DOI Link: https://doi.org/10.59671/cU81t
Vol.33, Issue.7, Part.1, July 2025, PP. 2-22

Metagenomic Investigation of Core Fecal Microbiota in Water Buffaloes

A.Ilgın KEKEÇ[1], H.Tuğba YÜKSEL DOLGUN[2], Serkan İKİZ[1], K. Serdar DİKER[2]

[1]Istanbul University-Cerrahpasa Faculty of Veterinary Medicine, Department of Microbiology, 34500, Buyukcekmece, Istanbul, Türkiye
[2]Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Microbiology, 09010, Efeler, Aydin, Türkiye

Received April 2025 Accepted June 2025 Published July 2025

ABSTRACT

This study aimed to investigate the fecal microbiota of buffaloes of different breeds, ages, and genders reared in Türkiye using metagenomic analysis.

For this purpose, a total of 96 stool samples were collected from four farms in Istanbul and Kırklareli, including 48 from buffalo calves and 48 from adult buffaloes. Among these 24 buffalo calves and 24 adult buffaloes belonged to the Italian Mediterranean breed while the remaining 24 of each group were of the Anatolian breed. Additionally, for both the Anatolian and Italian Mediterranean breeds, an equal number of males and females (12 each) were selected. After being transported to the laboratory under appropriate conditions, DNA was extracted from the samples. The samples were poolled into groups of four, resulting in a total of 24 pooled samples. From the extractions, 24 pools of 4 were created according to age and gender. Sequencing was performed using the Oxford Nanopore MinION, followed by bioinformatics analyses.

A total of 180 MB of sequencing data was obtained. Based on analysis, 489 operational taxonomic units (OTUs) were identified. Among the groups, the highest number of OTUs (275) was detected in Anatolian buffalo calves younger than 3 months (Group 1). In contrast, the lowest number of OTUs (167) was observed in pregnant females older than two years from the Italian Mediterranean breed. At the phylum level Firmicutes (68.3%), Bacteriodetes (29.4%) and Proteobacteria (1.8%) were found to be the dominant phyla across all groups. The Firmicutes/Bacteriodetes ratio was highest (2.99%) in Anatolian buffalo calves older than three months, while the lowest ratio (1.57%) was recorded in Italian Mediterranean buffalo calves of the same age group. Notably, the Proteobacteria rate reached 9.27% in female Anatolian buffaloes older than two years. At the family level, Ruminoccaceae (47.8%) was the dominant family across all groups. At the genus level, *Ruminnococcus* (39.6%) was the dominant genus in all groups except one, where *Lactobacillus* (20.4%) was the most abundant genus in buffalo calves younger than three months that were fed with milk and feed.

As a result, microbial colonization data were obtained for both buffaloes breeds in Türkiye. While this study contributes to the limited literature on this subject, further research is needed to support and expend these findings.

Key Words: Buffalo, Microbiota, Next generation sequencing, Third generation sequencing

INTRODUCTION

The term microflora, which has been used for many years to describe all microorganisms inhabiting a spesific environment, has increasingly been replaced by microbiota in recent years (Bleich and Fox, 2015; Diker, 2017). The complete genetic material of the microorganisms constituting the microbiota is referred to microbiome (Liu, 2016). Microbiota is a highly diverse community comprising viruses, bacteria, fungi and protozoa, with microbial cells outnumbering all the cells in the body. Although microorganisms are generally associated with diseases, they colonize various body sites, particularly mucosal surfaces, where they serve as barrier against infections (Tlaskalová-Hogenová et al., 2004; Luczynski et al., 2016). The bacterial composition of the microbiota, particularly the intestinal microbiota, is closely linked to disease pathogenesis, metabolic and physiological processes, including immune system function, as well as feed utilization and productivity in animals. Due to these positive roles, the microbiota often referred to as the “second brain” (Diker, 2017; Hashimoto-Hill and Alenghat, 2021; Küllük and Dalğın, 2021). Microbiota colonization is influenced by both individual and environmental factors, leading to significant inter-individual differences. Identifying the microorganisms that constitute the microbiota can help elucidate these differences and provide insights into the intricate relationship between health and disease (Altuntaş and Batman, 2017; Diker, 2017).

Although microbiota was initially studied using culture based methods, these techniques limit the isolation and identification of the millions of bacteria, both aerobic and anaerobic, present in the microbiota. Therefore, non-culture-based metagenomic methods became necessary (Fouhy et al., 2012; Hiergeist et al., 2015; Gürsoy and Otlu, 2017; Tural et al., 2024). Metagenomic methods involve determining the nucleotide sequence that make up DNA, which contains crucial genetic information, anda re referred to as sequencing (Kızmaz et al., 2017). Oxford Nanopore, a third generation sequencing method that is widely used, is preferred over older methods due to its ability to generate long reads in a short time. This approach provides, more efficient results in less time with reduced labor (Kekec et al., 2022; Schadt et al., 2010; Lee et al., 2016). Microbiome research, which began the Human Genome Project, has expanded into veterinary medicine. In addition to elucidating the relationship between disease and health, it now also serves the purpose of enhancing the productivity offspring and improving the quality of animal products such as meat, milk, eggs and wool (Turnbaugh et al., 2007; Altuntaş and Batman, 2017; Diker, 2017; Anonymous). In this regard, it also contributes to public health by improving the quality of animal products consumed as food for humans. Moreover, microbiota transplantation is being explored as a potential method for controlling and treating diseases in both humans and animals, offering a promising alternative to antibiotics. This approach is considered a potential solution to the global problem of antibiotic resistance (Hashimoto-Hill and Alenghat, 2021; Küllük and Dalğın, 2021; Gupta et al., 2015; Garmendia et al., 2012; Altuntaş and Batman, 2017; Amon and Sanderson, 2017).

Studies have primarily focused on the rumen, gastrointestinal system, and milk in cattle, the gastrointestinal system in chickens, and the rumen and milk

microbiota in buffaloes, with relatively limited research on fecal microbiota. In Türkiye, chickens, cows, sheep, goats and buffaloes are the primary animals raised from their products. Buffaloes are considered a favorable alternative for humans due to their ease of breeding, lower costs, and the superior quality of their milk and meat compared to cattle (Du et al., 2019; Siddikiy and Faruque, 2018; Cruz-Monterrosa et al., 2020; Sarıözkan, 2011; Pasha, 2013).

This study aimed to investigate the intestinal microbiota, often referred to as the "second brain", of Anatolian buffaloes, known for their high diseases resistance and ability to adapt to harsh environmental conditions, and Italian Mediterranean buffaloes, recognized for their high productivity traits (Sarıözkan, 2011). The stıdy also sought to explore potential differences in microbiota based on breed, gender, age, pregnancy and feed consumption, using Oxford Nanopore method, a third-generation sequencing technique.

