease in cats and dogs. CKD can be caused by primary glomerulopathies, nephroliths, renal dysplasia, polycystic kidney disease, pyelonephritis, renal carcinomas, nephrotoxic drugs and toxins. The fact that cats diagnosed with CKD and with shorter survival time have low or excess body weights suggests that there may be strong correlations between diet and CKD. In recent years, effects of nutrition on microbiota changes and the role of these changes in diseases have taken particular interest in veterinary medicine. This review article focuses on the curative role of dietary fiber intake, which targets the intestinal microbiota and aims to reverse dysbiotic factors in cats and dogs with chronic kidney disease.

Keywords: dietary fiber, prebiotics, renal diseases, microbiome

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Introduction

The complex relationship between nutrition and the development and prevention of various diseases in cats and dogs is one of the most popular topics in recent years. There is a rising incidence of kidney disease (CKD) in humans and is also quite common in cats and dogs. In a study conducted in the United Kingdom showed that chronic kidney disease was the most common cause of death in cats over 5 years of age, with a rate of 13.6% (O'Neill et al., 2015), and the prevalence of CKD in dogs was reported to vary between 0.5-7% (Lund et al., 1999).

Low and excess body weights were associated with shorter survival rate in cats diagnosed with CKD (Freeman et al., 2016). This correlation suggests that there might be strong connections between diet, and CKD management. On the other hand, the complex relationships between the microbiome and the metabolic and immune systems also play a direct role in the pathogenesis of diseases (Hoffmann et al., 2015). Dietary fibers are partially fermented parts of plants that cannot be digested hydrolytically, and dietary fiber cause effective changes in the microbiota and may indirectly affect morbidity and mortality (Kwon et al., 2022). Although the role of microbiota changes in the pathogenesis, treatment and prevention of diseases in pet medicine is not well understood and many studies today focus on this area. CKD causes significant quantitative and qualitative changes in the intestinal microbiota (Vaziri et al., 2012). Since uremia is one of the most serious complications of kidney diseases, increased urea flow into the intestines causes an increase in lumen pH and pathogenic bacteria, resulting in complications such as the formation of uremic toxins, translocation of endotoxins and the formation of secondary diseases. Uremic toxins hence are used as clinical biomarkers in CKD.

Kidneys are essential for homeostasis and kidney diseases affect many body systems. Kidneys are responsible not only for waste management and acid-

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base regulation, but also for many metabolic functions such as regulation of blood pressure (renin-aldosterone) and endocrine functions (erythropoietin and vitamin D). These interactions make kidneys more susceptible to dietary effects and they are significantly affected by eating habits. Chronic kidney disease is generally defined as a structural and functional disorder in one or both kidneys that lasts longer than 3 months (Bartges, 2012). This definition is similar in both humans and cats and dogs. Since chronic kidney disease is generally common in older animals, it is difficult to establish the differential pathology of mortality. The staging of CKD is described by IRIS (International Renal Interest Society), where stage 1 defines non-azotemic disease and stage 4 defines severe end-stage renal azotemia. Main causes of CKD are primary glomerulopathies, nephroliths, renal dysplasia, polycystic kidney disease, pyelonephritis, renal carcinomas, nephrotoxic drugs and toxins (Chacar et al., 2020). While the primary factor is often unknown, continuous damage to the kidney and infiltration of inflammatory cells creates irreversible inflammation and initiates renal fibrogenesis (Reynolds and Lefebvre, 2013). It has been reported that the source of inflammation in CKD might be the gastrointestinal system (Lau et al., 2015). Renal fibrosis is the most common pathological process in CKD, and so far no drug has been proven to cure fibrosis (Kawabata et al., 2023). CKD is a state of progressive loss, that is, it is characterized by a continuous decrease in kidney functions (Chen et al., 2020).

Plasma/serum creatinine (Scr) and blood urea nitrogen (BUN) concentrations are routinely used to estimate glomerular filtration rate (GFR) or to estimate and diagnose acute-chronic kidney diseases in veterinary clinics. GFR, known as a sensitive indicator of functional kidney, is defined as the volume of ultrafiltrate formed in the nephrons of both kidneys per unit of time (De Loor et al., 2013; Kerl and Cook, 2005). Decrease in glomerular filtration rate and tubular secretion leads to accumulation of nitrogenous waste and indirectly to uremia. The decrease in urea excretion from the kidneys causes an increase in the secretion of urea into the gastrointestinal tract, which is converted into ammonia by urease-produced bacteria (Carvalho et al., 2021). Many metabolic toxins accumulate in kidney diseases, and the most well-known toxins are Scr and BUN among 146 uremic toxins. Scr and BUN levels are affected by hydration and muscle mass. CKD can be detected earlier in cats and dogs with the increase in Symmetric Dimethylarginine (SDMA) levels than with the increase in Scr, thus provide opportunities treating animals when they have adequate nutrition. Diagnosis of the disease in advanced stages also limits survival time.

The gut microbiota is extremely diverse and consists of bacteria, archaea, viruses and eukaryotic organisms. The combined genetic potential of this endogenous flora is called the microbiome (Nicholson et al., 2012). Although bacteria are the most abundant group, it is very difficult to identify all bacteria with current molecular methods. The bacterial population is mostly concentrated in the large intestine. It is estimated that the microbial load in the intestine varies between 1012 and 1014, approximately 10 times higher than the host cells (Suchodolski, 2011). It was reported that the duodenum of cats contains higher numbers of bacteria than that of dogs, with anaerobic bacteria predominating (Johnston et al., 1993).

In recent years, the definition of gastrointestinal functionality has been encountered. Celi et al. (2017) defined gastrointestinal functionality as a stable state in which the microbiome and the intestinal tract are in symbiotic balance and the animal welfare and performance are not restricted due to intestinal dysfunction. The main components of this definition are gastrointestinal barrier and microbiota. According to Souza et al. (2023) higher α-diversity value and higher number of Faecalibacterium, Turicibacter, Blautia and Fusobacterium in dogs are biomarkers of gastrointestinal functionality. The dominant phyla in dogs and cats are Firmicutes (including Clostridia and Bacilli), Fusobacteria, and Bacteroides (Suchodolski, 2022). These are followed by the Proteobacteria and Actinobacteria phyla (Handl et al., 2011; Middelbos et al., 2010). However, the microbiome is dynamic and could change throughout lifetime in response to the environment, diet, stress, age, diseases and medication use. Additinoally, fecal bacterial profile analyses showed that each dog had a unique and relatively stable bacterial profile (Simpson et al., 2002). The disadvantage of fecal microbiome analyzes is that it is unclear what extent they reflect the microbiomes of the intestinal regions. Today, molecular methods targeting the 16S rRNA gene are accepted standard for identifying bacterial microbiota. The diversity of the microbiome is also associated with the host health, and loss of diversity is associated with diseases.

Changes in the diversity or composition of the intestinal microbiota are defined as dysbiosis. Cats suffering from CKD are known to have dysbiosis (Summers et al., 2019). It is currently thought to be only a symptom of diseases and actively contribute to pathologies, since dysbiosis has not yet been proven to be the cause of any disease. While dysbiosis exacerbates inflammation in susceptible individuals, normobiosis restoration is the desired result

(Suchodolski, 2016). Dysbiosis does not always involve pathogens because the absence of important commensals can also be detrimental in the absence of pathogens.

Chronic diseases are closely related to dysbiosis. Recently, many studies have been conducted on whether dysbiosis contributes to the progression of CKD. Wilkins et al. (2019) explained that the most common genera in kidney diseases are Bacteroides, Corynebacterium, Anaerococcus, Prevotella, Rothia, Sutterella, Eubacterium, Fusobacterium, Leptotrichia, Parabacteroides, Peptoniphilus, Porphyromonas, and Veillonella. Hanges in microbiota profile/diversity are closely related to uremic toxin formation levels (Hasegawa et al., 2017). In CKD, gastrointestinal tract environment modifiers such as urea, uric acid, trimethylamine-N-oxide (TMAO), indole, p-crysol, as well as dietary restrictions, slow colonic transit, drugs such as iron-containing compounds, phosphate binders and antibiotics are considered as causes of dysbiosis. (Jazani et al., 2019; Mafra and Fouque, 2015). The overgrowth of bacteria producing urease, indole and crysol-forming enzymes as a result of the flow of urea and other toxins into the gastrointestinal lumen increases the lumen pH and causes the microbiota composition to change. Protein fermentation leads to the formation of uremic toxins that lead to the progression of CKD. Indoxyl sulfate, derived from tryptophan metabolism by the intestinal flora, and p-cresol, produced from the partial breakdown of tyrosine and phenylalanine, are among the most studied uremic toxins (Summers et al., 2019; Wu et al., 2011). Since 90% of the indoxyl sulfate accumulated in the blood is bound to albumin of uremic patients, the main excretion is done through tubular secretion, and accumulation occurs as a result of decreasing urinary excretion as CKD progresses (Hasegawa et al., 2017; Niwa, 2010). Both indoxyl sulfate and p-cresol cause oxidative stress by inducing the production of reactive oxygen species and cause progression of nephrotoxicity (renal fibrosis, glomerular sclerosis) by functioning as nephro-vascular toxins (Dou et al., 2007; Enomoto et al., 2002; Niwa, 2010). It has also been shown that the increase in p-cresol occurs during uremia may contribute to immunosuppression in dogs with CKD by changing neutrophil function in dogs (Bosco et al., 2016). Indoleacetate and TMAO levels are also useful in the diagnosis and staging of CKD in cats (Nealon and Winston, 2023).

It was shown that fermentable fibers or oligosaccharides caused a decrease in blood urea levels (Younes et al., 1995). Furuse et al. (2014) found that addition of galactooligosaccharide (GOS) to the diet of nephrectomized rats significantly reduced cecal indole and serum indoxyl sulfate concentrations and ameliorated tubulointerstitial damage. The decrease in cecal indole level was explained by an increase in indole-negative bacteria (e.g., most species of Bifidobacteriaceae) and a decrease in indole-positive bacteria (e.g., some species of Clostridiaceae). Wang et al. (2020) reported that Eggerthella lenta and Fusobacterium nucleatum increased uremic toxin production in rats with CKD and contributed to the progress of kidney disease, and that probiotic intervention with Bifidobacterium animalis reduced the severity of CKD by decreasing both these species and toxin production (Wang et al., 2020). Souza et al. (2023) in their very recent study, it was reported that cassava fiber produced lower indole, phenol and p-cresol in dogs compared to a control diet containing no fiber. Kieffer et al. (2016) fed rats with CKD high levels of fermentable dietary fiber and they observed a decrease in cecal pH and serum/urine indoxyl sulfate, p-cresol levels and an increase in the Bacteriodetes-Firmucutes ratio. A study in humans also reported a decrease in p-cresol levels after consumption of yoghurt containing Lactobacillus and Bifidobacterium animalis species (Stuivenberg et al., 2023). Hall et al. (2020) showed that the diet containing soluble fiber (scFos) led to lower phenolic uremic toxin production compared to the diet containing insoluble (apple puree) fiber when consume similar total fiber ratios but a two fold difference in soluble fiber to CKD stage 1 and stage 2 cats. Ephraim and Jewell (2020) compared feeding high and low soluble fiber diets in dogs with CKD over a 10-week period, they showed that the high soluble fiber diet was more successful in reducing uremic toxins.

Inflammation is a mediator and common feature of the progression of chronic kidney disease and its complications (Vaziri et al., 2012). The main trigger of inflammation is the gastrointestinal system (Lau et al., 2015). Vaziri et al. (2012) showed that tight junctions were disrupted as a result of serious decreases in occludin, claudin-1 and ZO-1 protein expressions in the colonic mucosa of nephrectomized rats, and they also proved that that condition contributed to systemic inflammation with progression in CKD. Enterocytes and paneth cells regulate luminal bacteria by producing mucus and antibacterial factors in the intestine (Pelaseyed et al., 2014). When deprived of fiber, commensal bacteria damaged the intestinal protective mucus layer, paving the way for pathogen invasion (Desai et al., 2016). This situation also explains conditions such as increased intestinal permeability, systemic inflammation and microbial flora-induced endotoxemia associated with the uremic

result of high permeability, translocation of bacteria, bacterial products and toxins, a different blood microbiome profile is observed in patients with CKD compared to healthy individuals. Shah et al. (2019) performed a quantitative analysis of the blood microbiome in CKD patients for the first time and found a decrease in α diversity and an increase in the Proteobacteria phylum, Gammaproteobacteria class, Enterobacteriaceae and Pseudomonadaceae families, although β diversity was similar compared to healthy controls. Additionally, this study showed that progression in CKD or decline in GFR rate also leads to increases in the Proteobacteria phylum.

Dietary Fiber and CKD

Dietary fiber, first defined by Hipsley in 1953, is defined by AAFCO (Association of American Feed Control Officials) as the partially fermented components of feedstuffs that cannot be digested hydrolytically (Fahey et al., 2019; Hipsley, 1953). All currently known prebiotics are carbohydrates, and many different carbohydrate derivatives are marketed as prebiotics in worldwide (Ranganathan, 2018). While all prebiotics can be classified as fiber, not all fibers are prebiotic and prebiotic fibers provide effective changes in the microbiome (Brownawell et al., 2012). Short-chain fatty acids (SCFA) are formed as a result of the fermentation of dietary fibers by the microbiota. The most abundant of these carboxylic acids, which contain one to six carbon atoms, is acetate, followed by propionate and butyrate (Wong et al., 2006). Alexander et al. (2018). Compared the dietary intake of a non-prebiotic fiber product (cellulose) and a prebiotic (inulin) product and found that the latter fiber product caused higher increases in fecal short-chain fatty acids (SCFA), an increase in Firmicutes and a decrease in Proteobacters. Dietary fiber supplementation can affect bacterial populations by providing additional energy in the colon, and generally Lactobacillus and Bifidobacterium and butyrate-producing bacterial groups increase (Kafeshani, 2017; Simpson et al., 2002). Most fibers act by enriching fiber-fermenting SCFA-producing Firmicutes (Pilla and Suchodolski, 2021). Enterocytes and colonocytes provide 60-70% of their energy from SCFA oxidation (Jazani et al., 2019). Kawabata et al. (2023) suggested that SCFAs enter the bloodstream via monocarboxylate carriers and are absorbed and used by the kidney via the G-protein coupled receptor. They also reported that acetate reduces the production of reactive oxygen species by increasing the viability of human proximal tubule epithelial cells under oxidative stress.

The intestinal microbiota is sensitive to nutrients, and diet is the main regulator of its activity (Nicholson et al., 2012). While sharp changes in macronutrients are necessary for changes in bacterial taxa and bacterial metabolites, micronutrients change the microbiota function (Pilla and Suchodolski, 2021). David et al. (2014) showed that only 5 days of consumption of diets consisting entirely of animal or plant products caused effective changes in the microbial population and showed rapid adaptation of the microbiota.

The most studied dietary fibers today are inulin, lactulose, mannan-oligosaccharides (MOS), fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS). It is known that the genera responsible for metabolizing oligosaccharides are Lactobacilli and Bifidobacteria (Perini et al., 2023). It is thought that the fermentation of inulin-type fructans, which are rapidly fermented in the colon for SCFA production, in the large intestine occurs preferentially with Bifidobacterium (Roberfroid, 2005). It is known that there are decreases in Bifidobacaterium and Lactobacillus genera in CKD (Crespo-Salgado et al., 2016). Those studies also showed that dose-dependent effects of both FOS and inulin. Bifidobacteria are among the first to colonize the neonatal intestine, they play an important role in modulating both the metabolic and immune activities of the host. Inulin supplementation at a dose of 50 mg/kg for 21 days in British Shorthair cats resulted in increased Firmicutes and Actinomycetes, while Bacteroides and Proteus numbers decreased (Liang et al., 2023). Garcia-Mazcorro et al. (2017) reported that consumption of prebiotic mixtures of FOS and inulin up to 31 mg/kg did not significantly alter the abundance of most bacterial groups in the feces of healthy dogs. Most studies using FOS at concentrations of 1% or 2% on a dry matter basis showed no effects on important gastrointestinal variables such as SCFA, BCFA, fecal ammonia, fecal pH, fecal score, and fecal microbial population (Perini et al., 2023). In a meta-analysis study evaluating the use of prebiotics in dogs, it was stated that many prebiotic additives such as FOS, MOS, and inulin at 1.4% of the diet on a dry matter basis increased the number of Bifidobacterium and Lactobacilli species in the feces and this increase was correlated with the dose (Patra, 2011). Addition of FOS to the diet of Beagle dogs for 8 weeks increased the number of Bifidobacterium and Bacteroides species, while Clostridium perfringens decreased below the detection level after a 4-week period (Ide et al., 2020).

Restriction of fruits, vegetables and high-fiber foods is recommended for CKD patients to prevent excessive intake of potassium. Food intake surveys in human hemodialysis patients showed significantly

reduced intake of dietary fiber, an important source of fermentable carbohydrates, and substances such as potassium and vitamin C (Yang et al., 2018). This facilitates the transition from saccharolytic to proteolytic metabolism. Shortage of complex carbohydrates, required for the saccharolytic microbiota, may lead to a decrease in SCFA production and, as a result, a deficiency of nutrients required for colonocytes and Treg cells (Jazani et al., 2019). Bifidobacterium, Lactobacillus and Faecalibacterium are of high importance due to their in carbohydrate fermentation resulting in butyrate, which is the energy source for colonocytes (Turroni et al., 2011). Toxic end products were accumulated with increased metabolism of protein and other nitrogenous compounds as a result of lack of carbohydrate sources or high protein/carbohydrate ratio (Jazani et al., 2019; Montemurno et al., 2014). Proteolytic catabolism is leaded to higher ammonia concentration in the gastrointestinal tract, resulting in a shift to alkaline pH and an increase in proteolytic species. Imbalance in favour of proteolytic species will have a fundamental role in the progression of CKD as it will increase uremic toxin production (Yang et al., 2018).