MATERIAL AND METHODS

Samples

A total of 96 fecal samples were collected from four farms in Istanbul and Kırklareli, originating from 48 male and 48 adult buffaloes, all of which had not previously been treated with antibiotics. Of the 48 males, 24 were selected as Anatolian buffaloes (native breed) and 24 as Italian Mediterranean buffaloes. The buffaloes were sampled without gender discrimination, assuming they were less than one year old and had not yet reached sexual maturity. For the 48 adult buffaloes, 24 were selected as Anatolian buffaloes (12 females, 12 males), and 24 as Italian Mediterranean buffaloes (12 females, 12 males). The fecal samples used in the study were collected directly from the rectum between August 2019 and February 2020. Information on breed, age, gender, and pregnancy status was recorded for each animal. The collected fecal samples were immadiately transferred to tanks containing liquid nitrogen -196°C and were stored at -80°C until sequencing analysis was performed.

DNA Extraction

DNA isolation from the collected stool samples was performed using the Qiagen Stool kit (cat. No. 51504) according to the manufacturer's protocol. From the 96 DNA extracts obtained, 24 DNA pools were created, each containing equal amounts of DNA, categorized by breed, age, gender, nutrition and pregnancy status.

Microbiota Analysis

After pooling the DNA extracts, the DNA concentration of the pools was measured using the Thermo Fisher Scientific Qubit 2.0 Fluorometric Quantification device and Qubit™ 1X dsDNA HS Assay Kit (Q33230). The DNA concentrations were then adjusted following the Oxford Nanopore rapid sequencing 16S Barcoding Kit (SQK-16S024) protocol. Finally, barcoding and PCR procedures were performed as described in Tables 1 and 2.

Table1:PCRmix

LongAmp Taq 2X Master Mix	25 µl
Barcod Primer	1 µl
Template DNA	24 µl
Total	50 µl

Table 2: PCR cycle conditions

Temperture	Time	
95°C	3 min.	
95° C	15 s.	
62 °C	15 s.	15 cycle
65 °C	15 s.	
65 °C	5 min.	
+4 °C	∞	

Sample groups corresponding to the barcode numbers resulting from the barcoding PCR are provided in Table 3.

Table 3: Sample groups corresponding to barcode numbers.

BARCODES	GROUPS
BC 01	Anatolian buffalo calf, < 3 months, milk and feed (Group 1)
BC 02	Anatolian buffalo calf, < 3 months, milk and feed (Group 2)
BC 03	Anatolian buffalo calf, < 3 months, milk and feed (Group 3)
BC 04	Anatolian buffalo calf, < 3 months, milk and feed (Group 4)
BC 05	Anatolian buffalo calf, > 3 months, only feed (Group 5)
BC 06	Anatolian buffalo calf, > 3 months, only feed (Group 6)
BC 07	Italian Mediterranian calf, < 3 months, milk and feed (Group 7)
BC 08	Italian Mediterranian calf, < 3 months, milk and feed (Group 8)
BC 09	Italian Mediterranian calf, < 3 months, milk and feed (Group 9)
BC 10	Italian Mediterranian calf, < 3 months, milk and feed (Group 10)
BC 11	Italian Mediterranian calf, > 3 months, only feed (Group 11)
BC 12	Italian Mediterranian calf, > 3 months, only feed (Group 12)
BC 13	Anatolian buffalo, > 6-7 years, Female (Group 13)
BC 14	Anatolian buffalo, > 2 years, Female (Group 14)
BC 15	Anatolian buffalo, > 2 years, Female (Group 15)
BC 16	Anatolian buffalo, > 2 years, Male (Group 16)
BC 17	Anatolian buffalo, > 2 years, Male (Group 17)
BC 18	Anatolian buffalo, > 2 years, Male (Group 18)
BC 19	Italian Mediterranian buffalo, > 2 years, Female, **pregnant** (Group 19)
BC 20	Italian Mediterranian buffalo, > 2 years, Female (Group 20)
BC 21	Italian Mediterranian buffalo, > 2 years, Female (Group 21)
BC 22	Italian Mediterranian buffalo, > 2 years, Male (Group 22)
BC 23	Italian Mediterranian buffalo, > 2 years, Male (Group 23)
BC 24	Italian Mediterranian buffalo, > 2 years, Male (Group 24)

The products were purified using AMPure XP (Beckman Coulter®) after the PCR protocol. After analyzing the intensities of the purified products with Qubit 2.0 fluorometer (ThermoFisher Scientific®), all samples were collected in an eppendorf tube to create barcoded DNA pools. One microliter of RAP was added to the pool for the binding of the adapters and the mixture was incubated for 5 min. The product was purified with AMPure after the adapters had bound. Then, the loading buffer was prepared and the product was loaded onto the device following the protocol guidelines. The Flowcell (Flow Cell R9 Version Nanopore®) to be used was checked using the Minknow program, and the loading process was performed. The Flow Cell Priming Kit (Nanopore®) was used for the loading procedure and the process was completed according to the protocol. After the loading process was completed, the products were transferred to the Flow Cell MinION (Nanopore®) device and a 24-hour sequencing protocol was performed

using the MinKnow program. Bioinformatic analysis was performed on the obtained data. After the completion of sequencing, the data were converted from fast5 to fastq using guppy v3.1.5 software (base calling and demultiplexing). Barcode and adapter sequences were cleaned using Porechop v0.2.3. Additionally, 45 bases were deleted from both edges of the sequences to remove universal primers and tags. After cleaning the sequences, reads of 1350–1550 bp were filtered, and the remaining reads were excluded. The cleaned reads were analyzed using mothur v.1.39.5 platform (custom workflow analysis). Sequences were screened for chimeric structures, aligned, and distances between sequences and the similarity matrix were measured. Reads showing 99% or higher similarity were grouped together, thus forming OTUs. The generated OTUs were compared against the RDP 16S rRNA database and taxonomic annotations were assigned. Statistical results were obtained by associating the same sequenced OTUs. Graphs were generated using Minitab and R programs.

To determine the core microbiota from the comprehensive data obtained, taxa present at more than 1% were evaluated (O’donnell et al., 2017).

The obtained data were filtered based on family, genus and species and the variation between groups was examined. The similarity of the samples to each other was analyzed by determining their diversity and abundance, and dendograms were created.

Principle Coordinate Analysis (PCoA), a distance-based analysis that identified the maximum variance to detect the correlations between variables in multidimensional data, was used to perform principal component analysis of the samples at all levels and specifically at the genus level.

RESULTS

As a result of metagenome analysis, 180 MB of data was obtained. Based on these data, the OTU numbers from the processed pool samples were classified according to their distribution at the kingdom, phylum, class, order, family and genus levels and expressed as percentage value. Shannon and Chao graphs showing the similarities and alpha diversity of the groups, and Principle Coordinate Analysis (PCoA) graphs, depicting their beta diversity, were generated in both 2D and 3D formats. A total of 489 OTUs were detected across all groups. OTU numbers and taxonomic distribution according to groups are shown in Figure 1. The highest number of OTUs was observed in group 1 (Anatolian buffalo calves under 3 months old), with 275 OTUs, while the lowest number was observed in group 19 (2.5 years old, Italian pregnant), with 167 OTUs. For both breeds, the number of OTUs in buffalo calves was found to be higher than in adult buffaloes.

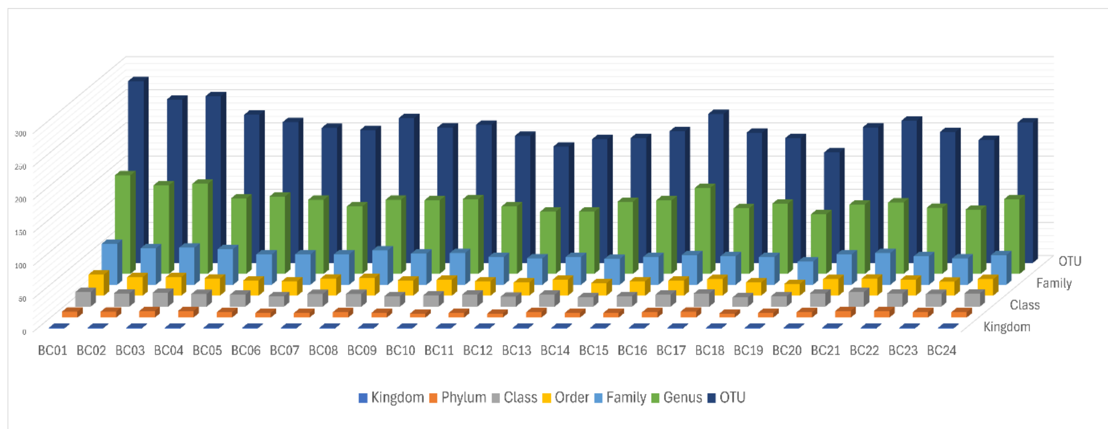

Figure 1: OTU numbers and taxonomic distributions by groups

When exemining the phylum distribution, Firmicutes was identified as the dominant phylum across all groups, with the highest proportion observed in group 19 (2-3 year old female Anatolian buffaloes) at 87.12%, and the lowest in group 13 (6-7 year old female Anatolian buffaloes) at 54% (average 68.3%) (Figure 2). Bacteriodes ranked second, with an average proportion of 29.45%. The lowest percentage was recorded in group 14 (female Anatolian buffaloes, average age of 2.5 years) at 8.9%, while the highest was detected in group 4 (2-3 month old Anatolian buffaloes) at 41.3%. The Proteobacteria phylum, which ranks third, was observed the highest rate (22.7%) in group 13 (6-7-year-old Anatolian female buffaloes), the lowest rate in group 8 (1.5-month-old Italian Mediterranean buffalo calves) with a rate of 1.125%, an average of 1.88%, while, it was not observed in groups 6, 7, 9, 10, 11, 12, 19, 21, 22, 23, 24. The Fusobacteria phylum was detected only in group 13 (6-7-year-old female Anatolian buffaloes). The percentage distribution of phyla across the groups is illustrated in Figure 2.

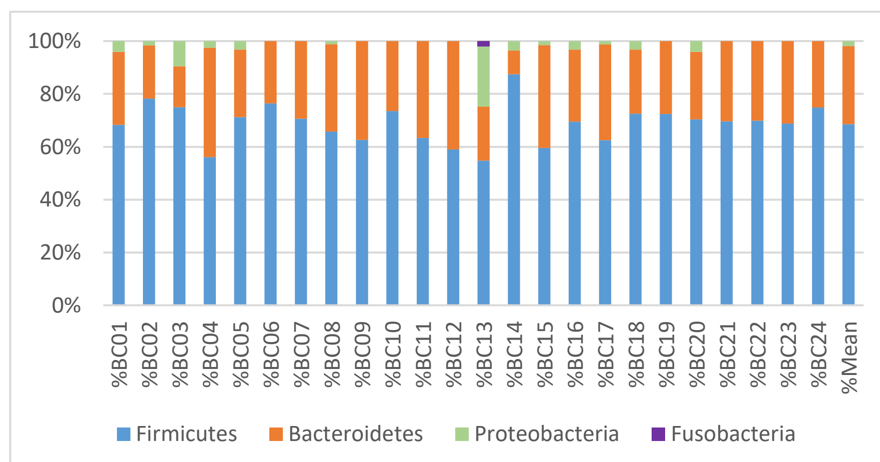

Figure 2: Percentage distribution and average percentage of the detected phylums by groups.

Firmicutes/Bacteroidetes ratios in Anatolian and Italian Mediterranean buffalo calves and adult buffaloes are presented in Table 4. Although no significant differences were observed between the ratios, the highest rate (2.99) was recorded in Anatolian buffalo calves older than 3 months that were fed only on feed. In contrast, the lowest rate (1.57) was observed in Italian Mediterranean buffalo calves older than 3 months.

Table 4: Firmicutes/Bacteroidetes ratios of Anatolian, Italian buffalo and buffalo calf groups.

		F/B Ratios			
Anatolian, < 3 months, Milk and feed	BC01	2,65	2,16	BC07	**Italian, < 3 months, Milk and feed**
	BC02			BC08	
	BC03			BC09	
	BC04			BC10	
Anatolian, > 3 months, Feed	BC05	2,99	1,57	BC11	**Italian, > 3 months, Feed**
	BC06			BC12	
Anatolian , > 2 years, Female	BC13	2,95	2,54	BC19	**Italian, > 2 years, Female**
	BC14			BC20	
	BC15			BC21	
Anatolian, > 2 years, Male	BC16	2,33	2,47	BC22	**Italian, > 2 years, Male**
	BC17			BC23	
	BC18			BC24	

Proteobacteria rates observed in Anatolian and Italian Mediterranean buffalo calves and adult buffaloes are presented in Table 5. In Anatolian buffaloes older than 3 months this rate was significantly lower at 1.82%, whereas a significantly higher rate of 9.27% was detected in female Anatolian buffaloes older than 2 years. Although no significant differences were observed in Italian Mediterranean buffaloes and buffalo calves, the highest rate (1.6%) was found in females over 2 years old (Group 19, 20, 21) among Italian Mediterranean buffaloes.