Microbiota and CKD

Removal of toxins such as urea, uric acid, creatinine, indole and phenols originating from the intestine using probiotics/prebiotics is called enteric dialysis (Prajapati et al., 2023). Nakabayashi et al. (2011) supplemented a synbiotic enriched with galactoolisaccharides to nine hemodialysis patients for two weeks and they observed a significant decrease in serum p-cresol levels but indoxyl sulfate and phenol levels were not affected.

While the amount of uremic toxin increases with constipation in people with CKD, only one study examined the relationship between CKD and constipation in cats and dogs. While 42% of cats with CKD had a defecation frequency of once a day or less, no difference for stool scores was observed between healthy cats and cats with CKD (Jones et al., 2022). Incubation with indoxyl sulfate and p-cresol sulfate in mice with CKD creates an abnormal contraction pattern in the intestines and doubles the gastrointestinal transit time (Hoibian et al., 2018). As CKD progresses, protein assimilation also deteriorates. Summers et al. (2020) found higher concentrations of fecal isovaleric acid correlated with p-cresol in 28 cats with CKD and concluded that this was evidence of protein malassimilation. Higher protein flow to the colon as a result of malassimilation supports the decrease of Bifidobacterium, which is a saccharolytic bacteria with high ability to ferment dietary fibers, and the proliferation of proteolytic bacteria (Summers et al., 2023). Prolonging the colonic transit time also reduces the amount of SCFA producing from carbohydrates entering the colon (Yang et al., 2018). Another effect of SCFAs is that they support regulate intestinal motility (Smith et al., 2013). SCFAs are both a direct energy source and signalling molecules that affect intestinal transit time through receptors (GPR41) on enteroendocrine cells (Samuel et al., 2008).

Similar pathways occur in the pathogenesis of multiple non-communicable diseases. Studies conducted in humans have shown that multimorbidity, that is, having two or more chronic conditions at the same time, is no longer an exception (Pietzner et al., 2021). Heart failure (HF) is often accompanied by CKD, and a 2-3 fold increase in heart rate is reported in patients (Hua et al., 2023). Sympathetic hyperactivity in CKD is observed even in the early stages (Kiuchi et al., 2020). When renal blood flow decreases, the renin-angiotensin-aldosterone(RAAS) system is activated. Angiotensin II release, which first aims to increase the GFR rate by narrowing the efferent arteriole, is a response to nephron loss, but incompatibilities in this response result in proteinuria and cause the development of hypertension (Lawson and Jepson, 2021). The kidneys are seriously affected by hypertensive damage. Hypertension due to the RAAS system cannot be created in germ-free mice (Karbach et al., 2016). It is also known that germ-free mice have lower blood pressure than conventional mice (Moghadamrad et al., 2015). Decreased diversity in the microbiota and changes in enterotype distribution are associated with both prehypertension and hypertension (Li et al., 2017). Liu et al. (2022) demonstrated for the first time the presence of live bacteria in the kidneys of hypertensive and normotensive (pre-hypertensive/spontaneous hypertensive) rats and reported that dysbiosis in the kidney microbiota was the cause of hypertension rather than its consequence. This study predicted that the source of these bacteria in the kidneys was the gastrointestinal tract. Addition of Tatar buckwheat, which has prebiotic/probiotic properties, to the diet affected the intestinal microbiota, reduced bacterial transport to the kidneys, and reduced blood pressure through the reorganization of the core microbiota in the kidneys.

Although both soluble and insoluble fibers have been shown to bind to minerals and inhibit absorption, properties that enhance mineral absorption have also been demonstrated for soluble fibers (Baye et al., 2017). Fiber is fermented in the colon and make the minerals ready for absorption (Metzler and Mosenthin, 2008). As a different way, the colon pH decreases with the formation of volatile fatty acids as

dietary fiber fermentation and lead to increase the solubility of phosphorus and ensure its absorption (Lopez et al., 2000). Varley et al. (2010) did not observe any changes in gastrointestinal pH by adding inulin to the diets of pigs and phosphorus and calcium utilization were not affected. In laboratory animals, dietary supplements containing acacia fiber were found to increase femur calcium, magnesium, phosphorus and zinc concentrations, whereas inulin supplementation did not have such an effect (Massot-Cladera et al., 2020). These conflicting results in both studies can be attributed to the use of low amounts of inulin addition to the diets. Because, Rideout and Fan (2004) showed reduced urinary P loss in pigs after addition of 50 g/kg inulin. Supplementation of different amounts of dietary FOS in healthy dogs increased the apparent total channel digestibility (ATTD) of calcium, magnesium, sodium, zinc, and iron compared to a diet without FOS (Pinna et al., 2018). In CKD, this mineral-fiber interaction becomes even more complex.

The use of fermentable fibers raises concerns about limiting the effectiveness of phosphorus-binding drugs because these drugs used in CKD allow higher amounts of dietary phosphorus to reach the colon. A thesis study conducted in human hemodialysis patients showed that inulin supplementation did not reduce fecal phosphorus excretion compared to the control group (Biruete, 2017). Very recently, Biruete et al. (2023) showed that fermentable dietary fibers increase circulating levels of Fibroblast Growth Factor-23 (FGF), thus affecting phosphorus homeostasis by reducing blood phosphorus levels. Hyperphosphotemia is a natural stimulant for FGF-23 and is aimed at correcting abnormal serum phosphate concentration. Recently, a high correlation between indoxyyl sulfate, phosphate, and FGF-23 has been demonstrated in cats (Liao et al., 2019). Additionally, in latter study, when indoxyl sulfate was controlled, the correlation between FGF-23 and phosphate became insignificant, whereas when FGF-23 was controlled, the relationship between indoxyl sulfate and phosphate remained significantly important. While the most important sources of phosphate in the diet are high protein ingredients and feed additives, there are huge difference between the two sources on their usability. While inorganic phosphates in additives are absorbed at rates more than 90%, 70-80% of the phosphates in animal proteins and only 50% of those in vegetables (Favero et al., 2021). Therefore, dietary change is essential to restrict phosphate absorption.

Dietary fibers should not be used as the sole tool in the management of kidney diseases. In special diets used for kidney diseases in cats and dogs, the protein, phosphorus and sodium content is reduced, while the calorie density and levels of potassium, B vitamins, antioxidants and ω-3 polyunsaturated fatty acids (PUFA) are increased (Machado et al., 2022). Diet modification studies in humans have been conducted in different groups. Results showed that the risk of death was higher in the very low protein (0.28 g/kg/day) intake in group of non-diabetic chronic kidney disease stage four people (Menon et al., 2009). Low-protein diets aim to correct metabolic acidosis, the most common complication of CKD, by reducing net endogenous acid production and potential renal acid load (Di Iorio et al., 2017). However, controlling daily intake and avoiding malnutrition is important. Hall et al. (2016) stated that a diet enriched with high-quality protein sources, like fish oil, antioxidants, L-carnitine, controlled sodium concentration, and fiber-rich fruits and vegetables was more likely to improve renal function in dogs with high serum SDMA but not azotemic.

Conclusion

The most important source of uremic toxins produced by the intestinal microbiota is diet, and reducing the source and amount of uremic toxins in CKD is the therapeutic goal. Indoxyl sulfate, p-cresol sulfate, and trimethylamine-N-oxide are the most studied uremic toxins in both humans and animals. Since our current knowledge shows a linear correlation between these toxins and CKD severity, it is very important to try to utilize the benefits of dietary fibers in disease management. Prebiotic dietary fibers might be highly beneficial agents in achieving this goal.

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Importance of routine health examinations for cats and cat-friendly practices*

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ABSTRACT

This review provides information on the importance of routine health examinations for cats and examines the role of cat-friendly clinic practices in reducing stress for feline patients during clinic visits. Cats instinctively hide their discomfort and illnesses, a behavior that aids survival in the wild. As such, preventive veterinary care is essential for their well-being. Educating cat owners about the importance of routine health examinations is crucial, as it leads to increased veterinary visits. Cats typically experience more stress than dogs during clinic visits, which can create challenging situations and potentially leave a negative impression on pet owners. Although the benefits of preventative health care for felines are well-established, barriers to veterinary care highlight the need for improvements in this area. The implementation of cat-friendly clinic practices and the expansion of accreditation programs in Turkey are expected to enhance the quality of veterinary care for feline patients by creating a more accommodating environment that meets their specific needs, thus reducing their stress and improving their experiences during routine health examinations and hospitalizations.

Keywords: cat, cat-friendly, practice, veterinary clinic

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Introduction

The bond between humans and their feline companions is a cherished one, marked by mutual affection and care. Yet, beneath the serene exterior of our cats lies a complex behavioral trait that can often mask their vulnerabilities—instinctively, cats conceal their discomfort and illnesses in ways that are closely linked to their personalities. For instance, a highly impulsive cat might react strongly to stressful stimuli in its environment, while a cat with low agreeableness might display irritability, which could be an indicator of underlying pain or illness (Litchfield et al., 2017). This characteristic poses a unique challenge for pet owners and veterinarians alike, as it can lead to delayed detection of health issues, underscoring the critical importance of routine health examinations for early intervention and treatment.

Cats are considered self-sufficient (Quimby et al., 2021), which presents a significant obstacle for veterinarians who must emphasize the critical nature of early and preventative healthcare to cat owners. Beyond the challenge of increasing cat owner awareness, the physical act of bringing cats to the vet poses its own set of difficulties. A cat's emotional health and well-being are closely linked to the environment in which they live and the interactions they experience (Taylor et al., 2022). They feel most secure when they have control over their environment, so being removed from their territory to a

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veterinary clinic-a place filled with unfamiliar smells and stimuli-can be extremely distressing for them. Even the simple act of placing a cat into a carrier may be met with resistance, highlighting the complexities of managing feline stress in relation to veterinary care. In Turkey, the engagement of cat owners with veterinary care services is notably low, with only 17% taking their cats for regular health check-ups. This is compounded by the fact that a mere 24% of pet owners act swiftly when clinical symptoms arise. The stress associated with veterinary visits is a significant barrier, with 45% of cat owners identifying the anxiety experienced before, during, and after examinations as the most challenging aspect of the process. It's important for veterinary clinics to be aware of these statistics and work towards creating a more welcoming and less stressful environment for feline patients.

Routine health care for cats

Routine health care refers to the non-emergency, general care that is needed to the cat healthy throughout its life. This includes routine veterinary care for vaccinations, parasite control, and dental care; proper nutrition; grooming; and protection from household hazards (Bukowski and Aiello, 2011). The implementation of routine health examinations for cats that appear to be in good health is imperative, as it substantially contributes to the advancement of early disease detection and facilitates the initiation of timely therapeutic measures (Paepe et al., 2013).

Cats with no clinical evidence of gastrointestinal, ocular, respiratory, skin or urogenital tract disease that are being administered internal and external parasite control are unlikely to be a zoonotic risk to their owners (Lappin et al., 2019).

Several key factors affecting a cat's wellbeing in the clinical environment

Understanding the wellbeing of cats in a clinical setting is crucial for providing optimal care. Recognizing this, experts have identified several factors that are essential for maintaining the health and happiness of feline patients. There are several key factors that veterinarians consider important for cat wellbeing, which are grouped into themes (Dawson et al., 2016). Each theme plays a significant role in ensuring that cats receive the care they need while minimizing stress and promoting a positive veterinary care experience. There are seven themes: 1. Physical environment of the clinic: This includes the clinic's layout, cleanliness, and the presence of facilities that cater to the specific needs of cats. 2. Routine animal care provided by veterinary team members: The skills and practices of the staff in handling cats, as well as the standard of care they provide. 3. Interactions between the patient, staff, and client: How the veterinary staff interact with both the cats and their owners can significantly affect the animal's stress levels and overall experience. 4. Clinic management: Effective management practices ensure that the clinic operates smoothly, which can reduce stress for both animals and humans. 5. Medical and surgical procedures: The competence with which procedures are carried out and the pain management protocols in place are crucial for animal wellbeing. 6. Staff attitudes and education: The knowledge and attitudes of the veterinary staff towards animal wellbeing can greatly influence their care for cats. 7. Communication between the veterinarian and client: Clear communication about the cat's health, treatment options, and wellbeing needs is essential.

Cat-friendly clinic practices

Numerous initiatives have been established by organizations to mitigate stress in companion animals. Launched in 2016, Fear Free offers training and certification for professionals in various pet-related fields, such as training, grooming, sitting, veterinary care, and shelters (Fear Free, 2022). In a more targeted effort, International Cat Care introduced the Cat Friendly Clinic (CFC) initiative in 2012, which sets forth guidelines specifically designed to alleviate stress in cats receiving veterinary care (CFC, 2023). Additionally, two new sets of Cat-Friendly Guidelines are focusing on veterinary interactions and the clinical environment were published in the Journal of Feline Medicine and Surgery in November 2022 (Taylor et al., 2022). The CFC initiative not only provides an accreditation program but also offers comprehensive recommendations for veterinary practices to adopt, aiming to lessen stress for cats at every stage of the veterinary visit, from travel to hospitalization (ISFM, 2020). Cat-friendly clinic practices and programs have become a certification program supported by associations globally (AAFP, 2022).

Cat friendly clinic accreditation program

The cat friendly clinic program has been developed by International Society of Feline Medicine (ISFM) as a global initiative and resource to help make veterinary clinics more welcoming to cats and their caregivers, to reduce the stress of veterinary visits for cats, and to make treating and interacting with cats easier for veterinary staff (CFC, 2023). Cat Friendly Clinic Program has different accreditation levels; Gold, Silver, Bronze. These accreditation levels are regulated according to the criterias of the in-clinic waiting room and/or reception area, examination and operation rooms, hospitalization and equipment are based on scientific principles aimed at reducing stress for cats in

veterinary clinics by the International Society of Feline Medicine (ISFM), the veterinary division of International Cat Care (Bessant et al., 2022).

In-clinic Waiting Room and/or Reception Area: Veterinary clinics must have a designated waiting area to minimize stress. Separators constructed from metal railings are permissible; however, it is crucial that the materials used are conducive to cleaning and disinfection to prevent both visual contact and the spread of infectious diseases. The dimensions of the hospitalization cage play a pivotal role in a cat's comfort and stress levels. A larger cage affords greater flexibility in layout, ensuring adequate separation between essential resources such as the bed, water bowl, food bowl, and litter box, thereby promoting freedom of movement and reducing stress during the cat's stay. For Silver level accreditation, the minimum cage size for day patients (up to 24 hours) is set at 2700 cm² in surface area (e.g., 45 x 60 cm) with a height of 39 cm. For cats hospitalized beyond 24 hours, the cage should have a minimum surface area of 3600 cm² (e.g., 60 x 60 cm) and a height of 55 cm. The Gold level standard necessitates physical separation between canine and feline hospitalization areas. The minimum cage size for Gold level day patients is 3600 cm² (e.g., 60 x 60 cm) with a height of 55 cm. For longer stays, the cage should measure at least 6300 cm² (e.g., 70 x 90 cm) with the same height. While the floor surface area is of utmost importance, the length of the cage is also a critical factor. It should accommodate additional structures like shelves, boxes, or carriers, providing the cat with alternative resting places apart from the cage floor.To ensure maximum comfort and stress reduction for hospitalized cats, it is essential to provide them with control over their environment. Both Silver and Gold level accreditations necessitate a soft and comfortable bed for the cat, distinct from disposable pads used on the hospitalization area's floor. The bed should occupy a significant portion of the floor space, offering a cozy resting surface. Food and water bowls must be positioned away from the resting area to maintain cleanliness and order. A secure hiding spot is crucial for the cat's sense of safety. Options such as a 'cat bed', 'igloo bed', or a sufficiently large cardboard box serve this purpose well. The ability to hide not only diminishes stress but also promotes the cat's overall well-being, a mandatory criterion for both Silver and Gold accredited clinics that provide overnight hospitalization. Furthermore, providing an elevated area for the cat to perch enhances its comfort. This could be a shelf within the cage, a dual-purpose box for hiding and sitting, or a cat carrier with one side open. Such arrangements cater to the cat's natural preference for higher vantage points, contributing positively to its hospital stay experience.

Conclusion

In light of the growing number of pet clinics, which now surpasses 1700 (Balaban, 2021), and the singular achievement of only one hospital in Turkey attaining Cat-Friendly Accreditation, it is evident that there is a disparity between the availability of pet care services and their utilization by cat owners. Despite the advancements in education, lifestyle, and economic conditions that have led to increased pet ownership, there remains a reluctance among cat owners to engage in regular clinic visits. These visits are pivotal for the execution of preventive medicine practices, which are integral to the health and welfare of feline companions. To address this, a multifaceted approach is necessary. Firstly, veterinary faculties should incorporate cat welfare training into their curricula to ensure that future practitioners are well-versed in the nuances of feline care. Secondly, existing and newly established clinics should be informed and motivated to pursue the ISFM Accreditation program, which signifies a commitment to cat-friendly practices. This includes ensuring that all staff members, particularly veterinary technicians, are trained in animal welfare. Furthermore, to cultivate a culture of responsible pet ownership, media campaigns should be launched to educate the public. Veterinarians should also be provided with marketing training to promote the benefits of the accreditation program, thereby incentivizing clinics to participate. Ultimately, the goal is to foster an environment where routine health examinations are not only less stressful for cats but also recognized by owners as a non-negotiable aspect of responsible pet care. By doing so, we can improve the overall quality of life for our feline friends and ensure that their health needs are met promptly and effectively.