Table 5: Proteobacteria rates seen in Anatolian and Italian buffalo and calf.

		Proteobacteria %			
Anatolian, < 3 months, Milk and feed	BC01 BC02 BC03 BC04	4,42	0,66	BC07 BC08 BC09 BC10	**Italian, < 3 months, Milk and feed**
Anatolian, > 3 months, Feed	BC05 BC06	1,82	0,41	BC11 BC12	**Italian, > 3 months, Feed**
Anatolian, > 2 years, Female	BC13 BC14 BC15	9,27	1,6	BC19 BC20 BC21	**Italian, > 2 years, Female**
Anatolian, > 2 years, Male	BC16 BC17 BC18	2,58	0,29	BC22 BC23 BC24	**Italian, > 2 years, Male**

When the results were analyzed at the family level (Figure 3), the Ruminococcaceae family was found to have the highest rate of 57.9% in group 6 (6-month-old Anatolian buffalo calves) and the lowest rate of 22.9% in group 13 (6-7 year old Anatolian female buffaloes). On average Ruminococcaceae was the most dominant family, with a rate of 47.84%. In contrast to the other groups, the Pasteurellacae family was found at a relatively high rate (18.9%) in group 13.

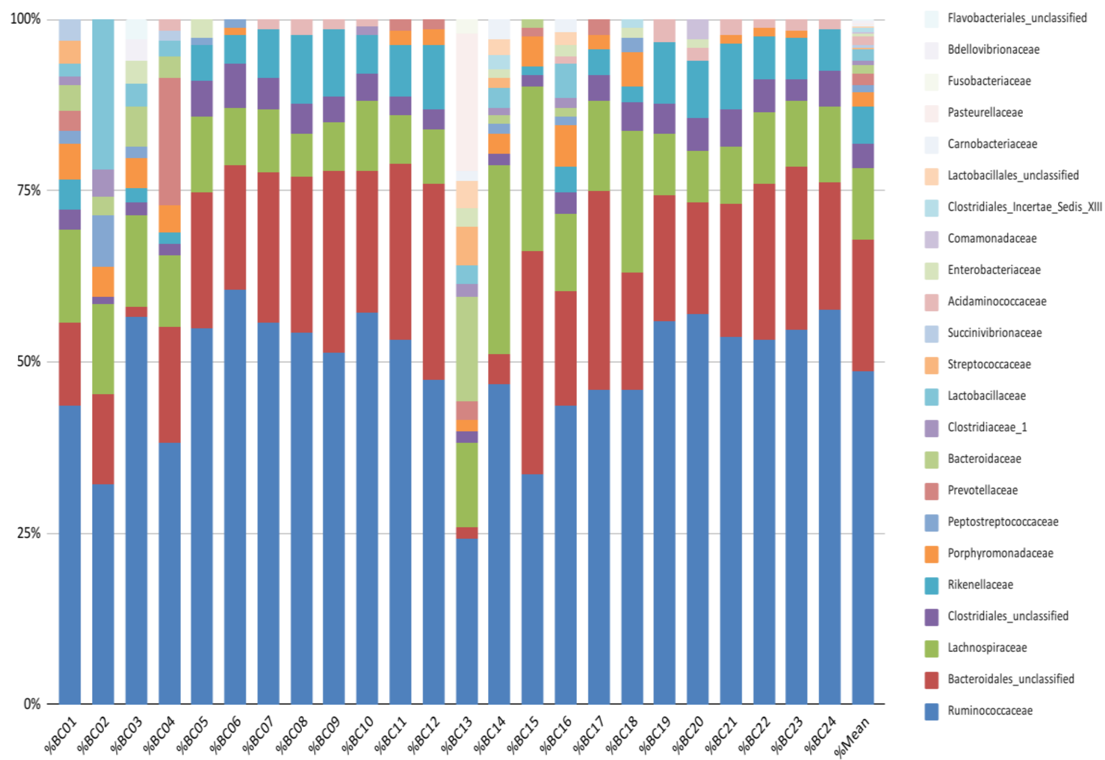

Figure 3: Percentage distribution and average percentage of the identified families by groups

When examining the genus distribution across the groups, the *Ruminococcaceae_unclassified* genus, which was dominant in all groups except for group 2 (2-month-old Anatolian females), exhibited the highest rate in group 6 (6-month-old Anatolian females) at 49.9%, and the lowest rate in group13 (6-7-year-old Anatolian females buffaloes) at 15%. On average, it accounted for 39.6% of the genus composition. In group 2, the *Lactobacillus* genus was identified as the dominant genus, constituting 20.4% (Figure 4). Furthermore, the *Bifidobacterium* genus, which is believed to be associated with the immune system, was not present in the core microbiota.