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JIVS

MicroRNAs as potential biomarkers in ruminant, avian and porcine

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ABSTRACT

In recent years, studies on microRNAs have increased considerably. miRNAs are small RNA molecules, ranging from 19 to 25 nucleotides in length, that control the suppression of target genes after transcription. MiRNAs serve as fine-tuning factors that influence the expression of up to 60% of all mammalian protein-coding genes. Unlike proteins, miRNA sequences are widely conserved across species. This conservation strongly suggests that miRNAs emerged early in evolution and maintain their functional importance. It has been revealed that these small structures containing a small number of nucleotides can act as critical points in the organism. While traditional cancer biomarkers are mainly produced by tumor tissues or normal embryo tissues, they are absent or present in small amounts in tissue organs and the blood of healthy adults. MiRNAs can be easily detected in the blood, making them selectable candidates as biomarkers for disease. The ruminant family, one of the most diverse subspecies of terrestrial mammals, lives in a wide variety of environments worldwide and is known to have a major impact on various ecosystems and industries, including agriculture, daily activities, and cultures. MiRNAs have a significant impact on the physiology of farm animals, biological development, and cell differentiation. In this review, we will examine miRNAs that have been identified as candidates or potential candidates for the diagnosis and treatment of diseases seen in ruminants, pigs, and avians in recent years. In this way, we will provide a perspective to prevent diseases that can cause great economic losses in veterinary medicine and the production industry.

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Introduction

Family numbers of miRNAs have exhibited significant growth in recent years. MiRNAs, which are RNA molecules with a length of 19 to 25 nucleotides, regulate the inhibition of specific genes following transcription (Lu and Rothenberg, 2018). MiRNAs are short, naturally occurring RNA molecules that are involved in regulating gene expression after the process of transcription. They are around 22 nucleotides long and function by binding to certain areas on mRNA molecules that are complementary to them. This binding leads to the repression of translation, preventing the target mRNA from being translated into protein. Each miRNA has several messenger RNAs (mRNAs) as its targets, and each mRNA has multiple miRNAs that bind to it. MiRNAs function as precise regulators for approximately 60% of all protein-coding genes in mammals. miRNA sequences exhibit a high degree of conservation across several species, in contrast to proteins. The evidence strongly indicates that miRNAs appeared early in their evolutionary history and have retained their functional significance (Antunes et al., 2020). These tiny

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structures, composed of a limited number of nucleotides, have been found to function as crucial focal areas inside the organism. Although tumor tissues or normal embryo tissues primarily generate traditional cancer biomarkers, these biomarkers are either absent or found in minimal quantities in the tissue organs and blood of healthy adults. MiRNAs may be readily identified in the bloodstream, rendering them viable contenders as biomarkers for illnesses (Wang et al., 2018). Our approach involves examining the miRNAs of ruminant pigs and avian species, which have been identified as diagnostic or treatment biomarkers, or have the potential to be biomarkers, in recent years based on veterinary medicine guidelines.

Ruminant

The ruminant family, one of the most diverse subspecies of terrestrial mammals, lives in a wide variety of environments worldwide and is known to have a major impact on various ecosystems and industries, including agriculture, daily activities, and cultures (Mendes, 2012). The ability of this group of animals to survive and reproduce on low-quality, low-protein, and high-fiber plant sources is an important factor in their success. Many of the various stakeholders believe that animal health is crucial to livestock production. However, there is disagreement among consumers, farmers, and veterinarians about what constitutes an acceptable health status (Ojo & Kreuzer-Redmer, 2023). Cell differentiation, biological development, and the physiology of farm animals are significantly affected by miRNAs (Wang et al., 2013). These processes include controlling muscle growth and hypertrophy, adipose tissue expansion, oocyte maturation, and early embryonic development. Recent studies have revealed the critical roles of miRNAs in sheep (Hou et al., 2018), goat (Zhong et al., 2020), and cattle rumen development as well as maintenance of intestinal homeostasis (Liang et al., 2014; Do et al., 2019).

Cattle

In a study, it was stated that mastitis, which is inflammation of the udder, is a challenging problem that causes high economic losses in dairy animals, and the complexity of the disease, the degree of economic losses, and the increasing importance of the dairy industry, together with public health concerns, necessitate the design of appropriate mastitis diagnostics that can provide rapid, accurate, and confirmatory diagnosis. It has been suggested that miRNAs may be potential biomarkers in the diagnosis of bovine mastitis (Li et al., 2015; Chakraborty et al., 2019). Again, in line with the results obtained in his study (Srikok et al., 2020), when MIR29B-2 is used together with the California mastitis test (CMT) and the number of days in milk (DIM) data, it is possible to screen and classify milk samples taken from cows as healthy, subclinical mastitis, or mastitis. This is applicable. It has been demonstrated that it appears to have sufficient discriminatory power to enable it to be used as a biomarker in cases where the condition of the milk sample cannot be determined based on CMT results.

In another study conducted by Lai et al., 2021) to detect bovine mastitis, although the sensitivity and specificity of miR-21 in serum were lower than miRNA biomarkers in milk, the significant increase of miR-21 in serum could reflect the effect of local inflammation on the systemic reaction.

Subclinical mastitis, that is, inflammation of the mammary gland without clinical symptoms, is known as one of the most common and costly diseases in dairy farming worldwide. Milk miRNAs encapsulated in extracellular vesicles (EVs) are proposed as potential biomarkers of diseases, including subclinical mastitis. In a study by Saenz-de-Juano et al. (2022), they looked at naturally infected and healthy mammary glands for subclinical mastitis. They found that bta-miR-223-3p was the miRNA that was most highly controlled in all of the individuals.

Given the importance of early disease detection to reduce the major financial and animal welfare impact of bovine mastitis worldwide, improved tools that can accurately detect early mastitis are urgently needed. The value of miRNAs as disease biomarkers has been demonstrated, but their potential to accurately detect early mammary inflammation has not been studied in detail. The study said that the rise in btamiR-223 and bta-miR-142-5p levels in the early stages shows that miRNAs can be useful as early diagnostic biomarkers of mastitis in cattle (Tzelos et al., 2022).

Studies examining the underlying molecular mechanisms associated with mastitis in high milk-producing dairy cattle by analysis of milk-released exosomal miRNA show that key target genes, including significantly downregulated miR-375, CTLA4, IHH, IRF1, and IL7R, can be upregulated. These genes are negative regulators of immune response pathways that may be associated with impaired inflammatory mechanisms in breast cells. In light of this information, a study conducted in dairy cattle stated that bta-miR-375 may be a promising biomarker for the development of mastitis in dairy cattle (Mahala et al., 2024).

In the review on the potential use of miRNAs in tissue and/or circulation as biomarkers in the assessment of health and welfare status in animal species, it was revealed which miRNAs control stress,

immunity, milk, genetic infrastructure, management, and environment, which are some of the most important factors in animal welfare (Miretti et al., 2020).

Bovine milk and colostrum provide essential nutrients and immunologically active factors that are beneficial to a newborn calf. Exosomes derived from milk and colostrum are known to be the most important for cellular communication. Researchers looked for and described exosomal miRNA in the milk and colostrum of Holstein and East Anatolian Red (DAK) cows. They found 795 miRNAs that were expressed differently in the two groups of cows. A total of 545 of these were determined to be known miRNAs and 260 were new miRNAs, and it was stated that the data obtained from this study will make useful contributions to potential miRNA biomarker studies (Özdemir, 2020).

In a research project investigating embryonic development in cattle, significant downregulation of several DNA damage response (DDR) genes tested after increasing miR-30c or decreasing CDK12 expression suggested a possible role of miR-30c in regulating embryonic development through DDR pathways (Lin et al., 2019).

Extracellular vesicles (EVs), found in various biological fluids and especially reproductive fluids, have attracted considerable attention due to their possible role in cell-to-cell communication. Among the different bioactive molecule cargos of EVs, miRNAs are emerging as promising diagnostic biomarkers with high clinical potential. In line with this information, the presence of large amounts of miRNA in the fluids of the bovine reproductive system was revealed. 310 of these were characterized in oviduct fluids and 351 in uterine fluids. In this study, which was characterized and also looked at the level of differentiation, it was revealed that miRNAs play an important role in cell signalling, intercellular junctions, and related pathways such as reproductive functions that may play a role in the modulation of the oviduct and uterus throughout the cycle, as well as in the processes of embryonic existence and development (Hamdi et al., 2021).

MiRNAs modulate male fertility by regulating gene expression. Menezes et al. (2020) used RT-qPCR to look at the changes in sperm miR-15a, miR-29b, and miR-34a from bulls that were high fertile (HF) and low fertile (LF). MiRNA levels of miR-15a and miR-29 were found to be higher in LF sires compared to those present in HF sires. Finding miRNAs in the spermatozoa of fathers with different levels of in vivo fertility will help us understand how the ability to fertilize changes in cattle and other mammals. These possible biomarkers can be used as fertility markers in reproductive biotechnology to check the quality of semen and guess if a male will be fertile.

They looked at how miR-224 might help control adipogenic differentiation in bovine preadipocytes in their study (Zhang et al., 2019). Comparative transcriptome analysis between castrated male cattle with increased intramuscular fat (IMF) and intact male cattle revealed that miR-224 and lipoprotein lipase (LPL) were aberrantly expressed and negatively correlated, and LPL was the predicted protein of miR-224. They revealed that this was their goal. When miR-224 was overexpressed or silenced, qRT-PCR showed a negative regulatory effect on LPL. The mRNA expression levels of fat formation-associated biomarkers C/EBPα, C/EBPβ, PPARγ, FASN, and PLIN1 decreased when miR-224 was overexpressed, while the opposite effect occurred when miR-224 was inhibited, which was followed by adipogenic differentiation. All together, the results show that miR-224 controls the adipogenic differentiation of pre-adipocytes in cattle by focusing on LPL. It was concluded that this provides insight into the molecular basis of IMF accumulation in cattle.

The presence and biodegradable potential of miRNAs in other biofluids have been less studied, especially in the veterinary field. External miRNAs are an example of this; they are largely identified in rats and humans but have not been in cattle. As a result of research (Shaughnessy et al., 2020) in cattle, they have discovered that hsa-miR-548d-5p, hsa-miR-2113, and hsa-miR-1244 could be a potential bioeffect for diagnosing chronic granulomatous inflammation of the colon caused by Mycobacterium avium subspecies paratuberculosis (MAP).

Endometritis, seen as inflammation of the endometrium, can affect fertility and is known to cause serious economic losses in the dairy industry. Exosomes and exosomal miRNAs, which are widely found in various tissues and body fluids, have been shown to play an important regulatory role in immune responses. However, with the study on the mechanisms by which miR-218 regulates the release of cytokines and chemokines in endometritis, MiR-218 has been stated to be a potential biomarker for the detection of endometritis (Wang et al., 2020).

During a research study involving Piedmontese, which have double muscle groups in beef cattle breeding, miRNAs expressed in skeletal muscle were analyzed. miR-10b, miR-126-5p, miR-143, and miR-146b were found to be significantly up-regulated, while miR-21-5p, miR-221, miR-223, and miR-30b-5p were found to be significantly down-regulated. It has been reported that miR-23a is expressed at high levels in all groups created from these miRNAs in animals (Tewari et al., 2021).

As reported in the research by Huang et al. (2022), two small RNA libraries from fatty (S01) and normal livers (S02) from Holstein DairyCow (HDC) were analyzed by deep sequencing. A total of 12,964,411 and 15,426,289 clean reads were obtained, representing 370 known and 182 novel miRNAs, respectively. As a result of the analysis, 66 upregulated and seven downregulated differentially expressed miRNAs (DIE-miRNAs) were identified. DIE-miRNAs showed that ethyl lipid metabolism and cocaine addiction are closely related to liver metabolism. It has been reported that these findings will provide valuable information for further functional validation of miRNAs between normal and fatty livers, as they may benefit new attractive miRNA biomarkers for disease detection in HDC.

A study in Japanese black cows compared the circulating miRNAs of non-pregnant and pregnant animals, investigated miRNAs as biomarkers for early pregnancy diagnosis, and established a measurement system that included selecting an appropriate reference miRNA and determining the effect of hemolysis on miRNA measurement. By using microarray analysis, both pregnant and non-pregnant cows' plasma contained a total of 124 miRNAs. Levels of five circulating miRNAs were detected to be significantly higher in pregnant cows than in non-pregnant cows. 24 miRNAs were detected only in the pregnant group. showed that miR-2455 was a suitable reference miRNA in the plasma of non-pregnant and pregnant Japanese black cows, and miR-19b, miR-25, miR-29a, and miR-148a were significant reference miRNAs. In this study, four miRNAs, miR-19b, miR-25, miR-29a, and miR-148a, were detected at high levels in the plasma of pregnant Japanese black cows. Since these miRNAs are less affected by hemolysis, it has been demonstrated that they can potentially be used as biomarkers for early pregnancy diagnosis in cattle (Ono et al., 2022).

Senecio spp. It is known to be one of the most common plant-related poisonings in cattle. In a study that performed miRNA profile analysis to determine potential diagnostic biomarkers for Senecio brasiliensis poisoning, it was reported that miR-122, miR-885, and especially miR-21 could be potential biomarkers with high specificity and sensitivity in the detection of poisoning (Winter et al., 2022).

An investigation was carried out on the circulating expression levels of miRNAs that might serve as potential candidates for early pregnancy diagnosis in pregnant and near-pregnant heifers. The study showed that the Let-7d-5p miRNA could be used as a sign of early pregnancy and give information about how the fetus and cow's cells and molecules interact in the early stages of pregnancy. It has been demonstrated that it is of critical importance (De Los Santos Funes et al., 2023).

Subacute ruminal acidosis (SARA) is known to be a metabolic disease frequently seen in dairy cows fed with high-yield, concentrated feeds. A pilot study was conducted by Ojo et al. (2023) to determine that miRNAs circulating in the blood of cows could be used as potential biomarkers to detect animals with metabolic disorders such as SARA. According to the results of the study, bta-miR-30b-3p and bta-miR-2285 in the bloodstream were found to be good candidates for biomarkers that could help diagnose SARA.

It is known that endometritis is one of the most important diseases causing infertility in mammals. A study that looked at the endometrial transcriptome and miRNA profiles in cattle with clinical and subclinical endometritis disease found that the levels of miR-146a and miR-223 expression profiles went up significantly. As a result of this development, it has been stated that the relevant miRNAs can be safe biomarkers to distinguish between healthy and endometritis cattle (Shokri et al., 2023).

Sheep

Breeding stress-tolerant animals and mitigating the negative effects of environmental and pathogenic stress factors is a potential strategy to aid the health of ruminants. In the study conducted by Naylor et al. (2020), stress was administered to 15 female lambs with an intravenous bolus of lipopolysaccharide (LPS; 400 ng/kg) to evaluate a comprehensive set of circulatory mediators released in response to acute immune stress to identify candidate biomarkers that can be used in the selection of stress-resistant animals. Stress was applied, and blood was collected from the carotid artery at 0, 2, 4, and 6 hours after the LPS challenge to identify and monitor candidate stress biomarkers. The temperature was also recorded over time. Biomarker responses were evaluated with a repeated measures model to compare time points with baseline values, and miR-145, miR-233, and miR-1246 expression levels increased and remained high during the study.

In the study that analyzed potential miRNAs participating in metabolism with extracellular vesicles in the presence of inflammation in sheep, it was shown that miR-26a-5p, among miRNA regulators in the biological process of inflammation, may have an important role in the resolution of inflammation (Ciliberti et al., 2023).

In a study to detect miRNA expression in the proteom of the early antral follicles in sheep, based on in silico analyses, specific miRNAs associated with

genes corresponding to the most abundant proteins in the sheep follicle (VIM, LMNA, ACTB, and HSPA5) were studied, and five key mIRNS potentially linked to the oocyte mayosis (hsa-let-7b-5p; hsa-mir-221-3p; Hsamir -17-5p; HS-mir-24-3p and Hsa-Mir-107) were identified (Otávio et al., 2023).

Avian

Stress-induced immunosuppression is one of the serious threats to the poultry industry, especially evident for young chickens. In a study conducted to investigate the molecular mechanism of stress-related immunosuppression in chickens, dexamethasone (Dex) was injected into the chicken thymus to suppress the immune system of chickens. Then, miRNA expression analysis was applied to the thymuses. As a result of the analysis, it was stated that the differentially expressed miRNAs ggamiR-2954, gga-miR-146b-3p, gga-miR-106-3p, and gga-miR-214 would provide a basis for revealing the molecular regulation mechanism of immunosuppression in poultry (Zhou et al., 2019).

Marek's disease virus (MDV) is known to be the causative agent of Marek's disease (MD), a complex pathology characterized by paralysis, immunosuppression, and T-cell lymphogenesis in chickens. MD is controlled in poultry production through in-egg or incubation-administered vaccines, and these practices have been shown to protect against lymphoma formation but not superinfections caused by MDV field strains. In the study, where full transcriptomic and proteomic analyses of chicken serum exosomes obtained from commercial leghorn and broiler Marek's disease virus vaccine studies were performed, it was stated that cellular cargo miRNAs showed different patterns in vaccinated chickens, suggesting that miRNAs target the MAP kinase, cellular proliferation pathway. It has been reported that the miRNA set in unvaccinated tumor chickens showed significant targeting of phosphoinositol signaling. As a result of these comprehensive analyses, bioinformatic analyses of miRNAs and predicted miRNA targets showed that there were more tumor suppressor miRNAs in VEX compared to TEX (Neerukonda et al., 2019).

Coccidiosis caused by Eimeria spp. infection in broiler chickens continues to be one of the most important diseases in terms of production and economy today. Considering that the development of a genetic biomarker panel for subclinical infection will be an important biological tool for the management of broiler flocks, the study by Giles et al. (2020) analyzed the expression of miRNAs to determine their potential in diagnosing coccidiosis in broiler flocks. As a result of analyses conducted clinically or among chickens infected with Eimeria maxima and Eimeria acervulina, Gallusgallus (gga)-miR-122-5p, gga-miR-205b, and gga-miR-144-3p showed that they can be used in the diagnosis of subclinical coccidiosis.

In the study conducted by Chen et al. (2022), the expression levels of circRNAs, miRNAs, and mRNAs in the spleen obtained from commercial dual-purpose Sasso T445 breed chickens infected and uninfected with Eimeria tenella were analyzed. In conclusion, among these, it has been shown that circMGAT5 can inhibit the activation of macrophages through the circMGAT5-miR-132c-5p-MMD (macrophage differentiation-associated) axis to participate in the immune response caused by Eimeria infection.

Circular RNAs (circRNA) are molecules that result from backsplicing events that connect a downstream 5' splice site to an upstream 3' splice site. Little is known about the potential pathogenesis-inducing function of circRNAs in chickens. circDNAJB6, found in chickens, is a stable and newly conserved circular RNA that is mainly expressed in the stomach, lung, spleen, and thymus. In light of this information, the study conducted by Tan et al. (2024) investigated the function of circDNAJB6, which can serve as potential biomarkers and act as potential targets for the treatment of bacterial infection. It has been found to interact with five miRNAs: 3p, gga-miR-1306–5p, gga-miR-6549–5p, and gga-miR-1684a–5p.

Young peregrine falcons (Falco peregrinus), which are wild birds, were studied to see how the endocrine system and immune system are connected. The study found that exposure to perfluoroalkyl acids (PFAAs) has big effects on both systems, and the researchers wanted to look at these effects in terms of miRNAs. As a result of the analyses, plasma miRNA-155 counts were identified to have significant negative relationships with PFAAs concentrations (Sun et al., 2021).

Porcine

Zearalenone (ZEN), a mycotoxin with estrogenic effects, is known to pose a risk to animal health. It is not easy to detect and diagnose the disorders caused by this effect. For this reason, in a study conducted to investigate the effects of ZEN on pigs, miRNA expression profiles in pig jejunum and serum were examined. As a result of the investigations, it was stated that ssc-miR-135a-5p, ssc-miR-432-5p, ssc-miR-542-3p, and ssc-miR-493-3p profiles may be effective when comparing pigs exposed to ZEN and pigs not exposed to ZEN (Grenier et al., 2019).

Research on miRNAs and porcine oocyte maturation and the molecular mechanisms behind this complex

process has not been fully elucidated. To elucidate these mechanisms, in the study conducted by Hu et al. (2020), exosomal miRNA was obtained from porcine follicular fluid (PFF), and the differentiation levels of miRNAs were examined. As a result of the analyses, it was reported that miR-125b, let-7d-5p, miR-200b, miR-26a, and miR-92a may be potential biomarkers for the molecular identification of high-quality oocytes.

Early pregnancy diagnosis in sows, which can greatly increase the efficiency of the swine industry, is an important issue that needs to be examined and researched. Regarding this, (Zhou et al., 2020) examined the expression levels of miRNAs released into the bloodstream through exosomes in their study. They identified a large number of miRNAs in twenty-eight different ways between the pregnant and control groups. As a result, they successfully determined the exosomal miRNA profiles circulating in the serum of pigs in the early period of pregnancy and showed that miR-92b-3p and miR-17-5p can be used as potential circulating biomarkers for early pregnancy diagnosis.

It is known that impairment in fetal skeletal muscle growth developing in the uterus can lead to decreased birth weight and decreased carcass quality in pigs. Recently, their research showed the part that miRNAs and the genes they target play in the development of skeletal muscles during pregnancy and in the cause of intrauterine growth restriction (IUGR) (Ali et al., 2021b). In this study, they performed miRNA analysis in the longissimusdorsi muscle (LDM) of the pig. As a result, miR-140, miR-186, miR-101, miR-15, miR-24, miR-29, miR-449, miR-27, miR-142, miR-99, miR-181, They identified 13 miRNAs significantly associated with fetal weight, including miR-199 and miR-210. They reported that the miRNA profile of skeletal muscle can be used to predict fetal weight, and fetal weight-related miRNAs can serve as potential biomarkers of prenatal fetal health and growth (Ali et al., 2021a).

In a study to measure the sperm quality of sexually mature pigs, samples of seminal plasma surrounding the sperm were studied. miRNA expression profiles were examined in extracellular vesicles (SP-EV) obtained from seminal plasma. ssc-miR-205, ssc-miR-493-5p, and ssc-miR-378b-3p, which are expressed differently in high-quality and low-quality semen, are related to cellular localization (nuclear and cytosol) and molecular functions (acetylation, Ubl conjugation, and protein kinase). It has been revealed that it potentially impairs sperm quality by allowing gene targeting (Dlamini et al., 2023).

Porcine reproductive and respiratory syndrome (PRRS) is a serious infectious disease in the swine industry and is known to cause serious economic losses in current swine production worldwide. It is clear that there is no effective anti-viral strategy to prevent this disease. A study that tried to find a solution to this problem found that PRRSV infection lowers the expression of ssc-miR-124a and that ssc-miR-124a has a strong antiviral effect by decreasing the expression of CD163. Overall, these results suggest that ssc-miR-124a may be an important therapeutic target for the development of new anti-PRRSV therapies (Li et al., 2021).

Among the miRNAs examined in the porcine cardiopulmonary bypass model, which was created to investigate the protective effects of polarized cardioplegia (STH-Pol-B) and hyperkalemic cardioplegia (STH2-B) after cardiac surgery, miRNA-708-5p was significantly lower in STH-Pol-B hearts (P =.019) and miRNA-122 expression was found to be significantly higher (P =.046). In line with these results, it is stated that polarized and depolarized arrests are not superior to each other and confirm their potential (Santer et al., 2019). In a similar study, regarding the relationship between cardioplegia and temperature, only ssc-miR-451 was expressed differently between STH2-hot and STH2-cold, and these data were reported for the first time that cardiopulmonary bypass and the temperature of the cardioplegic solution affected the expression of miRNAs in the left ventricular tissue. shows. Consequently, specific miRNAs have been reported as potential therapeutic targets to limit ischemia-reperfusion injury in patients undergoing cardiac surgery (Kiss et al., 2020).

Clinical signs are typically how veterinarians diagnose inflammation in pigs. However, it is known that this method is not very reliable due to subjective clinical interpretations. In a study conducted to diagnose this inflammation response with miRNAs at the molecular level, inflammation was induced in pigs with Escherichia coli lipopolysaccharide (LPS). For inflammation responses, whole blood samples were taken from pigs after 0, 1, 3, and 8 hours, and miRNA expression levels in plasma were measured. As a result of the investigations, they revealed that the expression of ssc-let-7e-5p, ssc-mir-22-3p, and ssc-miR-146a-5p are the miRNAs that change most significantly over time (Swain et al., 2021).

In the study conducted by Bilinska et al. (2023), eight miRNAs (miR-21a, miR-26b, miR-30a, miR-92a, and miR-146a) were detected in pork in vitro in order to understand the importance of extracellular miRNAs in cell differentiation processes. MiR-148a, miR-199, and miR-383a were determined. Adipogenesis analysis revealed that miRNAs (miR-21a, miR-92a), especially those associated with the inflammatory process, were expressed at high levels in differentiated adipocytes and were also secreted by the cells. These findings

may provide an important basis for understanding the role of extracellular miRNAs in cellular differentiation processes, and they have been reported to provide important guidance for the discovery of potential therapeutic and diagnostic targets.

African swine fever (ASF) is known to be a serious viral disease in domestic and wild pigs with a high mortality rate, for which no effective vaccine or antiviral drug is available (Dixon et al., 2019). MiR-451 is one of the most representative miRNAs described to present upregulation in ASF-infected pigs 3–7 days after infection (Núñez-Hernández et al., 2017; Pang et al., 2023). In the study for the diagnosis of this disease, a colorimetric detection strategy was aimed at the detection of an ASF-related miRNA based on isothermal rolling circle amplification (RCA) and salt-induced gold nanoparticle aggregation developed by Chi et al. (2024). As a result of the experiments, it was stated that its repeatability was satisfactory. This newly developed method has been reported to be successfully applied to the detection of ssc-miR-451 in pig serum samples.

Metabolites and miRNAs in plasma were looked at as possible biomarker candidates in a pilot study to learn more about molecular pathophysiology in a minipig model with the goal of finding lethal radiation early. The pilot study analyzed changes in miRNA and metabolites immediately after total body irradiation (TBI). As a result, it has been shown that 92 miRNAs can be potential biomarkers in the early diagnosis of radiation (Chakraborty et al., 2023).

The molecular mechanisms of deoxynivalenol (DON), which is a common mycotoxin worldwide and affects human and animal health, in living organisms have not been fully investigated. In the study conducted by Segura-Wang et al. (2021), the effects of this toxin on piglets were found to be associated with miRNA expression responses. As a result of the data obtained, only slight changes in miRNA expression were detected in the liver tissue of pigs, and ssc-miR-10b was down-regulated in the liver of piglets exposed to DON.

In the research undertaken by Lecchi et al. (2020), it was investigated whether the acute pain associated with castration and tail docking of male piglets could modulate the expression of salivary miRNAs, and the results obtained showed that miR- in the saliva of piglets castrated and tail docked without the application of painkillers. They showed that 19b, miR-27b, and miR-365 increased significantly. They stated that research on this subject should be expanded so that the detected miRNAs can be used as potential biomarkers.

In the study conducted by Li (2021), the place and importance of miRNAs in this production mechanism in the development of back fat in Landrace and Neijiang pigs were investigated. Eleven miRNAs that are unique to Landrance pigs and thirty-five miRNAs that are unique to Neijang pigs were identified among the miRNAs that were screened. It was reported that miR-1-3p can regulate lipid accumulation and synthesis and may serve as a potential marker for pig breeding as a consequence of the study.

It is known that intramuscular fat (IMF) content is a very important parameter in evaluating pork quality. Studies on this subject show that miRNAs play a role in IMF accumulation. In this research, a new miRNA that is involved in IMF adipogenesis in pigs was looked at in terms of the growth and differentiation of pre-adipocytes inside the muscle. As a result of the study, miR-146a-5p was determined to target SMAD4 and TRAF6 to inhibit porcine intramuscular adipogenesis by attenuating TGF-β and AKT/mTORC1 signaling, respectively. These findings imply a new miRNA biomarker that regulates intramuscular adipogenesis to improve pork quality (Zhang et al., 2021).

Conclusion

In overall, the rapidly expanding domain of miRNA investigation has unveiled the crucial function that these diminutive RNA molecules perform in controlling the expression of genes in numerous species. The extensive preservation of miRNA sequences indicates that they originated early in the course of evolution, highlighting their fundamental significance. Due to their capacity to regulate the expression of a considerable number of protein-coding genes in mammals, miRNAs are indispensable participants in cellular processes. MiRNAs are exceptionally promising candidates for disease biomarkers due to their distinctive properties, especially in the context of avians, swine, and ruminants. In contrast to conventional cancer biomarkers, miRNAs are readily detectable in the bloodstream, providing a non-invasive and conveniently accessible diagnostic approach. This has the capacity to prevent diseases that present substantial economic risks to the veterinary medicine and diverse manufacturing sectors. The review explores the effects of miRNAs on the cell differentiation, homeostasis, and biological development of farm animals belonging to the diverse ruminant family. Through the identification of particular miRNAs that are linked to diseases in these animals, scientists have the potential to establish diagnostic and therapeutic interventions. The ramifications transcend the domain of veterinary medicine, exerting an impact on sectors including agriculture and advancing knowledge regarding the

complex interconnections between miRNAs and the well-being of various species. This overview provides an extensive examination of the latest developments in the identification of miRNAs for the purpose of diagnosing and treating diseases that impact important livestock species. The results of this study not only provide insights into the possible uses of miRNAs in the field of veterinary medicine, but also underscore their wider importance in protecting the well-being and financial sustainability of sectors that depend on these creatures.

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Avian IgY antibodies and its immunotherapeutic applications

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ABSTRACT

Antibodies, also called immunoglobulins, are specialized proteins produced by the immune system in response to the presence of pathogens or foreign substances in the body. These unique proteins are commonly used for diagnostic and therapeutic purposes because they easily bind to antigenic molecules. Polyclonal antibody production currently involves the use of laboratory animals such as rats, rabbits, sheep, goats, and horses. However, the manufacture of these antibodies generally involves practices that cause pain to animals, such as prolonged bloodletting. In recent years, isolating antibodies from egg yolk following hyperimmunization of chickens has emerged as a popular approach for producing significant amounts of antibodies. This approach combines the principles of natural passive immunity and artificial passive immunity. To ensure a continuous accumulation of antibodies in egg yolks, chickens are regularly immunized with specific antigens. Egg yolk antibodies, known as IgY, are extracted and used for immunotherapy and immunodiagnostic purposes in human and animal applications due to their promising antibacterial properties. The antibacterial properties of egg yolk antibodies have been a significant focus in IgY studies. Several reports have shown that IgY helps prevent bacterial transmission or infection in vivo. The production of IgY against mammalian antigens has a higher success rate than IgG production. This is because of the phylogenetic difference between mammals and chickens. Furthermore, these antibodies have a more comprehensive range of antigenic epitope recognition and can respond to more than one species, making them more versatile. This study compiles information on the properties, mechanisms of action, and uses of egg yolk antibodies based on existing literature on IgY technology.

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Introduction

Klemperer (1893) conducted an experiment demonstrating that specific antibodies (Abs) produced after immunization chickens could be transferred to the egg yolk. This finding gained popularity as animal welfare became an increasingly prominent ethical issue in the scientific community. Klemperer's findings were first made known worldwide with the publication of the study titled 'Principles of Humane Experimental Technique' conducted by Russell and Burch in 1959 (Russell and Burch, 1959). Subsequent research by numerous scientists over the next two decades further emphasized the significance of these findings.

In 1992, a research team funded by the German Government and with experience in egg yolk antibodies was established. This team investigated the comparative effectiveness of chicken antibodies with traditionally used polyclonal antibodies produced from mammals, specifically rabbits. As a result of this

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research, it is noteworthy that chicken antibodies were found to be more effective than mammalian antibodies because they contain highly phylogenetically protected antigens. Over six years, IgY technology gained increasing acceptance. Within a few years, it became an accepted alternative to traditional procedures and has been reported to cause less stress to animals (Schade et al., 2000).

The term 'IgY technology' first appeared in 1996 to describe the production and uses of egg yolk antibodies. That same year, the European Center for the Validation of Alternative Methods (ECVAM) recommended using egg yolk-produced IgY antibodies instead of mammalian IgG antibodies to reduce the pain associated with invasive antibody sampling. In addition, it was reported in the ECVAM workshop that using chickens for antibody production improves animal welfare by eliminating the need for blood collection and reduces animal use since chickens produce more antibodies than mammals (Schade and Hlinak, 1996). In 1999, The Swiss Government Veterinary Office (Office Vétérinaire Fédéral) approved IgY technology as an alternative method to support animal health (Schade et al., 2000).

The use of IgY antibodies in basic research has become increasingly common due to their advantages over mammalian antibodies. IgY does not bind to mammalian rheumatoid factors or Fc receptors, making it a preferred choice. However, it should be noted that using chicken antibodies can sometimes result in false positive results in immunochemical tests (Schade et al., 2000).

Studies have shown that the yield of IgY antibodies is considerably higher than that of IgG antibodies. According to Gottstein and Hemmeler (1985), an immunized chicken can produce 18 times more antibodies than an immunized rabbit in one month. Another study reported that a chicken can produce the same amount of antibodies per week as approximately 100 ml of serum or 200 ml of whole blood (Larsson et al., 1993). Similarly, it has been reported that up to 1500 mg of IgY can be collected monthly, of which an average of 2% to 10% is specific IgY (Schade and Hlinak, 1996). During the same period, IgG antibodies collected approximately 200 mg and constituted only 5% of the specific antibody (Tini et al., 2002). Laying hens can produce more antibodies than 4 rabbits in the same period. They are also called 'small factories' due to their ability to produce over 20 g of IgY in approximately one year (Xu et al., 2011). Egg yolk antibodies have the advantage of inducing a lower antigenic response, making them more suitable for long-term production than rabbits (Gassmann et al., 1990). Additionally, IgY technology allows for accessible collection of antibodies from egg laying hens, eliminating the need for painful blood collection. Thus, it meets the principle of reducing practices that cause pain, which is the primary goal of animal welfare (Larsson et al., 1993; Tini et al., 2002). Similarly, keeping chickens is a more cost-effective and manageable alternative to using laboratory mammals for research purposes (Gassmann et al., 1990). The production of IgY is considered a more sterile and efficient system compared to the production of mammalian IgG. IgYs are a sustainable alternative to antibiotics because they do not have any adverse effects, such as resistance or toxic residues (Coleman, 1999).

This study describes IgY technology, including its production, structure, components, and mechanisms of action. Additionally, it discusses the use of egg yolk antibodies in preventing and treating pathogenic infections.

Properties of IgY antibody

Structure

Chickens possess three distinct classes of immunoglobulin: immunoglobulin Alpha (IgA), immunoglobulin Mu (IgM), and immunoglobulin Upsilon (IgY). The IgA and IgM found in chickens are structurally and functionally equivalent to their mammalian counterparts. IgY, the dominant antibody group in blood serum and egg yolk, was initially classified as IgG due to its similar function to mammalian IgG antibodies (Gadde et al., 2015). However 1969, researchers recommended that the immunoglobulins found in chicken egg yolk and serum should be named IgY instead of IgG due to structural differences (Leslie and Clem, 1969). IgY antibodies are also accepted as the evolutionary ancestor of IgG and IgE and are the predominant serum antibodies in amphibians, reptiles, and lung-breathing fish, except birds (Leslie and Clem, 1969; Warr et al., 1995).