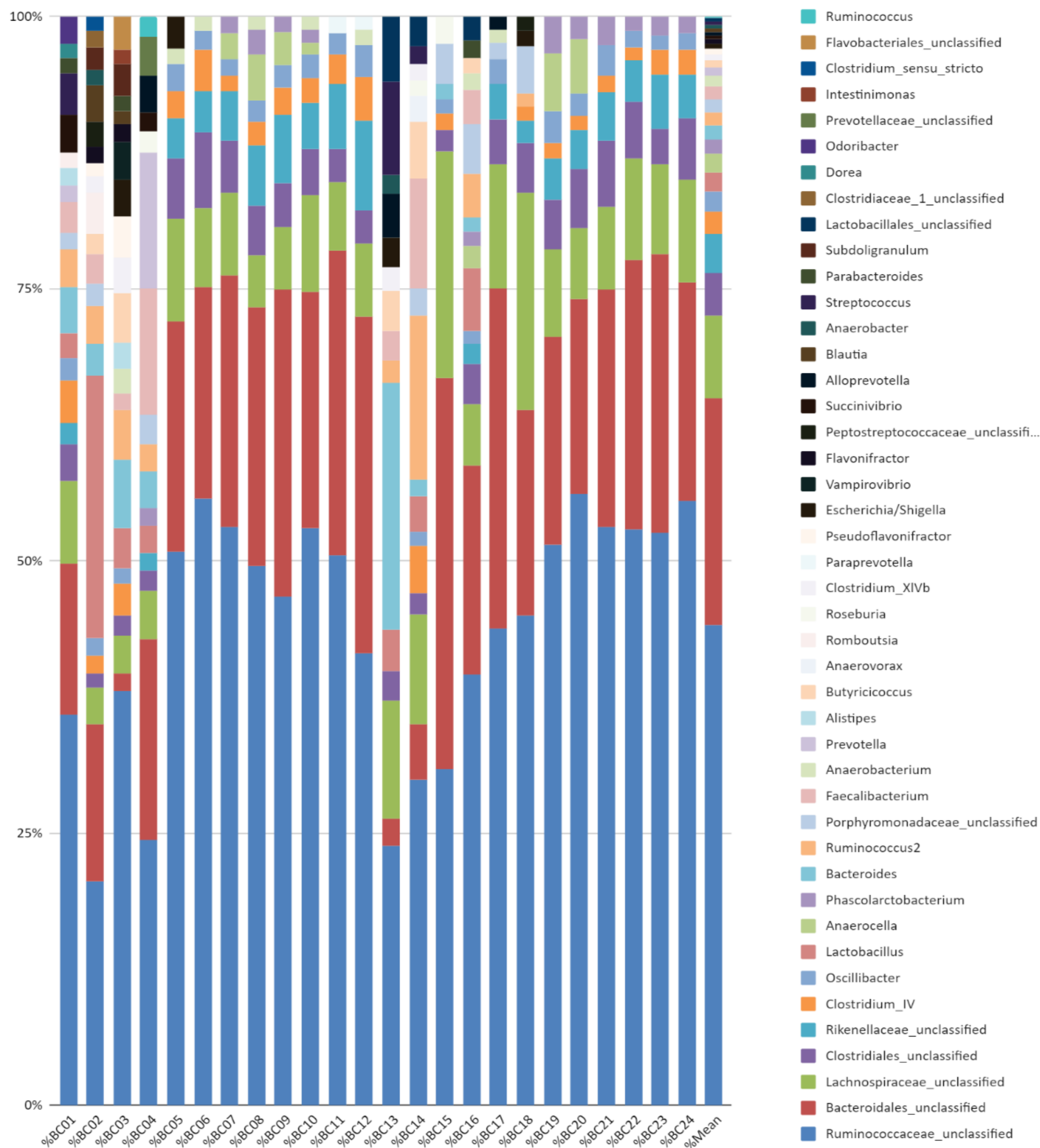

Figure 4: Percentage distribution and average percentage of the detected genera by groups.

Table 6: The availability of breeds thought to be effective on milk quality.

	BC 13 (%)	BC 14 (%)	BC 15 (%)	BC 19 (%)	BC 20 (%)	BC 21 (%)
Dorea	0,317259	0,963785	0,124952	0,141941	0,045796	0,124987
Sutterella	0,348985	0,029206	0,11952	0	0	0
Parasutterella	0	0	0	0,005257	0	0

The presence of Anatolian and Italian Mediterranean breed-spesific genera, which are thought to influence milk quality but are not included in the core microbiota (below 1%) is provided in Table 6.

Shannon and Chao graphs were analyzed to assess the richness and evenness of the groups. According to the Shannon index, group 20 exhibited greater richness compared to the other groups, whereas group 2 (2-month-old Anatolian buffalo calves fed with milk and feed) and group 12 (average 5-month-old Italian buffalo calves fed only feed) showed lower richness. In contrast, the Chao index indicated that group 2, group 9 (Italian buffalo calves fed with milk and feed for 2 months) and group 12 had a more uniform distribution than the other groups (Figures 5, 6).

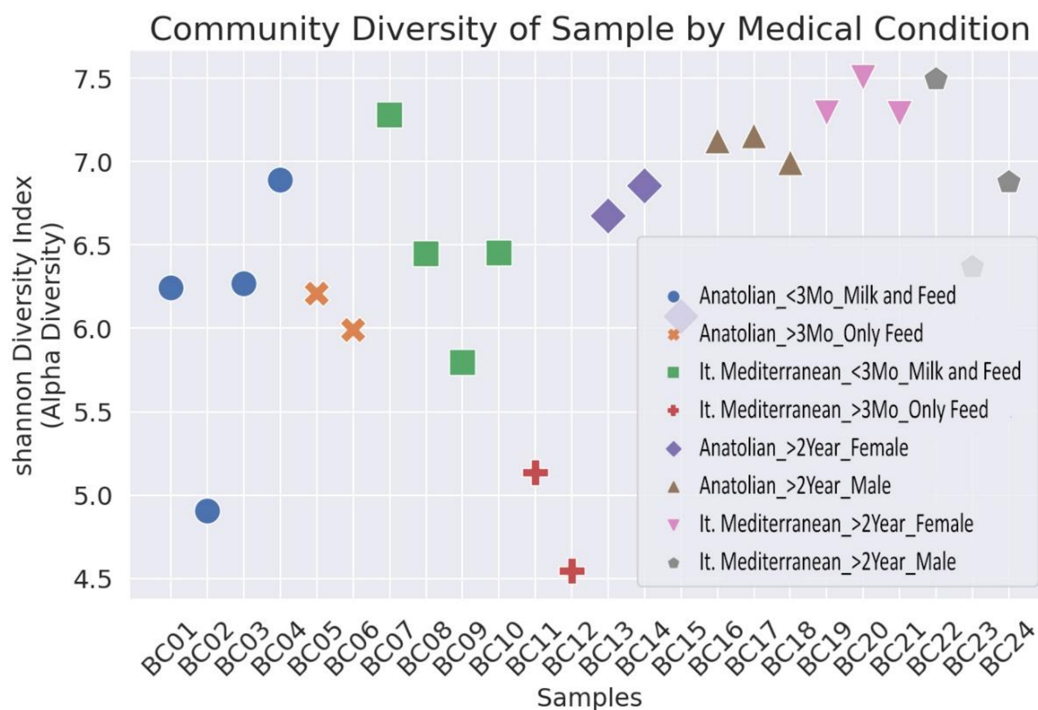

Figure 5: Specimens and their health status according to the Shannon diversity index.

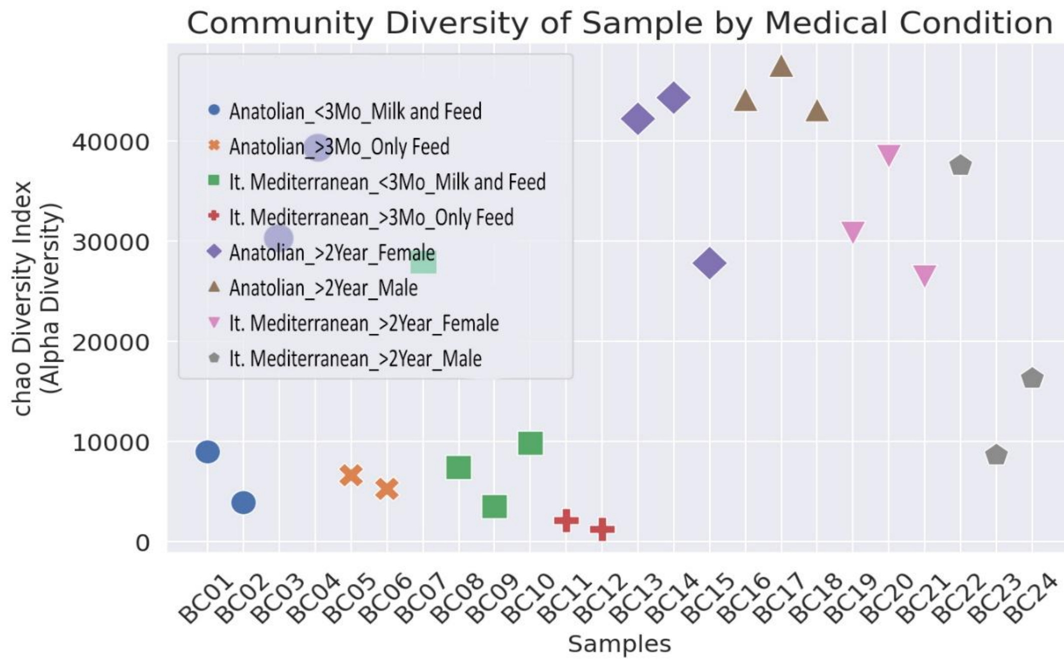

Figure 6: Samples and their health status according to the Chao diversity index.

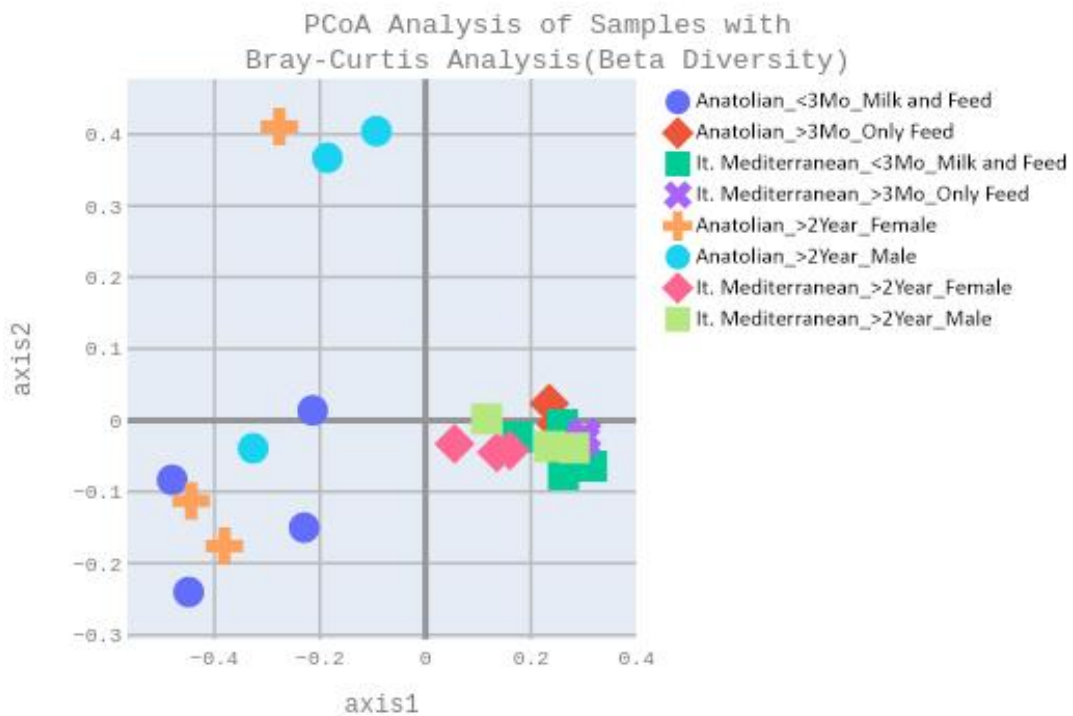

Figure 7: Beta diversity, Bray-Curtis, 2D Principal Coordinate Analysis (PCoA) plot.

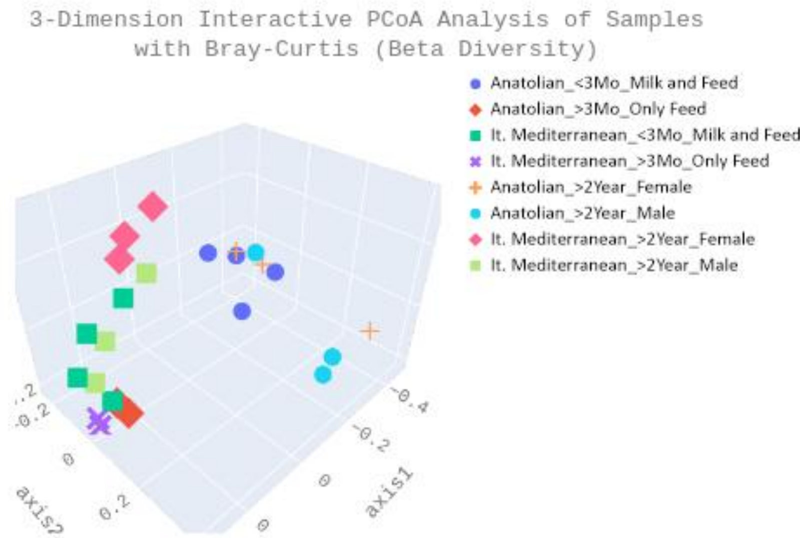

Figure 8: Beta diversity, Bray-Curtis, 3D Principal Coordinate Analysis (PCoA) plot.

The 2D and 3D PCoA graphs of the samples are presented in Figure 7 and Figure 8. When the three-dimensional Basic Component Analysis chart is examined from different perspectives; it is observed that althoug the clusters of groups 19, 20 and 21, comprising Italian Mediterranean breed and female buffaloes over two years old, are in close proximity, distinct differences exist among them. Despite the animals in groups the 19 and 20 (2.5 years old) belonging to a similar age category, group 19 (pregnant buffaloes) formed a cluster that was considerably distant from group 20. In contrast, groups 20 and 21 (5-7 years old) were clustered relatively closer together.

DISCUSSION

As a result of the sequencing analyses conducted in this study, highly detailed data were obtained, particularly regarding OTU numbers. The total number of OTUs identified represents the diversity of microbial units detectable in the intestinal microbiota of buffaloes. In total, 489 OTUs were detected. OTU numbers were found to be higher in buffalo calves compared to adult buffaloes. The highest number of OTUs was observed in Anatolian buffaloes (Group 1), aged between 10 days and 2 weeks, whereas the lowest number of OTUs was detected in pregnant Italian Mediterranean buffaloes (Group 19), aged 2.5 years. However, several studies (Dill-McFarland et al., 2017; Ren et al., 2021) suggest that microbial diversity tends to increase with aging. Shabana et al., (2021) reported that OTU numbers increased with age in sheep, while in goats, OTU numbers declined as lifespan progressed. This finding suggested that age-related variations in OTU

numbers may be species dependent. Additionally, these differences have been attributed to factor such as the physiological state and immune status of the host, interactions between symbiotic bacteria and the intestinal epithelium, pH levels, oxygenation, nutrient profile, and dietary transition rates (Amin and Seifert, 2021).