It has been reported that within the egg matrix, IgA and IgM antibodies are concentrated in the egg white, while IgY antibodies are concentrated in the egg yolk (Gadde et al., 2015). The structure of the IgY molecule is with two heavy chains (H) weighing 65 kilodaltons (kDa) each and two light chains (L) weighing 25 kDa each (Pereira et al., 2019). IgY has a larger molecular mass (~180 kDa) than IgG (~150 kDa). The IgY antibody's heavy chain is represented by the letter Y, or the twentieth letter of the Greek alphabet, upsilon (υ) (Warr et al., 1995; Gadde et al., 2015). Figure 1 illustrates the molecular structures of IgG and IgY.

The light chain of IgY is similar to that of IgG, as it consists of a constant region (CL) and a variable region (VL). However, the heavy chains of IgY and IgG differ.

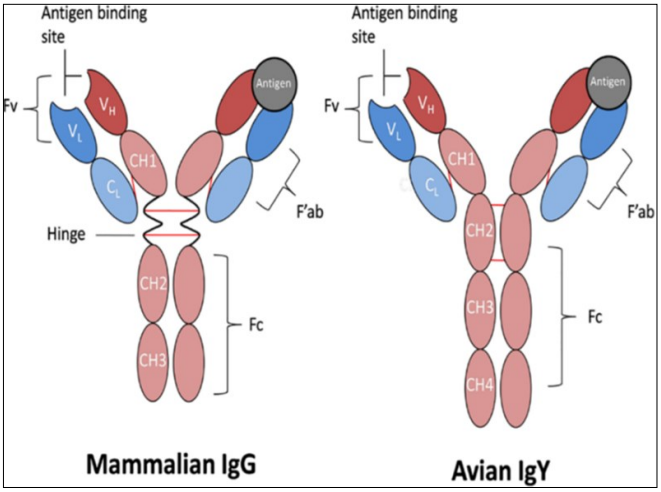

Figure 1. Molecular structures of IgG and IgY

The diagram shows the disulfide bonds connecting the heavy and light chains, indicated by red lines. IgY has an additional CH4 domain, similar to IgE, but lacks the folding region found between CH1 and CH2 in IgG (Lee et al., 2017).

IgG has three constant regions (CH1-CH3) in its heavy chain, while IgY has four constant regions (CH1-CH4) (Pereira et al., 2019). Several studies reported that the CH3 and CH4 regions of IgY correspond to the CH2 and CH3 regions of IgG (Parvari et al., 1988; Fellah et al., 1993; Magor et al., 1994; Warr et al., 1995). However, the CH2 region of IgY does not have a corresponding domain in IgG. This is because it has been replaced by the folding region (Parvari et al., 1988; Fellah et al., 1993). It is important to note that IgY is less flexible than IgG because IgY lacks the folding region located between the CH1 and CH2 domains found in IgG (Shimizu et al., 1992). Additionally, the short proline and glycine-rich regions located at the borders of the CH1-CH2 and CH2-CH3 domains provide a limited degree of inflexibility to the IgY molecule, which does not have a folding region (Warr et al., 1995). Structural and phylogenetic differences between IgY and IgG have distinct effects on their biochemical and molecular interactions. Egg yolk antibodies do not activate rheumatoid factors or mammalian Fc receptors (Larsson et al., 1992). Table 1 summarizes the differences between IgY and IgG.

Stability

Numerous studies have demonstrated that egg yolk antibodies can be stored for extended periods without any activity loss. According to Larsson et al. (1993), egg

Table 1. Characteristics of IgY compared to IgG (Zhang et al., 2021)

Features	IgY	IgG
Species	Birds, reptiles, amphibians and lungfish	Mammals
Antibody subclasses	None	IgG_1, IgG_2, IgG_3 ve IgG_4
Antibody source	Egg	Blood serum
Antibody sampling	Meets 3R animal welfare principles	It can be painful
Average antibody per animal	50-100 mg/ egg yolk	5 mg/ml/blood, up to 40 ml blood collection per month
Monthly antibodies per animal	1000-2800 mg/chicken/month	200 mg/rabbit/month
Amount of antigen-specific antibodies	Total antibody %0,5-10	In serum 50-200 µg/ml
Molecular weight (kDa)	180	150
Isoelectric point	5,5-7,6	up to 8.5
pH stability	pH 3,5-11/ decisive	pH 2-11/ decisive
Proteolytic degradation	Pepsin, papain	Pepsin, papain, tripsin ve kimotripsin
Heat stability	65 °C decisive > 2 month 100 °C > 6 minute 4 °C > 6 month	Generally higher than IgY
Chain type/number of areas/folding area	υ chain/3 fixed fields/none	ɣ chain 2 fixed fields / there is
Binding to Fc receptors	No	Yes
Cross-reactivity with rheumatoid factor	No	Yes
Cross-reactivity with human anti-mouse antibody (HAMA)	No	Yes

yolk antibodies can be stored at +4°C for approximately 5 to 10 years without severe loss of activity. The study also reports that IgY antibodies remained effective after being stored for 6 months at 24°C or 1 month at 37°C. The most effective method for extending the storage time of IgY antibodies is to freeze them at -20°C. It has been reported that IgY antibodies may lose over 50% of their activity when stored for extended periods at -70°C (Schade et al., 2000). Furthermore, it has been reported that the stability of IgY is not significantly affected by lyophilization and freezing processes unless they are repeated multiple times (Shimizu et al., 1988). Many research mentions a minimum loss of antibody activity, but some researchers have reported a significant decrease in IgY solubility after lyophilization (Chansarkar, 1998; Sunwoo et al., 2002). In addition, IgY antibodies can be stored for an extended period without activity loss when spray dried. Moreover, spray drying has been reported as a more cost-effective alternative to lyophilization (Yokoyama et al., 1992).

Egg yolk antibodies are heat-stable. However, their activity and stability decrease when exposed to high temperatures for extended periods. Lösch et al. (1986) reported that IgY activity did not decrease when eggs were boiled for six minutes, but antibody activity decreased after ten minutes. Similarly, another study reported that egg yolk antibodies maintained around 50% of their antibody activity after being heated at 70° C for 30 minutes but only retained 10% of their activity when heated at 80°C for 10 minutes (Hatta et al., 1993). However, according to Shimizu et al. (1992), IgY antibodies can remain active even after being heated to 70°C for 15 minutes, suggesting they can withstand temperatures between 60°C and 70°C.

Egg yolk antibodies exhibit stable activity within the pH range of 4 to 11, and the activity ceases completely at pH 3 Shimizu et al. (1988 and 1992). Similarly, it has been observed that the activity of IgY is irreversibly lost when the pH drops to 3.5 (Lösch et al., 1986; Schmidt et al., 1989; Hatta et al., 1993; Lee et al., 2002). It is reported that the faster decrease in activity of IgY compared to mammalian IgGs may be due to conformational changes that affect the antigen binding site (Shimizu et al., 1993). Reports suggest that the acid-induced inactivation of IgY can be accelerated by complex carbohydrates, sugars, or stabilizers such as sorbitol (Shimizu et al., 1994; Lee et al., 2002). It has been reported that under alkaline conditions, the conditioning of IgY antibodies is stable up to pH 11 but drops significantly at pH 12 and above (Lösch et al., 1986; Shimizu et al., 1992). The effectiveness of IgY, when taken orally, depends on its ability to resist enzymatic degradation in the stomach and bowel. Researchs indicates that while IgY antibodies show some resistance to trypsin and chymotrypsin, they are mainly susceptible to pepsin digestion, especially in acidic environments (Shimizu et al., 1988; Otani et al., 1991; Reilly et al., 1997; Jaradat and Marquardt, 2000). The proteolytic activity of the pepsin enzyme on IgY ends at pH 5. In their study, Schmidt et al. (1989) reported that trypsin had a more substantial inactivating effect on IgY antibodies than chymotrypsin. Furthermore, in the same study, in vitro, incubation of egg yolk, egg suspensions, and isolated IgY antibodies with trypsin and chymotrypsin for 3 hours was reported to result in complete inactivation of IgY antibodies by trypsin and significant inactivation by chymotrypsin. However, an increase in antibody activity of egg yolk and egg suspensions has been reported, attributed to other protein components in the egg acting as a buffer. Therefore, the stability of IgY in the intestine depends on various factors, including age, health status, nutritional factors, stomach pH, and enzyme levels.

Research on the stability of egg yolk antibodies has mostly been conducted in laboratory settings. However, several studies have shown that orally administered antibodies maintain their biologically active form after passing through the intestine (Reilly et al., 1997; Carlander et al., 2000; Berghman et al., 2005). Conducting additional in vivo studies to demonstrate the stability of IgY in feed, egg yolk, and whole egg matrices is crucial for the potential commercial use of IgY.

Mechanism of effect

The mechanism behind orally administered egg yolk antibodies has yet to be fully understood. However, this mechanism is known to be largely the result of antigen-antibody interaction (Rahman et al., 2013). Various theories have been suggested to explain the protective effect of IgY. The primary mechanism involves antibodies binding to specific antigens on pathogens, disrupting their biological functions or ability to proliferate. IgY antibodies bind to bacterial antigenic structures, including flagella, fimbria, outer membrane proteins, and lipopolysaccharides, preventing their adherence to the intestinal wall (Peralta et al., 1994; Tsubokura et al., 1997; Jin et al., 1998; Yokoyama et al., 1998). IgY inhibits the adherence of pathogens to intestinal cells. This reduces the proliferation and colonization of pathogens within the cells (Jin et al., 1998; Sugita-Konishi et al., 2000; Lee et al., 2002; Sunwoo et al., 2002; Girard et al., 2006). Additionally, IgY antibodies

2002; Girard et al., 2006). Additionally, IgY antibodies have other mechanisms of action on antigens, such as bacterial agglutination resulting in immobilization and death, neutralization of toxins (Wang et al., 2011), and inhibition of enzyme activity (Rahman et al., 2013). It has been reported that binding specific IgY to bacteria can alter cellular signaling events, thereby reducing toxin production and release (Xu et al., 2011).

Production of IgY

When producing egg yolk antibodies, it is possible to use various immunization protocols and recommendations. However, it has been reported that antibody titers can be affected by various factors, including the type of antigen, route of administration, type of adjuvant used, frequency of immunization, age, strain, and stage of animal development (Schade et al., 2005; Chalghoumi et al., 2009). Specific protocols have been implemented to obtain IgY by controlling these variables (Schade et al., 2005; Michael et al., 2010). Agent-specific IgY can be produced in chickens using a variety of antigenic structures, including complex antigens like bacteria and parasites, as well as simpler antigens such as proteins, peptides, polysaccharides, and nucleic acids (Chalghoumi et al., 2009; Michael et al., 2010). The data showed that chickens generally produced specific antibodies after immunization with protein antigens. However, it is reported that the lowest antigenic molecular weight required to obtain a sufficient immune response is similar in mammals and chickens and is approximately in the range of 5 to 10 kDa (Schade et al., 2000).

Additionally, adjuvants are required to trigger the formation of high antibody titers. Among the adjuvants used for this purpose, Freund's Complete Adjuvant (FCA) is considered the "gold standard" and is the most used adjuvant for immunization purposes (Schade et al., 2000). However, although FCA has the strongest effect on the formation of high levels of antibody titers, it can cause severe inflammation in the areas where it is applied (Chalghoumi et al., 2009). Some studies have shown that chickens better tolerate immunization with Freund's Complete Adjuvant (FCA) than mammals and does not cause tissue injury in chickens (Gassmann et al., 1990; Bollen et al., 1996). However, other studies contradict these results (Wanke et al., 1996; Olbrich et al., 2002).

Freund's Incomplete Adjuvant (FIA), which does not include mycobacteria, is one of the best alternatives to FCA (Schade et al., 2000). For immunization applications, it is recommended to use FCA for the first immunization and FIA for subsequent immunizations to prevent inflammation in the injection area (Reddy et al., 2013; Łupicka-Słowik et al., 2014). In young chickens, antigens are usually administered intramuscularly (i.m.) into the breast muscle to produce egg yolk antibodies. Subcutaneous injection through the neck can cause unnecessary stress to the animal. Injection in the leg muscle can cause lameness and should be avoided (Schade et al., 1996). Alternatively, antigens can be administered orally for a non-invasive approach (Thibodeau et al., 2017).

The number of required immunizations varies depending on the adjuvant, antigen characteristics, and application dose. To achieve the desired level of antibodies, it is recommended to administer at least two immunizations with an interval of four or six weeks before the chickens enter the ovulation period. After the final immunization, IgY titers should be measured 14 days later (Pereira et al., 2019). If the antibody titres are below the desired level, increasing the frequency of immunization during the ovulation period is recommended (Schade et al., 1996). The titers of IgY increase from the 14th day following the immunization (Sui et al., 2011; Wen et al., 2012) or from the fifth week after antigen inoculation (Grzywa et al., 2014). After a gradual increase, the antibody titer reaches its peak and stabilizes. It then gradually decreases (Wen et al., 2012). However, it has been reported that egg yolk antibodies can be kept at high titers for more than 150 days with booster immunization applications (Meenatchisundaram et al., 2011).

The process of extraction IgY involves removing the lipids and extracting the antibodies to produce a water -soluble form. Various methods exist for extracting IgY from egg yolk, including the polyethylene glycol precipitation Methot, water dilution method, ammonium or sodium sulfate precipitation methot, dextran sulfate precipitation, pre-cooled propane and acetone method and water dilution, ultrafiltration method. Polson's polyethylene glycol precipitation method (Palson et al., 1980) is widely accepted among these methods (Schade et al., 2000). However, the appropriate method depends on the purpose, cost, and available technology. It may be necessary to purify to varying degrees (Chalghoumi et al., 2009). In research, IgY production is primarily carried out in chickens. This process can also be applied to other birds, such as geese (Fink et al., 2017) and quails (Najdi et al., 2016), using a similar immunization protocol for chickens.

Immunotherapeutic applications of IgY

Polyclonal egg yolk antibodies, produced specifically

against contagious diseases, can reduce the likelihood of microbial resistance. For this reason, specific IgY antibodies are a suitable option for antimicrobial use in both animal and human health, particularly against resistant bacteria (Rahman et al., 2013). The antibacterial effects of IgY against gastrointestinal agents have been extensively studied. Orally administered IgY has been shown to protect against gastrointestinal pathogens such as human (HRV) and bovine Rotaviruses, Salmonella spp., and enterotoxigenic Escherichia coli (Karlsson et al., 2004). HRV is a leading cause of acute gastroenteritis in children, resulting in over one million deaths per year on average. A study on mice infected with Rotavirus found that IgY derived from eggs inoculated with three different serotypes (mouse, monkey, and human) effectively relieving diarrhea (Yolken et al., 1988; Hatta et al., 1993).

Several studies have been conducted on the protective effect of IgYs against the influenza virus. In a study conducted in Vietnam, yolk antibodies against the avian influenza A (H5N1) virus were isolated from eggs sold in markets. The study's results showed that administering IgY against H5N1 intranasally to mice before and after infection with H5N1 and H5N2 prevented the onset of the disease (Nguyen et al., 2010). Wen et al. (2012) investigated the protective effect of IgY against Influenza B virus. The results showed that intranasal administration of anti-Influenza B IgY prevented the development of influenza in mice before exposure to the virus.

Additionally, it reported that it alleviated the disease in mice treated after infection. In addition, IgYs have been produced that are effective against Newcastle disease virus (NDV), Infectious Bursal Disease virus (IBDV), Influenza, and Reovirus, which cause infection in poultry (Aizenshtein et al., 2016). These studies demonstrate that egg yolk antibodies offer protection against viral agents, and passive immunization with IgY may be possible.

Enterotoxigenic Escherichia coli (ETEC) remains a significant health concern due to diarrhea. ETEC is a common cause of enteric Colibacillosis in newborn piglets, calves, children, and travelers to developing regions. It causes an average of one million deaths per year (Mine et al., 2002). A study examined the protective effect of anti-ETEC IgY against ETEC infections in piglets and calves. It was found to offer a prophylactic and therapeutic approach that can control diarrhea caused by infection in both. The oral administration of anti-ETEC IgY has been successful in preventing gastrointestinal infections in animals (Ikemori et al., 1992; Yokoyama et al., 1992).

Salmonella spp. is a common cause of foodborne illness. The Salmonella agent possesses several surface components, including outer membrane proteins associated with virulence, flagella, lipopolysaccharides, and, in certain strains, fimbrial antigens (Mine and Kovacs-Nolan, 2002). A study was conducted to control Salmonellosis by investigating the protective effect of specific egg yolk antibodies produced against the agent's outer membrane proteins, lipopolysaccharides, or flagellar antigens in calves and mice. The research report indicates that chickens treated with anti-Salmonella IgY antibodies had a higher survival rate (Yokoyama et al., 1998). Additionally, it has been reported that IgY inhibits the adhesion of Salmonella Enteritidis to human intestinal cells in vitro (Sugita-Konishi et al., 1996).

Additionally, administering egg yolk antibodies against Clostridium difficile spores orally has been observed to delay the onset of diarrhea and reduce its recurrence in mice treated before infection (Pizarro-Guajardo et al., 2017). The therapeutic effect of IgY against Helicobacter pylori has also been extensively researched. It has been reported that anti- H. pylori egg yolk antibodies inhibit the growth of H. pylori in vitro and reduce gastric inflammation in mice (Malekshahi et al., 2011).

The protective effect of egg yolk antibodies against pathogens that cause respiratory tract infections has also been studied. It has been determined that IgYs produced specifically for the purpose of combating Mycobacterium tuberculosis cause dose-dependent proliferation of peripheral blood mononuclear cells in rats. RT-PCR analysis revealed increased mRNA amounts of interleukin-2 (IL-2) and interferon-gamma (IFN-ɣ). The results suggest that the anti-M.tuberculosis IgY has significant potential as an immunotherapeutic that stimulates the immune response (Sudjarwo et al., 2017). Additionally, specific IgYs produced against multidrug-resistant strains of Acinetobacter baumannii have been reported to halt bacterial growth in mice in laboratory environments, significantly reduce the death rate of infected mice, and alleviate lung inflammation (Shi et al., 2017).