In studies on the intestinal microbiota of ruminants, the dominant phylum has been identified as Firmicutes accounting for 81% of the microbial composition. This phylum is followed by Bacteroidetes, with a reported dominance ranging from 18% to 33%, while Spirochetes, Tenericutes, and Actinobacteria are also recognized as dominant phyla (Myer et al., 2015; 2017; Durso et al., 2017). In a study conducted in China, Firmicutes, Bacteroidetes, Tenericutes and Proteobacteria were reported as the dominant phyla in the buffalo intestinal microbiota, collectively comprising 95.38% of the total sequences identified (Zhang et al., 2017).

In this study, the Firmicutes phylum was identified as the dominant phylum, comprising 68.3% of the total microbiota, regardless of age, breed, or gender. This was followed by Bacteroidetes (29.45%) and Proteobacteria (1.88%). The Fusobacteria phylum (0.88%) was detected exclusively in the Group 13 (6-7 year old female Anatolian buffaloes). As the relative abundance of the Tenericutes phylum was below 1%, it was not considered part of the core microbiota. It has been reported that the composition of starter feed plays a crucial role in shaping the intestinal microbiota (Arshad et al., 2021). Accordingly, the absence of certain phyla may be attributed to variations in the composition of the starter feed.

Firmicutes have been reported to play a key role in energy extraction from food and are considered more efficient in this process compared to Bacteroidetes. Furthermore, Firmicutes are known to influence calorie intake, weight gain, and fat storage, and are therefore associated with obesity and diabetes (Magne et al., 2020). Proteobacteria, while typically present at low levels in the healthy gut, are known to increase during infections. Indeed, they are regarded as a hallmark of microbial dysbiosis (Shin et al., 2015).

Fusobacteria represent an opportunistic phylum naturally present in the rumen microbiota of ruminants. In other words, when the balance of the rumen microbiota is disrupted, Fusobacteria can proliferate excessively, leading to conditions such as acidosis and liver abscesses (Nagaraja et al., 2005). In a study conducted by Smith and Thornton (1993), it was reported that foot diseases caused by *Fusobacterium necrophorum* in ruminants may be linked to an increased presence of *Fusobacterium* in the gastrointestinal system. In the present study, unlike other groups, the Fusobacteria phylum was detected in the group of 6-7 year old female buffalos of the Anatolian buffaloes, with relative abundance of 1.93%. This finding suggests a potential increase in the risk of acidosis and foot diseases in these animals.

In both animal and human studies, changes in body weight have been associated with alterations in the proportions of Firmicutes and Bacteroidetes (Ley et al., 2005; 2008). In women, lower alpha diversity indices and a reduced abundance of Bacteroidetes were observed in overweight and obese individuals compared to those with normal weight. While the abundance of *Bacteroides*

remained unchanged in women, it was found to decrease in men as body mass index (BMI) increased (Ley et al., 2005).

In the present study, the Firmicutes/Bacteroidetes (F/B) ratio was higher in Anatolian buffaloes compared to Italian Mediterranean buffaloes. Additionally, when adult buffaloes were examined, gender was also found to be an influencing factor, with females of both exhibiting a higher F/B than males. These findings suggest that genetic factors related to breed and gender may play a role in shaping the microbiota composition.

Jami et al., (2013) reported a positive correlation between the F/B ratio in the gastrointestinal tract and the fat content in milk, indicating that an increase in Firmicutes or a decrease in Bacteroides leads to higher fat concentration in milk. In the present study, the F/B ratio of lactating Anatolian buffaloes was higher than that of Italian Mediterranean buffaloes, suggesting that the fat content of Anatolian buffalo milk may be greater. This observation aligns with the findings of Zhang et al. (2017). Furthermore, it was observed that the relative abundance of Proteobacteria was higher in Anatolian buffaloes and water buffaloes than in Italian Mediterranean buffaloes and water buffaloes. This suggested that Anatolian buffaloes and water buffaloes may be more susceptible to intestinal dysbiosis.

At the family level, Ruminococcaceae (57.9%) was identified as the dominant family across all groups. However, unlike other groups, the Pasteurellacae family was detected at a relatively high rate in 6-7-year-old female Anatolian buffaloes (Group 13). This findings may be associated with genetic factors related to breed, age and gender. A study on lambs also highlighted that variations in microbial colonization may be linked to dietary differences (Yang et al., 2018).

At the genus level, *Ruminococcaceae_unclassified* was the most dominant, followed by *Bacteroides_unclassified* and *Lachnospiraceae_unclassified*. However, in group 2 (2-month-old Anatolian calves fed with milk and feed), the dominant genus was *Lactobacillus*. A study on cattle reported that *Bifidobacterium* and, in particular, *Lactobacillus* were highly abundant in the fecal microbiota of milk-fed calves, as expected (Alipour et al., 2018). However, in the present study, the absence of *Bifidobacterium* and *Lactobacillus* in the other groups, despite their known association with the mucosal immune response, suggests a potential deficiency in immunity among young animals in the study gropus.

Buffaloes are the second largest source of milk in the world after cows. Buffalo milk has a higher solids content compared to cow milk. However, buffalo milk is a richer source of fat, protein, lactose and minerals than cow milk, so it is considered more nutritious for humans (El-Salam et al., 2011; Park and Haenlein, 2013 pp: 519-553).

The intestinal microbiota plays a crucial role in nutrient digestion, regulation of host fat storage, stimulation of intestinal epithelial renewal, and modulation of the immune system maturation (Andersson et al., 2008; Cho and Blaser, M. J., 2012). Additionally, it has been established that the fecal microbiome is associated with the mammary gland and its microbiota (Jiménez et al., 2008; Williams et al., 2020). Previously studies investigating mechanisms underlying differences in milk composition between cows and buffaloes have demonstrated

that the biosynthesis of amino acids, such as valine, leucine and isoleucine, as well as pathways related to pantothenate, and CoA biosynthesis, and biotin metabolism, are positively associated with spesific bacterial genera, including *Parabacteroides, Dorea, Sutterella,* and *Parasutterella* in the fecal microbiota. Furthermore, positive correlations have been reported between milk fat content and pathways involved in lipopolysaccharide biosynthesis, amino acid biosynthesis, and valine, leucine, and isoleucine biosynthesis (Zhang et al., 2017). In the present study, while *Sutterella* and *Dorea* were not part of the core microbiota (i.e., present at <1%), they were found in greater abundance in lactating Anatolian buffaloes compared to Italian buffaloes. However, the *Parasutterella* genus was not detected in lactating animals of either breed.