Additionally, the antibacterial effect of IgY against infections in aquatic creatures has also been investigated. IgY was produced against Vibrio spp., the leading cause of death of shrimps (Litopenaeus vannamei). It has been reported that using IgY in powder form, which contains anti-Vibrio IgY, significantly reduces the mortality rate of shrimps infected with V. harveyi and V. parahaemolyticus (Gao et al., 2016).

Another study investigating the effectiveness of IgY

produced against Propionibacterium acne bacteria reported that specific antibodies could be a potential alternative to antibiotics in the treatment of acne. (Revathy et al., 2014). Moreover, it examined the protective effect of egg yolk antibodies against Pseudomonas aeruginosa, which causes opportunistic infections and develops resistance to many antimicrobials. The research findings indicate that anti-P. aeruginosa IgY can enhance the cellular immune response, suggesting its potential use for prophylaxis in cystic fibrosis patients (Thomsen et al., 2016). IgY antibody purification has been evaluated for use in anti-venom serotherapy, specifically for Bothrops sp. venom. Anti-venom egg yolk antibodies have been produced and reported to effectively neutralize the venom with minimal side effects in mice (Araújo et al., 2010). In another study, anti-venom IgY was produced against the venom of Bothrops atox, also known as the Peruvian snake. The anti-venom IgY has been reported to demonstrate significant cross-reactivity with the venom of Bothrops brazili (Mendoza et al., 2012).

Conclusions

It is clear that the immunotherapeutic and immunoprophylactic use of IgY antibodies, as described above, to control various infectious diseases is beneficial. IgY antibodies, which can be produced and used similarly to mammalian IgG antibodies, are considered a valuable alternative therapeutic tool for treating many diseases in humans and domestic animals. It may also provide a new avenue for producing antidotes against natural toxins and serve as a diagnostic tool. Additionally, passive immunization with agent-specific IgY is a safer and non-toxic alternative to antibiotics and preserves fan welfare. In the future, IgY technology is expected to have increased benefits in both research and universal applications in medicine. However, this technology also has limitations. The principle of passive immunization provides short-term protection, which requires continuous application of antibodies in adequate doses. This can be costly for large-scale production. Therefore, new methods must be developed to produce high-quality antibodies economically and efficiently.

Moreover, precautions should be taken to increase the stability of egg yolk antibodies in the intestines and at high temperatures. Further researchs should be conducted on selecting adjuvants and immunogens to immunize chickens. This will increase the capacity to produce specific IgY antibodies against multiple pathogens simultaneously. Additionally, the use of new immunogens should be considered, as it can reduce the need for purification procedures and protein expression.

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The effects of culture media and media components on the development of rat embryos

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Review Article

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ABSTRACT

After in vitro culture of rat embryos, blastocyst rates are lower than the other species because of the embryonic block observed in the 2- or 4-cell stages in vitro. Optimal culture media and systems that provide variable physiologic needs in the different stages of rat embryos. The modifications of rat embryo culture media could have a positive effect on increasing the blastocyst rates. However, since the results of rat embryo studies are changed depending on factors like strains preferred, maintenance conditions and different commercial products added to the culture media, the success rate of producing healthy newborns for reproductive biotechnological studies has not yet reached the desired level by using current embryo culture media. Understanding the needs of rat embryos cultured from zygote to blastocyst stage in vitro is important for successful advanced studies such as cloning and transgenesis. The purpose of this review is the effects of different culture media and media components on the preimplantation stages of rat embryos and get a perspective for developing the culture media.

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Introduction

The laboratory rats are one of the most preferred species among laboratory animals for different research areas such as toxicology, biomedical engineering, biology, genetics, medicine and reproductive biotechnology (Iannaccone and Galat 2014, Hickman et al. 2017). There are similarities between rats and humans genetically, anatomically, and physiologically. Therefore, the rats can also contribute to cancer and other human disease studies (Iannaccone and Galat 2014, Agca 2019).

There are many difficulties due to the embryonic metabolism and culture conditions while developing the rat embryos from the zygote to the blastocyst stage. An embryonic developmental block occurs in in vitro culture and is generally caused during the zygotic gene activation phase (ZGA) which is happened in different embryonic stages for different mammalian embryos (Telford et al. 1990, Yamada and Nishikimi 1999) and ZGA happens in 2- or 4-cell stage in rat embryos (Mayer and Fritz 1974). To overcome in vitro embryonic developmental arrest, a lot of studies have been carried out (Zhang and Armstrong 1990, Kishi et al. 1991). On the other hand, the metabolism and developmental biology of rat embryos are not well understood because of the difficulties of getting high-quality fertile embryos through superovulation procedures (Miller and Armstrong 1981, Brison and Leese 1991). More efficient embryo culture media has been developed when starting to understand rat preimplantation embryo metabolism and physiology (Summers and Biggers 2003, Vajta et al. 2010, Men et al. 2023). To understand better the metabolism and in

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vitro culture needs of rat embryos, a retrospective examination should be done for rat embryo culture media in the present review, and it can help in getting perspective.

The overview of rat embryo culture media

The mammalian embryo culture media show similarity with physiologic salt solutions such as Earle Balanced Saline Solution, Tyrode solution and KRB (Krebs-Ringer bicarbonate); M16 media is based on Tyrode solution and Whitten's medium is derived from KRB (reviewed in Summers and Biggers 2003).

There was not any embryo culture medium that developed rat embryos successfully before the 90s (Miyoshi 2016), and some culture media has been improved with modifications after understanding the energy metabolism and physiology of mammalian embryos (Men et al. 2023). mKRB medium (modified Krebs-Ringer bicarbonate medium) has been developed by Toyoda and Chang (1974) and used as an in vitro fertilization medium in rats. Kishi et al. (1991) have used HECM-1 (chemically defined hamster 1-cell embryo culture media) to develop rat preimplantation embryos. Miyoshi et al. (1994) have developed mHECM-1 (modified hamster 1-cell embryo culture media) for rat embryo culture. Miyoshi et al. (1995a) has been studied on special media to develop rat embryos and designed R1ECM (rat 1-cell embryo culture medium). R1ECM has been improved by same group (Miyoshi et al. 1995b), and the media renamed as mR1ECM (modified rat 1-cell embryo culture medium). After the success of KSOM medium (Potassium simplex optimized media) in mouse embryo culture, KSOM-R was designed by Nakamura et al. (2016) for rat embryos. Also, M16 medium has been used experimentally to develop rat embryos (Popova et al. 2011).

Media components

Glucose and phosphate: Glucose and phosphate were thought to be the main reasons for the occurrence of developmental embryonic block of the preimplantation rat embryos cultured in vitro, although they are the components of oviduct fluid (Kishi et al. 1991, Miyoshi et al. 1994, Tsujii and Nakamura 1999). Moreover, glucose did not play a role in the rat embryos in the early division stages from 1-cell to morulae, neither promoting nor inhibiting effects have been shown (Miyoshi et al. 1994). However, preimplantation rat embryos in the later stages were affected positively by lower glucose concentrations in vitro. Some studies have shown that glucose uptake increased in the blastocyst stage for rat embryos (Brison and Leese 1991) and glucose enhanced the speed of embryonic development (Matsumoto and Sugawara 1995).

It has been reported that inorganic phosphate-included embryo culture media prevent the in vitro development of hamster and rat embryos critically (Schini and Bavister 1988, Yamada and Nishikimi 1999). Although low amounts of phosphate (0.001-0.01 µM) did not inhibit the embryonic development of rat embryos from zygote to blastocyst stage in the glucose-added HECM-1, 0.1 µM phosphate concentration blocked the development of 4-cell rat embryos to the morulae stage. The development of 2-cell rat embryos to the 4-cell stage was limited when increasing phosphate concentration, and 1-cell embryos did not proceed beyond to 2-cell stage in higher concentrations of phosphate (Miyoshi et al. 1994, Miyoshi et al. 1995a). It has also been reported that 1-cell rat embryos were not affected negatively by the low amount of phosphate and embryos, after they reached the 2-cell stage, developed to the blastocyst stage in the medium without phosphate (Matsumoto and Sugawara 1995). Miyoshi and Niwa (1997) however indicated that rat embryos beyond the 8-cell stage were not affected negatively by phosphate, and blastocyst formation and cell numbers increased in the culture media supplemented with phosphate. It has been shown that rat 1-cell embryos failed to reach the blastocyst stage in phosphate-supplemented KSOM-R, rather they were blocked at the 2-cell stage (Nakamura et al. 2016).

Amino acids: The importance of amino acids in the development of rat embryos was shown in different studies (Biggers et al. 1997, Ohboshi et al. 1998, Leese et al. 2021). Ammonium which is released after metabolizing amino acids has been reported to inhibit embryonic development of rat embryos cultured in vitro (Gardner and Lane 1993). Ho et al. (1995) found positive effects of low amounts of amino acids on mouse embryos; they also emphasized that embryonic development can be affected by the ammonium amount which can be adjusted by the volume of the media and embryo number per drop. Adding an Eagle's essential and nonessential amino acids has a beneficial impact on rat embryo development as same as the other species (Miyoshi et al. 1995a, Summers and Biggers 2003, Men et al. 2023).

For modeling the rat oviductal fluid, which is rich in taurine in all periods from the estrus cycle to the first three days of gestation, and contains high amounts of glycine, glutamate and alanine, the KSOM-R medium was developed based on KSOM-AA (amino acid added potassium simplex optimized media) (Nakamura et al. 2016). Adding amino acids to the

culture media has been shown to enhance the in vitro development of rat embryos and increase the blastocyst rate (Nakamura et al. 2016).

Protein sources: It has been known that the protein sources in embryo culture such as FBS (Foetal Bovine Serum Albumin), L-glutamine and BSA (Bovine Serum Albumin) are beneficial for mammalian preimplantation embryos (Kuran et al. 2001); contrary to this, there are opinions for that these sources have not positive effect on blastocyst formation (Gómez and Diez 2000). However, R1ECM which contains glutamine has been reported to support the development of rat embryos from morulae to blastocyst stage substantially, and it can be said that glutamine is an important chemical for in vitro culture of rat embryos (Miyoshi et al. 1995a). FBS, due to phosphate and growth factors it contains, has a deleterious effect on rat embryos from 1-cell to morulae (Han and Niwa 2003).

BSA which contains albumin can be added in embryo culture media as a resource of amino acids, fatty acids, and a small amount of vitamin sources, used to ease embryo manipulations, and additionally, used to be bound toxic heavy metals such as zinc and copper in culture media (Bavister 1995, Kito et al. 2008). Albumin is known to have both beneficial and detrimental effects in mammalian embryo culture (Bavister 1995). BSA has been understood to promote in vitro embryonic development of many mammalian embryos in the first cleavage stages and it is often used in mouse embryo culture media (McKiernan and Bavister 1992, Han and Niwa 2003, Zhou et al. 2003). Although hamster embryos are well developed in protein-free culture medium, it has been reported that BSA should not be considered completely ineffective for mammalian preimplantation embryos (McKiernan and Bavister 1992). Zhou et al. (2003) however reported that BSA harms rat embryos after the 1-cell stage. In contrast, it has been shown that BSA stimulated blastocyst formation and cell numbers in 2-cell rat embryos (Kito et al. 2008).

Phenol red: Phenol red is used as a pH indicator in cell culture media. Nakamura et al. (2016) reported that phenol red prevents blastocyst development of rat embryos in KSOM-R, thereby it has an estrogenic-like effect (Berthois et al. 1986, Ernst et al. 1989).

Osmolarity

The osmolarity of culture media is as important as media contents for all cell and embryo cultures. It has been shown that the role of the osmolarity of culture media in rat embryos is important (Miyoshi et al. 1994). Changing osmolarity depending on the presence of glucose and amino acids has been reported as a substantial factor in embryonic development in rats (Miyoshi et al. 1994, Ohboshi et al. 1998).

Miyoshi et al. (1994, 1995a) arranged medium osmolarity by changing NaCl concentrations and adding sorbitol and investigated the effects of osmolarity on rat embryo development. They found that rat embryos were sensitive to osmolarity and the optimal medium osmolarity for rat embryos was 244-246 mOsm. Sperm penetration in in vitro fertilization of rat embryos has been successfully achieved by increasing the NaCl concentration of mR1ECM (Oh et al. 1998). This situation has shown that rat embryos in early preimplantation stages could be developed in high osmolarity culture media as distinct from other species (Agca 2019). However, although the mR1ECM medium has a low amount of NaCl and its osmolarity is 246 mOsm, it can be increased the rate of blastocyst formation (Miyoshi et al. 1995a). It has been reported that KSOM-R (260 mOsm) was a supporting media for rat embryos, although it has a low osmolarity in comparison with embryo culture media used in other species (Nakamura et al. 2016, Men et al. 2020, Men et al. 2023). Considering all of these, it is possible to say that rat embryos have a different metabolism from other species (Popova et al. 2011).

The evaluation of rat embryo culture media success

Many researchers prefer to use mice in embryologic studies because mouse embryos are well known, and culture systems for mouse embryos successfully work. On the other hand, although rat, hamster, and rabbit embryos do not have improved embryo culture systems, using these animals in research is important for understanding the development of human preimplantation embryos (Seshagiri and Vani 2019). For this reason, it is very important to examine and develop the media used in the in vitro culture of rat embryos from past to present.

Kishi et al. (1991) have been reported that rat embryos cultured in HECM-1 were developed from zygote to blastocyst stage, but the blastocyst rate was low (9.9%). The more glucose-added HECM-1, which has low osmolarity by reducing the NaCl concentration and excluding amino acids from the medium, enhanced the development of rat embryos; after these changes, the rearranged medium was called mHECM-1 (Miyoshi et al. 1994). However, blastocyst formation (Table 1) in mHECM-1 has been shown that it does not better than mR1ECM or KSOM-R. Therefore, although it is not the first choice, mHECM-1 use as a culture medium may be preferred (Miyoshi et al. 1994, Miyoshi et al. 1995a, Nakamura et al. 2016).

Table 1. The development of rat embryos cultured in mHECM-1 medium.

References	Rat Strain	% zygote development to		
		≥2- cell	≥ 4- cell	Blastocyst
Miyoshi et al. 1994	Wistar	100	98	61
Matsumoto and Sugawara 1995	Wistar	94.4	93	71

Both in vitro fertilized or cultured rat zygotes cultured in mKRB (Table 2) have been reported to be blocked in the 2- or 4-cell stage (Toyoda and Chang 1974, Kishi et al. 1991). Moreover, in vivo developed rat embryos (obtained in 2- and 4-cell stages) could not arrive beyond the 4-cell stage when cultured in mKRB medium (Kishi et al. 1991). Rat 1-cell embryos obtained in vivo and developed in vitro have been reported that embryonic development was affected positively by transferring them to mR1ECM medium after being precultured with mKRB (Miyoshi et al. 1997). In similar research, short-term cultured rat embryos in mKRB before being cultured in mR1ECM have found that the blastocyst formation rate was higher than cultured only in mR1ECM (Kaneko et al. 2009). Therefore, it is possible to say using only mKRB medium as a culture media is not enough to develop rat embryos; however, if it is combined with other culture media, it can contribute to the development of rat embryos.

mR1ECM which is the most successful culture media than others has been reported as the only option to develop rat embryos for a long time (Nakamura et al. 2016), and is still used as one of the first choices as a culture medium due to obtaining very high blastocyst rates (Iannacone et al. 2001, Men et al. 2020). Although the number of live pups from the rat preimplantation embryos after in vitro culture in mR1ECM has been low (Miyoshi et al. 1995a, Kato et al. 2004, Nakamura et al. 2016), the rat preimplantation embryos have been developing in the mR1ECM medium successfully (Table 3).

KSOM medium which has high success rates in mouse embryo culture has been promising in developing rat embryos due to it can be editable for many species (Nakamura et al., 2016). The blastocyst

Table 2. The development of rat embryos cultured in mKRB medium and the collection time of embryos.

References	Rat Strain	Collection time of embryos	% embryos development to		
			≥2- cell	≥ 4- cell	Blastocyst
Kishi et al. 1991	Wistar	1-cell	76.2	4.1	0
Kaneko et al. 2009	Wistar	1-cell	90	42	28
Kishi et al. 1991	Wistar	2-cell	-	0	0
Kishi et al. 1991	Wistar	4-cell	-	0	0

Table 3. The development of rat 1-cell embryos cultured in mR1ECM medium.

References	Rat Strain	% zygote development to		
		≥ 2- cell	≥ 4- cell	Blastocyst
Miyoshi et al. 1995b	Wistar	100	73	58
Oh et al. 1998	Wistar	100	94.6	84.3
Han and Niwa 2003	Wistar	100	98	92
Iannaccone et al. 2001	Wistar Furth	56	35	0
Iannaccone et al. 2001	Lewis	13	13	9
Iannaccone et al. 2001	F344	72	42	12
Iannaccone et al. 2001	PVG	88	31	6
Kato et al. 2004	(SD x DA) x Wistar	99	41	33
Iannaccone et al. 2001	Sprague Dawley	96	94	76
Men et al. 2020	Sprague Dawley	95.9	61	18

Table 4. The development of 1- cell rat embryos cultured in KSOM-R medium.

References	Rat Strain	% zygote development to		
		≥2- cell	≥ 4- cell	Blastocyst
Nakamura et al. 2016	Wistar	~60	~85	~50
Men et al. 2020	Sprague Dawley	100	18	46

rates were found higher when 1-cell rat embryos (obtained 10-12 h after the ovulation) were cultured firstly in KSOM (for 18 h) and after in mR1ECM medium (Miyoshi et al., 1995b) than when cultured only in mR1ECM medium (Zhou et al. 2003). KSOM does not contain amino acids; KSOM-AA was developed after the importance of amino acids in the development of preimplantation embryos was understood. Moreover, KSOM-R which is based on KSOM-AA was designed by Nakamura et al. (2016) for rat embryos. In studies using mR1ECM and KSOM-R (Table 4) media, it has been shown both culture media promote the development of rat embryos cultured in vitro (Nakamura et al. 2016, Men et al. 2020, Men et al. 2023). However, the first research that compared these two culture media presented blastocyst rates of KSOM-R was better than mR1ECM (Men et al. 2020).

Conclusion

Rat embryo culture is not yet well understood, and the culture media need to improve. It can be possible to conclude that all present media for rat embryo culture are not optimal yet or that use of any of these media is not optimal for every strain or every research.

Since rat embryo culture media has been developed from the mouse embryo culture media, they all have similar contents. However, when compared with mouse embryo culture media, rat embryo culture media must have critical differences. Although these two species have similar genetic backgrounds, the present review shows that the effects of some of the culture media components on rat embryos are different from mouse embryos. Understanding these alterations is important to improve the current rat embryo culture media. Doing more investigations that compare mouse and rat embryos may help to understand rat embryos' needs in vitro.

Strain difference affects the success of culture media on the development of rat embryos in vitro; thereby, research with different rat strains may help to achieve optimal culture media or to understand which media is better for which strain.

mR1ECM medium has been successful in developing rat embryos from the 1-cell stage to the blastocyst stage, however, embryos developed in mR1ECM have a low capability of development in vivo and survival after embryo transfer. The in vitro development capability of culture media should support the subsequent in vivo development capability too. Therefore, it is possible to say that the mR1ECM medium still needs to be improved. Furthermore, although the results show that KSOM-R is successful in the development of rat embryos and their survival ability after embryo transfer, it needs more investigation for different strains.

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Prevalence of *Rotavirus*, *Coronavirus*, *Cryptosporidium* spp., *Escherichia coli* K99 and *Giardia lamblia* in neonatal calves with diarrhea in Burdur and its districts

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ABSTRACT

The aim of this study is to determine the prevalence of pathogens such as *Rotavirus*, *Coronavirus*, *Cryptosporidium* spp, *Escherichia coli* K99, and *Giardia lamblia* in neonatal calves with diarrhea in Burdur and its districts. The study material consisted of 96 diarrheic calves aged between 1 and 28 days from different cattle farms in the Burdur region. Fecal samples were collected, and the causative agents were identified using rapid diagnostic kits (BoviD-5 Ag Test Kit- BIONOTE). In the study, among the 96 diarrheic calves, a single enteropathogen was detected in 61 (63.54%), and 25 (26.04%) of these calves were positive for *Cryptosporidium*, 12 (12.5%) for *E. coli* K99, 11 (11.45%) for *Rotavirus*, 7 (7.29%) for *Coronavirus*, and 6 (6.25%) for *Giardia lamblia*, respectively. Multiple enteropathogens were responsible for diarrhea in 18 calves, and 2 (2.08%) of them were *Cryptosporidium* spp.+*Giardia lamblia*, 8 (8.33%) *Cryptosporidium* spp.+*Rotavirus*, 3 (3.12%) *Coronavirus*+*Rotavirus*, with 1 (1.04%) *Coronavirus*+*Cryptosporidium* spp., 2 (2.08%) *Rotavirus*+*E. coli*, and 1 (1.04%) of them was *E. coli*+*Cryptosporidi*um spp.+*Rotavirus*. Among the 96 diarrheic calves with mixed infections or a single enteropathogen, 38 (39.58%) had *Cryptosporidium*, 15 (15.62%) had *E. coli* K99, 26 (27.08%) had *Rotavirus*, 12 (12.50%) had *Coronavirus*, and 8 (8.33%) had *Giardia lamblia*. However, in 17 of the 96 diarrheic calves, neither mono- nor multiple enteropathogens were detected, suggesting that other factors might have caused diarrhea. Finally, this research provides valuable information for faster diagnosis, prevention, control, and treatment of enteropathogens causing diarrhea in neonatal calves in the Burdur region, contributing to reducing calf losses. It is believed that the research findings will shed light on future studies.

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Introduction

Diarrhea is a symptom that occurs due to infectious and non-infectious causes and is characterized by the volume of the stool, the amount of liquid it contains, and the increase in the frequency of excretion (Kozat, 2000; Geletu et al., 2021; Özbek et al., 2024). Neonatal calf diarrhea (NCD) poses a significant problem in cattle breeding, especially in newborn calves. Diarrhea causes loss of productivity and deaths in newborn calves and negatively affects the livestock sector economically (Khan and Khan, 1991; Hall et al., 1992; Radostits et al., 1994; Lorenz et al., 2011a). During pregnancy, ruminants do not transfer immunoglobulin from the mother to the fetus. For this reason, calves are usually born hypogammaglobulinemic or

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agammaglobulinemic and receive the necessary immunoglobulins through colostrum and milk after birth (Arda, 1994; Yılmaz and Akgül, 2014; Kozat, 2019).

Neonatal calves begin to produce their immunoglobulins on the 10th day after birth and reach normal plasma immunoglobulin levels by 60 days of age. This may cause infectious diarrhea to be common in newborn calves (Çakıroğlu et al., 2010; Yılmaz and Akgül, 2014; Kozat, 2019). During the first 28 days of newborn calves' lives, enteropathogens such as *Rotavirus*, *Coronavirus*, *Escherichia coli* (*E. coli*) and *Cryptosporidium*, alone or as mixed infections, are common factors that cause diarrhea in neonatal calves (Kozat, 2000). Among these enteropathogens, it has been reported that *Bovine Rotavirus* is the most common viral agent in neonatal calf diarrhea, along with *Bovine Coronavirus* (Woode et al., 1982; Snodgrass et al., 1986; Clark, 1993). Among bacterial factors, *E. coli* is an important enteropathogen in neonatal calves. Furthermore, *Cryptosporidium* spp. leads to a protozoal infection. The prevalence and zoonotic importance of parasitic agents such as *Giardia* spp. are increasing day by day. In addition to viral, bacterial and parasitic factors, non-infectious factors such as unfavorable shelter conditions, animal breeders with low education levels, insufficient colostrum intake and neglect of umbilical cord care are also effective in neonatal calf diarrhea (Kozat, 2000). In Turkey, many important studies are carried out to obtain healthy calves and protect them from diseases. In this context, it has been observed that research on the causes and factors that cause diarrhea, especially in calves, has increased rapidly in recent years (Kozat, 2000; Özkan and Akgül, 2004; İçen et al., 2013).

Rotaviruses destroy enterocytes during their journey from the oral tract to the small intestine, causing shedding in the intestinal cavity (Geletu et al., 2021). As a result of the replication of these viruses, disruptions occur in the digestion and absorption mechanisms (Snodgrass et al., 1980; Saklı, 2017).

Coronaviruses cause diarrhea due to decreased absorption and digestion, resulting in water and electrolyte loss (Clark, 1993). Diarrhea caused by Coronaviruses has a more severe course compared to Rotaviruses.

E. coli is another agent and is usually transmitted to calves orally during the neonatal period, but rarely it can also be transmitted through the umbilical cord. One of the most common enteropathogens that cause diarrhea is Enterotoxigenic *E. coli* (ETEC) (Şen et al., 2013). *Cryptosporidium* is transmitted to animals via the fecal-oral route (Olson et al., 1997). Infected calves expel oocysts along with their feces. Oocysts cause villous atrophy, degeneration and subsequent inflammatory changes, causing diarrhea (Sanford and Josephson, 1982). Giardia infection usually occurs at the end of the neonatal period and usually causes acute diarrhea (O'Handley and Olson, 2006).

The aim of this research was to identify specific enteropathogens and their prevalence in diarrhea occurring in neonatal calves in Burdur districts, and to provide a guide to veterinarians on prevention and control strategies. Besides evaluating the possible relationship between factors and fecal pH value, as well as investigating the existence of a potential correlation between fecal pH and calf ages were also aimed. The results of the study were intended to contribute to an observable reduction in deaths from neonatal calf diarrhea.

Material and Method

Animal material

This research was conducted on calves with neonatal diarrhea, which were reported to veterinary clinics operating in Burdur province and its districts (Ağlasun, Bucak, Çavdır, Gölhisar, Karamanlı, Kemer, Merkez, Tefenni, Yeşilova) between December 2022 and July 2023, and were determined by visiting various enterprises in the region. A total of 96 calves with neonatal diarrhea were evaluated, 47 of which were male and 49 were female. The calves included in the study consist of Holstein, Montofon, Simmental, Jersey and crossbreeds. When calves were included in the study, their previous illnesses and medication treatments were taken into consideration. During the clinical examination of the sick calves, the consistency, odor, content and color of the feces and the frequency of defecation were noted in detail. In addition, important factors such as the calves' age, breed, whether they received colostrum, body temperature, heart rate, dehydration degree, and diarrhea duration were evaluated.

Animals and ethical approval

Approval for this study was obtained from the Animal Experiments Local Ethics Committee of the Van Yüzüncü Yıl University (Date: 01.12.2022, Number: 2022/12-05).

Detection of enteropatogens in stool samples

Detailed systemic clinical examinations were performed on the diarrheal calves used in the study. During the examination, the consistency and content of the stool, the condition of the mucosa and the color of the conjunctiva were evaluated. In addition, clinical characteristics such as body temperature, skin elasticity, position of the eyeball in the orbit and

sucking reflex of the calves were examined. Rectal faecal samples were taken with a new plastic glove from each individual calf in accordance with the technique. Rapid test kits (Rapid BoviD-5 Ag Test kit; Cat. No: RC1302DD-Republic of Korea) were used to detect enteropathogens in stool samples. Before the test, kit box was carefully opened and materials such as test devices, assay diluent, and dropper were removed and checked for completeness. Then, a sample was taken from the diarrheal calf using a sterile fecal container and wearing gloves. The feces taken with the sampling apparatus were mixed with the assay diluent liquid and a homogeneous mixture was obtained by ensuring that the sample was completely dissolved. The sample taken from the resulting mixture with a dropper was carefully added to the sample section of the testing device and the process was completed. The test result was obtained by waiting 5-10 minutes for the sample to react with the test device. The test result was seen as agent negative or positive (Figure 1).

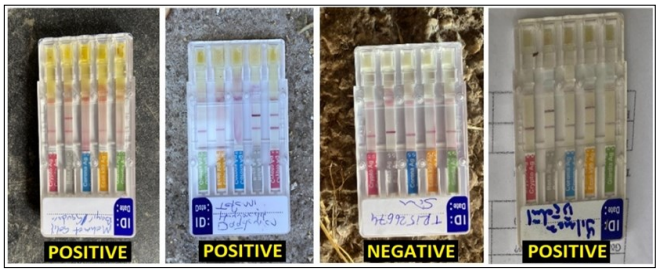

Figure 1. Positive and negative test results

Statistical Analysis

The obtained data were used to determine the rate and frequency of occurrence of the factors for descriptive statistics. SPSS (version-21) statistical package program was used in the calculations.

Results

Clinical findings

In the study, yellow watery, yellow gruel-like, yellow-green watery, green watery, green gruel-like, grey-white, brown, bloody, and mucous appearances were detected when the color, content and consistency of diarrhea were examined. The relationship between the appearance of feces and the factors is shown in Table 1.

Biochemical findings

In 17 of the diarrheal calves, none of the enteropathogens that could be determined by the test kit used were detected and this group was defined as other factors. While a single enteropathogen was detected in 61 of the remaining 79 calves, more than one enteropathogen was found in 18. Among the 61 calves in which a single enteropathogen was detected, the most common ones were *Cryptosporidium* spp. in 25, *E. coli* K99 in 12, *Rotavirus* in 11, *Coronavirus* in 7, and *Giardia lamblia* in 6 calves with diarrhea. According to the test results, mixed enteropathogens were detected in this study: *Cryptosporidium* spp.+ *Giardia lamblia* in 2 calves, *Cryptosporidium* spp. +

Table 1. The relationship between the color appearance of feces and the etiological agent

Agent	Yellow juicy	Yellow gruel-like consistency with blood	Yellow green liquid	Yellow green gruel-like consistency	Grayish-yellow liquid	grayish-dun liquid	other color	Total
Bovine Bovine rotavirus	5	3	1				2	11
Bovine Bovine coronavirus	2	1			2		2	7
E. coli	10	2						12
Giardia lamblia	2		1				3	6
Cryptosporidium spp	3	4	7	8	1	2		25
Bovine coronavirus + Bovine rotavirus	2				1			3
Bovine rotavirus + Cryptosporidium spp			2	1	4	1		8
Cryptosporidium spp + *Giardia lamblia*		1	1					2
Cryptosporidium spp+*Bovine coronavirus*	1							1
Bovine rotavirus +E. coli	1				1			2
Bovine rotavirus+Bovine coronavirus + Cryptosporidium spp.	1							1
Bovine rotavirus + Cryptosporidium +E. coli		1						1
Other factors (Unknown)	2	5	1	1	2	1	5	17

Rotavirus in 8 calves, *Coronavirus* + *Rotavirus* in 3 calves, *Coronavirus* + *Cryptosporidium* spp. in 1 calf, *Rotavirus* + *E. coli* in 2 calf, *E. Coli* + *Cryptosporidium* spp. + *Rotavirus* in 1 calf and *Coronavirus* + *Rotavirus* + *Cryptosporidium* spp in 1 calf. In addition, *Rotavirus* was detected in 27.08%, *Coronavirus* in 12.5%, *E. coli* in 15.62%, *Cryptosporidium* spp. in 39.58% and *Giardia lamblia* alone or simultaneously with other enteropathogens in 8.33% of the 96 examined calves (Table 2). The relationship between age and the prevalence of enteropathogens detected in calves with neonatal diarrhea in the Burdur region. The distribution of infectious agents detected in the study according to the ages of newborn calves with diarrhea is shown in Table 3 and Table 4. The age ranges where enteropathogens are most commonly seen are given in Table 5 and Figure 2. The relationship between age and the prevalence of enteropathogens detected in calves with neonatal diarrhea in the Burdur region.

Table 2. Ratio of etiological factors

Agent	Presence rate (%)
Cryptosporidium spp	25/96 = %26.04
E. coli	12/96 =%12.5
Rotavirus	11/96 = %11.45
Coronavirus	7/96 = %7.29
Giardia lamblia	6/96 = %6.25
Cryptosporidium+Rotavirus	8/96 = %8.33
Cryptosporidium+Giardia lamblia	2/96 = %2.08
Rotavirus+Coronavirus	3/96 = %3.12
Rotavirus + Coronavirus + Cryptosporidium spp	1/96 = %1.04
Rotavirus+Cryptosporidium+E.coli	1/96 = %1.04
Cryptosporidium spp+Coronavirus	1/96 = %1.04
Rotavirus+E. coli	2/96 = %2.08
Other factors	17/96 = %17.70

Table 3. Occurrence rate of infectious factors alone

Agent	Count	Percentage distribution
Rotavirus	26	%27.08
Coronavirus	12	%12.5
E. coli	15	%15.62
Cryptosporidium spp.	38	%39.58
Giardia lamblia	8	%8.33

Table 4. Age distribution according to factors in calves with diarrhea

Agent \ Age distribution	1-7 days	7-15 days	15-28 days	Total
Rotavirus	9	2	0	11
Coronavirus	6	1	0	7
Giardia lamblia	0	2	4	6
Cryptosporidium spp.	7	17	1	25
E. coli	11	1	0	12
Rotavirus + Cryptosporidium spp.	1	5	2	8
Cryptosporidium spp. + *Giardia lamblia*	0	1	1	2
Cryptosporidium spp. + *Coronavirus*	0	0	1	1
Coronavirus + Rotavirus	2	1	0	3
Rotavirus + E. coli	2	0	0	2
Rotavirus + Cryptosporidium spp. + E. coli	1	0	0	1
Rotavirus + Coronavirus + Cryptosporidium spp.	0	1	0	1
Other factors (unknown)	4	10	3	17
Total	42	41	13	96

Table 5. Minimum and maximum age range at which infectious agents are seen

Agent	Youngest age (d) (1-7)	Oldest age (d) (15-28)
Rotavirus	2	10
Coronavirus	2	10
E. coli	2	8
Giardia lamblia	12	26
Cryptosporidium spp.	6	16
Coronavirus + Rotavirus	2	8
Rotavirus + Cryptosporidium spp.	8	20
Cryptosporidium spp.+Giardia lamblia	9	16
Cryptosporidium spp+Coronavirus	17	17
Rotavirus + E. coli	3	4
Rotavirus + Coronavirus + Cryptosporidium spp.	14	14
Rotavirus + Cryptosporidium spp.+ E. coli	3	3
Other factors	2	25

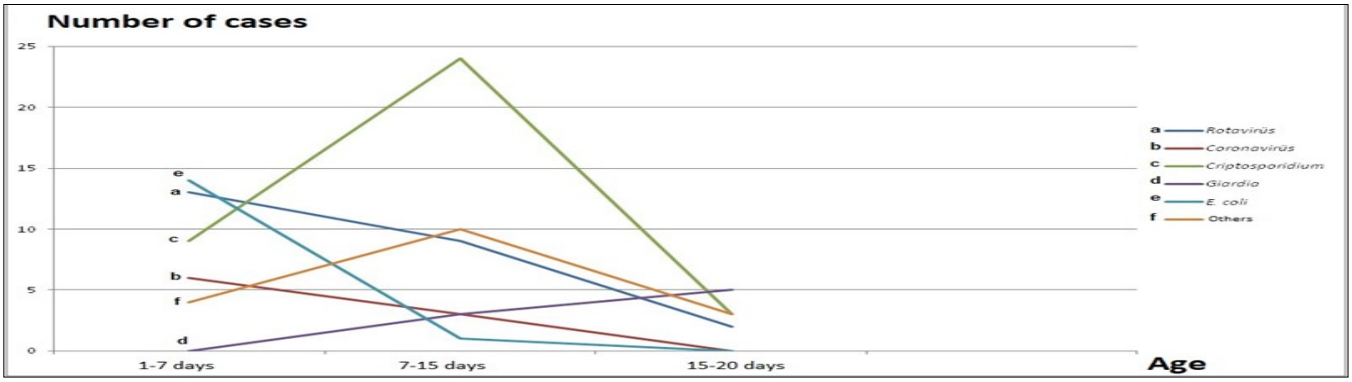

Figure 2. Relationship between age and prevalence of enteropathogens

The distribution of infectious agents detected in the study according to the ages of newborn calves with diarrhea is shown in Table 3 and Table 4. The age ranges where enteropathogens are most commonly seen are given in Table 5 and Figure 2.

Discussion

Despite modern veterinary practices, it is a fact that calf diarrhea still causes economic losses in cattle farms. In addition to diarrhea treatments, determining etiological factors and taking preventive measures is becoming increasingly important.

This study aims to provide a perspective on treatment strategies by determining the etiological factors affecting neonatal calf diarrhea in Burdur province and its districts. Diarrhea in newborn calves is a serious problem in the cattle industry, with high morbidity and mortality (Walker, 1998). Worldwide, one of the main causes of calf deaths and financial losses to the cattle industry is losses due to diarrhea (Wudu et al., 2008; Kozat, 2019). It is stated in many studies that various factors play a role in the emergence of diarrhea and that environmental factors, the effects of infectious agents, nutrition and many factors affecting the immune system interact in a complex manner (Waltner-Toews et al., 1986).

Protective measures starting from the pregnancy period are of great importance to reduce the frequency of calf diarrhea (Kozat, 2019). It has been emphasized in many studies that taking the right precautions in cases of diarrhea, especially giving colostrum on time and sufficiently, plays a critical role in strengthening the immune system of calves (Göncü, 2013; Şen et al., 2013). In addition to colostrum, improving the housing conditions of enterprises and ensuring hygiene can make significant contributions to the growth of calves in a healthier and more disease-resistant manner (Kozat, 2000). Within the scope of this study, we evaluated the reasons why some enterprises encounter calf diarrhea less frequently during our field visits to enterprises. They stated that the vaccination practices they carried out especially when the mother was in the dry period were effective in this case. It has been observed that in farms where these vaccination applications are carried out successfully, the immune systems of calves are strengthened and health problems such as diarrhea are prevented. It has been determined that adequate colostrum intake, as well as appropriate shelter conditions and balanced nutrition, are of great importance in raising healthy and disease-resistant calves during the neonatal period.

Breeders should be informed about the amount and timing of colostrum to be given to calves with neonatal diarrhea. However, despite these positive practices, it was determined that the housing conditions of the calves were not sufficient in most of the farms where the disease was detected during the field study.

Living conditions cause calves to decrease their resistance to diseases and cause health problems such as diarrhea, as they are not sheltered in a hygienic environment and proper care. These research findings are in line with the statements of previous researchers (Waltner-Toews et al., 1986; Göncü, 2013; Şen et al., 2013) about the importance of colostrum, as well as the findings of researchers about care and hygiene conditions (Kozat, 2000; Kozat and Tuncay, 2018).

Findings of this study support the data and emphasize that raising a healthy calf requires a multi-factor approach.

According to statistical data on diarrhea cases, the rate of diarrhea cases in newborn calves is over 50%, and the calf mortality rate varies between 1.5 and 8% depending on these cases (Frank and Kaneene, 1993). Similarly, in Turkey, calf diarrhea is accompanied by high levels of morbidity and mortality. This situation is still an important problem today due to reasons such as high treatment costs, poor performance and death which cause economic losses (Uzlu et al., 2010; Taş and Kozat, 2023).

According to recent research, cases of diarrhea in calves in the first four weeks after birth have been associated with various pathogens (Table 1). Microorganisms such as *Rotavirus*, *Coronavirus*, *E. coli*, *Cryptosporidium* and *Giardia* are stated as the most important causes of calf diarrhea (Khan and Khan, 1991; de La Fuente et al., 1998; Langoni et al., 2004; Lorenz et al., 2011b). It is emphasized that there is no single reason for the occurrence of neonatal calf diarrhea, instead, more than one factor plays a role, and regular and accurate fluid-electrolyte treatment is as important as an effective chemotherapy treatment to reduce high mortality rates due to diarrhea (Cho and Yoon, 2014).

According to the analysis results of this study, 61 of 96 calves had a single enteropathogen. Among these calves, *Cryptosporidium* spp. was detected in 25 (26.04%) of them, *E. Coli* K.99 in 12 (12.5%), *Bovine rotavirus* in 11 (11.45%), *Bovine coronavirus* in 7 (7.29%), and *Giardia lamblia* was detected in 6 (6.25%) of these calves.

Additionally, it was revealed that mixed infections played a role in 18 (18.75%) of 96 calves with diarrhea (Table 3). In this study, it was revealed that the enteropathogens detected in neonatal calves with diarrhea were similar to the infectious agents identified by previous researchers (Khan and Khan, 1991; de La Fuente et al., 1998; Langoni et al., 2004;

Lorenz et al., 2011b; Cho and Yoon, 2014).

It has been determined that providing appropriate hygiene conditions, as well as considering multiple etiological factors, plays a critical role in controlling and preventing the disease. These findings emphasize the need for a holistic approach to effectively manage diarrheal cases. Diarrhea occurring in neonatal calves significantly affects animal productivity losses and calf mortality rates. Therefore, it is of great importance to quickly identify the factors that cause diarrhea and apply effective treatment methods in order to reduce diarrhea-related losses and alleviate its effects (Kalınbacak, 2003; Murat and Balıkçı, 2012; Kozat and Tuncay, 2018). For this purpose, rapid diagnostic test kits are widely used (Al and Balıkçı, 2012; Kozat and Tuncay, 2018; Bal, 2019).

Immunochromatographic ready-made diagnostic kits help quickly and effectively detect the causes of diarrhea in newborn calves. These kits have an important role in obtaining rapid results under field conditions, being low-cost, easy to apply, and in detecting more than one enteropathogenic agent (Kozat ve Tuncay, 2018; Taş ve Kozat, 2023).

In this study, a commercial immunochromatographic test called Rapid Diagnostic Test (BoviD-5 Ag Test Kit - BIONOTE) was used to quickly determine the factors that cause diarrhea in newborn calves in the Burdur region. This test delivered the opportunity to detect *Rotavirus*, *Coronavirus, Cryptosporidium*, *E. coli* K99 and *Giardia lamblia* from fresh stool samples in a short time of 5-10 minutes.

According to the results of the study, in cases where a single enteropathogen was detected in 61 calves with diarrhea, the most common ones were *Cryptosporidium* in 25 (26.04%), *E. coli* K99 in 12 (12.5%), *Rotavirus* in 11 (11.45%), and *Rotavirus* in 7 (11.45%). In 6 cases (7.29%), *Coronavirus* and 6 cases (6.25%) were caused by *Giardia lamblia* (Table 3). In addition, among the 96 calves with diarrhea, 38 (39.58%) had *Cryptosporidium*, 15 (15.62%) had *E. coli* K99, 26 (27.08%) had *Rotavirus*, 12 (12.50%) had Coronavirus (12.50%) had *Cryptosporidium* (8.33%) and *Giardia lamblia* agents were detected (Table 4).

Many studies have been conducted on *Rotavirus,* which causes diarrhea in newborn calves in Turkey. Burgu et al. (1995) reported in a study that the rotavirus rate was determined to be 33.6%. In the research of Al and Balıkçı (2012), this rate was found to be 30%. In a different study, Alkan (1998) detected 53% *Rotavirus* in Ankara. In another study conducted in Ankara, Alkan et al. (1992) detected *Rotavirus* at a rate of 26.8%. In the study conducted by Erdoğan (2003) in Kars, this rate was found to be 26.9%. In a study conducted in Manisa, the rate determined by Bal (2019) was reported as 14%. In the study conducted in Van, Çabalar et al. (2007) detected *Rotavirus* at a rate of 17.97%. In this study, the *Bovine rotavirus* incidence rate was found to be similar compared to other researchers, but it was determined to be higher than the results obtained from some researchers (Çabalar et al., 2000; Bal, 2019). It is thought that these differences may result from regional climate and environmental conditions. Additionally, it is taken into consideration that differences in the age range of calves may also affect these results. *Rotavirus* infections can often occur as mixed infections (Garcia et al., 2000). In this study, mixed *Rotavirus* infection was detected in 26 of 96 calves (27.08%) in total. The single infection rate was found to be 11.45%.

In addition, it has been determined that the stool in diarrhea due to *Rotavirus* infection is generally yellow and watery. These findings show that *Rotavirus* infection may begin at an early age in neonatal calves and that the risk of disease continues at young ages. The findings of this study support the data of previous researchers (Rodger et al., 1982; Walker et al., 1998).

In many studies conducted on *Bovine Coronavirus* in Turkey, different rates were obtained in different regions. While the *Coronavirus* rate was determined as 13% in Al and Balıkçı's (2012) study in Elazığ. In other studies, *Coronavirus* rates are as follows: 7% in the study conducted in the Siirt region (Kozat and Tuncay, 2018), 9.35% in the Tokat region (Kaya and Coşkun, 2018), 9% in the Manisa region (Bal, 2019), 9% in the Sivas region (Kuliğ and Coşkun, 2019), 25% in the Muş region (Taş and Kozat, 2023). These results show that geographical differences and climatic conditions in Turkey may affect the spread of *Bovine Coronavirus*.
In this study, the diarrheal conditions of neonatal calves aged 1-28 days were examined in detail, and in total, *Coronavirus* was detected alone in 5 of 96 neonatal calves with diarrhea, and the number of infected calves reached 12 with mixed cases. In this context, while the rate of *Coronavirus* being found alone was 5.20%, the rate of mixed infection increased to 12.5%. These results reveal that *Coronavirus* rates may vary in studies conducted in different regions. In this research, it was observed that the smallest Coronaviruses were seen in calves that were 2 days old and the largest were 17 days old. This highlights that *Coronavirus* is a significant health threat to calves and the importance of preventive and therapeutic interventions initiated early in the disease. Additionally, diarrhea in *Coronavirus* infected animals has been observed to be yellow-green in color. In this study, it was determined that 7 of the stool colors of

12 sick animals diagnosed with *Coronavirus* were yellow, 3 were green and the other 2 were different colors. This information represents an important observation in terms of evaluating the clinical symptoms.

Cryptosporidium species are zoonotic protozoans that cause gastrointestinal infections in various species (Guerrant, 1997; Nguyen et al., 2007). Many studies have been conducted in different regions regarding the prevalence of *Cryptosporidium* spp in Turkey. 26.7% in Karacabey-Bursa (Burgu, 1984), 7% in Sivas (Kuliğ and Coşkun, 2019), 5.12% in Siirt (Kozat and Tuncay, 2018), 10.7% in Aydın (Özlem et al., 1997), 7.2% in Elazığ (Özer et al., 1990), 25.7% in Kars (Arslan et al., 2003), 27.33% in Konya (Sevinç et al., 2003), 35.8% in Ankara. (Sahal et al., 2005), 22.8% in Erzurum (Sarı et al., 2008), 20.7% in Nevşehir (Şimşek et al., 2012), 11.21% in Tokat (Kaya and Coşkun, 2018), 5.12% in Muş (Taş and Kozat, 2023).

In this study, while only *Cryptosporidium* infection was detected in 13 (13.54%) of 96 calves with neonatal diarrhea aged 1-28 days, it was determined that 38 (39.58%) had other factors along with *Cryptosporidium* infection. This shows that *Cryptosporidium* can be effective alone or in combination with other factors to cause diarrhea. It is stated that there are many risk factors affecting the prevalence of *Cryptosporidium* spp. Factors such as the number of animals in the enterprise, the age of the animals, whether they have diarrhea or are healthy, shelter type, suckling status, litter type and water source. It may have an impact on the prevalence of *Cryptosporidium* infections (Brook et al., 2008; Trotz-Williams et al., 2008). When we look at *Cryptosporidium* infections in Turkey and around the world, we see that the prevalence rates are different. It is thought that the reasons for these differences may be related to the geographical and climatic characteristics of the region where the studies were conducted, animal breeding practices, isolation of sick animals and the effect of environmental factors.

In addition, it was stated in the study that *Cryptosporidium* infection had been seen in certain enterprises before, but the disease was prevented by using a drug called halofuginone. Although *Cryptosporidium* infection has been seen in calves between the ages of 3 and 20 days, it has generally been observed that the calves are between the ages of 7-15 days. These findings show that *Cryptosporidium* infection can be seen in neonatal calves in the first week and is more common in calves in the first week or two. *Escherichia coli* infections are considered one of the leading causes of calf diarrhea, which usually occurs within 2-10 days after birth, and can rarely be seen within the first 24 hours after birth (Bilal, 2007). *Escherichia coli*, which is frequently encountered in the neonatal period, especially in 1-7 day(s) old calves, stands out as one of the main causes of diarrhea worldwide (Rodostitis et al., 2007). It has also been stated that enteropathogenic *E. coli* can be seen in neonatal calves within the first 30 days (Rodostitis et al., 2007).

E. coli infection rates also vary in studies conducted in Turkey. For example, in a study conducted in Siirt and its surroundings, the incidence of *E. coli* alone was reported as 6%, while the incidence rate in mixed was reported as 18% (Kozat and Tuncay, 2018). In a study conducted in the Tokat region, the rate of *E. coli* in calves with diarrhea was found to be 7.48% (Kaya and Coşkun, 2018). In Elazığ, the prevalence of *E. coli* in calves with diarrhea was determined as 16.66% (Al and Balıkçı, 2012). In this study, only *E. coli* infection was detected in 12 of 96 calves with neonatal diarrhea aged 1-28 days, and other factors were observed in 3 of them along with *E. coli* infection. While the rate of *E. coli* being found alone was 12%, the rate of being found together with other enteropathogens increased to 15.62% (Table 3, Table 4). It was determined that the majority of the feces were yellow and watery (Table 1) and the age range of 14 out of 15 *E. coli* infected calves was 1-7 days old (Table 5). This study confirms that the prevalence of *E. coli* infection varies between countries and regions and is frequently seen in the neonatal period, especially in calves 1-7 days old. Additionally, it was determined that *E. coli* infection was seen in calves that were at the youngest age of 2 days old and in calves that were at the oldest age of 8 days old (Table 5). These findings show that *E. coli* is a common infectious agent in calves during the neonatal period and that the risk of infection is higher in the first week.

Giardia lamblia is becoming increasingly important as it causes growth retardation in farm animals, reduces feed utilization and causes economic losses by causing diarrhea (O'Handley et al., 2003). *Giardia lamblia*, which causes significant economic losses in calf diarrhea in Turkey, is among the etiological factors that are generally ignored in some studies (Ayan et al., 2016). In another study, the presence and prevalence of *Giardia* in the feces of 10,672 cattle between the ages of 1-730 days was evaluated and *Giardia* was detected in 1,236 feces. It was determined that *Giardia* agents continued to spread as cysts in 1,184 of the animals in which *Giardia* was detected (Mark-Carew et al., 2010). In studies conducted in Turkey, Göz et al. (2006) detected *Giardia* at a rate of 14.7% in 231 newborn and young calves aged between 1 day

231 newborn and young calves aged between 1 day and 8 months in Van, and Kaya and Coşkun (2018) detected *Giardia* at a rate of 16.82% in 107 neonatal calves with diarrhea in Tokat. In this study, *Giardia* infection was detected in 6 of 96 diarrheal neonatal calves aged 1-28 days, and other factors were observed in 2 of them along with *Giardia* infection. While the rate of *Giardia* being found alone was 6.25%, the rate of being found together with the mixture increased to 8.33% (Table 3, Table 4). In this study, it was observed that the *Giardia* agent was seen in neonatal calves between 9 and 26 days old, but was mostly seen in calves older than 15 days (Table 4, Table 5). These results suggest that *Giardia* infection may be a cause of diarrhea in neonatal calves in Burdur and its districts and that the risk of infection may increase, especially after 15 days of age.

This research examined the cases of diarrhea in newborn calves in Burdur and its surroundings and identified the important enteropathogens that cause diarrhea. It was concluded that diarrhea in newborn calves poses a significant problem for the cattle industry, causing high mortality and yield losses. Rapid Diagnostic Test (BoviD-5 Ag Test Kit - BIONOTE), an immunochromatographic test, was used to detect the factors that cause diarrhea. Thanks to this test, the pathogens *Rotavirus, Coronavirus, Cryptosporidium, E. coli* K99 and *Giardia lamblia* were quickly detected. According to the research results, the infectious agents were determined to be *Cryptosporidium, Rotavirus, E. coli* K99, *Coronavirus* and *Giardia lamblia*, respectively. *Cryptosporidium, Rotavirus, E. c*oli K99, *Coronavirus* and *Giardia lamblia* infections were determined to be the most common factors in neonatal calf diarrhea. A difference was observed between the rates of occurrence of these factors alone and in mixed forms. *Cryptosporidium* and *Rotavirus* infections were determined to be the most common factors in neonatal calf diarrhea. Considering that these factors can be seen in the first two weeks of the neonatal period, the importance of preventive measures in the emergence of diarrhea in neonatal calves was emphasized. The study pointed out that the shelter conditions and hygiene of businesses are critical in the emergence of diarrhea.

As a result, in order to control the cases of diarrhea in newborn calves in Burdur and its districts, and to reduce economic losses, the factors must be identified quickly and accurately. The application of effective treatment methods, producer training for raising healthy and productive animals in the cattle industry, animal care and nutrition, and standardization of shelter conditions should be taken. It was emphasized that it is important to make this possible and take protective measures. It is thought that this study will shed light on future research.

Conflict of interest

In this study, I declare that there are no conflicts of interest among the authors.

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