The Shannon and Chao diversity indices are widely used in metagenomic analyses to essess microbial richness and divesity within sequence datasets. These indicates account for both species abundance and evenness, where higher values indicate a community with a well-balanced distribution of species. The Shannon index ranges from one (in cases where a single dominant species dominant species is present) to the total number of species observed (when all species are evenly distributed). In the present study, the highest microbial richness, as measured by the Shannon index, was observed in group 20, while lower richness was detected in group 2 (2-month-old Anatolian calves fed with milk and feed) and group 12 (average 5-month-old Italian calves fed only with feed). According to the Chao index, Groups 2, 9 (2-month-old Italian calves fed with milk and feed) and 12 exhibited a more uniform distribution of microbial taxa compared to the other groups.

Bray-Curtis dissimilarity analysis is a beta diversity metric used to assess microbial abundance differences between samples based on read count data. This analysis quantifies the dissimilarity in microbial composition (e.g. at the species level) between two or more samples, producing a value between 0 and 1. A value of 0 indicates tha two samples share identical species at the same abundance, whereas a value of 1 signifies completely distinct microbial compositions. In this study, PCA and PCoA plots were generated to visualize these differences. The most striking finding from these analyses was the distinct clustering of Group 19, which comprised pregnant animals. Pregnancy induces numerous immunological, metabolic and hormonal changes essential for maintaining and supporting this unique physiological state in the host (Milani et al., 2017; Susic et al., 2020). Prevous Studies have reported a reduction in alpha diversity (diversity within a sample) in the intestinal microbiome during pregnancy, accompanied by an increase in beta diversity (diversity between samples) (Koren et al., 2012; Nuriel-Ohayon et al., 2016; Catalano and Shankar, 2017). Deng et al. (2019), compared the vaginal and fecal microbiota of pregnant and non-pregnant cows and found that, while the vaginal microbiome remained relatively stable, significant alterations were observed in the fecal microbiota. These findings align with the results of the present study, in which pregnant animals exhibited a distinct microbiota composition and a lower OTU count. It is also important to note that this study utilized fecal samples, which are representative of the entire intestinal microbiome, and employed non culture-based analytical methods. Therefore, the results obtained are considered

consistent with those reported in current studies that use comparable methodologies.

CONCLUTION

The result of this study suggest that the absence of genera such as *Lactobacillus* and *Bifidobacterium* in the core intestinal microbiota of the animals sampled indicates a potentially weakened immune system, which could be strengthened throug supplementation. The findings emphasize that host genetics and environmental factors play a significant role in shaping the intestinal microbiome research holds promise for addressing challenges in animal husbandry, growth, and health.

Furthermore, it can be predicted that establishing intestinal microbiota colonization in neonates through competitive exclusion early in life may help prevent disease development and reduce the need for antibiotics treatments. This approach could contribute to mitigating the global threat of antibiotic resistance, by decreasing the reliance on antibiotics.

Additionally, future studies utilizing next-generation sequencing methods to identifiy resistomes and transferable gene regions containing resistance genes could provide valuable insights into the presence and potential spread of antibiotic resistance.

ACKNOWLEDGMENT

This study was supported by Istanbul University-Cerrahpaşa Scientific Research Projects Unit (Project No: 32577) and TUBITAK 2211 Domestic Postgraduate Scholarship Program.

We would like to thank Prof. Dr. Şükrü KIRKAN from the Department of Microbiology of Aydın Adnan Menderes Faculty of Veterinary Medicine and Prof. Dr. Ömür KOÇAK from the Department of Animal Science of Istanbul University-Cerrahpaşa Faculty of Veterinary Medicine for their information and assistance in the realization of this study.

DATA AVAILIBILITY

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

REFERENCES

Alipour, M. J., Jalanka, J., Pessa-Morikawa, T., Kokkonen, T., Satokari, R., Hynönen, U. ve diğ., 2018, The composition of the perinatal intestinal microbiota in cattle. Scientific Reports, 8(1), 1-14.

Altuntaş, Y. ve Batman, A., 2017, Mikrobiyota ve metabolik sendrom. *Turk Kardiyoloji Dernegi Arsivi*, 45(3), 286–296.

Amin, N. ve Seifert, J., 2021, Dynamic progression of the calf's microbiome and its influence on host health. *Computational and Structural Biotechnology Journal*, 19, 989-1001.

Amon, P. ve Sanderson, I., 2017, What is the microbiome? *Archives of Disease in Childhood-Education and Practice*, 102(5), 257-260.

Andersson, A. F., Lindberg, M., Jakobsson, H., Bäckhed, F., Nyrén, P. ve Engstrand, L., 2008, Comparative analysis of human gut microbiota by barcoded pyrosequencing. Public Library of Science One, 3(7), e2836.

Anonymus: NIH Human Microbiome Project. Erişim adresi: https://hmpdacc.org/hmp/ Son erişim Tarihi: 02.03.2022

Arshad, M. A., Hassan, F. U., Rehman, M. S., Huws, S. A., Cheng, Y., & Din, A. U. (2021). Gut microbiome colonization and development in neonatal ruminants: Strategies, prospects, and opportunities. Animal Nutrition, 7(3), 883-895.

Bleich, A. ve Fox, J. G., 2015, The mammalian microbiome and its importance in laboratory animal research. *Institute for Laboratory Animal Research Journal*, 56(2), 153–158.

Catalano, P. M. ve Shankar, K., 2017, Obesity and pregnancy: mechanisms of short term and long term adverse consequences for mother and child. Bmj, 356.

Cho, I. ve Blaser, M. J., 2012, The human microbiome: at the interface of health and disease. Nature Reviews Genetics, 13(4), 260-270.

Cruz-Monterrosa, R., Mota-Rojas, D., Ramirez-Bribiesca, E., Mora-Medina, P. ve Guerrero-Legarreta, I., 2020, Scientific findings on the quality of river buffalo meat and prospects for future studies. *Journal of Buffalo Science*, 9, 170-180.

Deng, F., McClure, M., Rorie, R., Wang, X., Chai, J., Wei, X. ve diğ., 2019, The vaginal and fecal microbiomes are related to pregnancy status in beef heifers. Journal of Animal Science and Biotechnology, 10(1), 1-13.

Diker, K. S., 2017, Hayvanlarda mikrobiyom-hayvan mikrobiyomu, Veteriner Hekimler Derneği Dergisi, 88(2), 122–132.

Dill-McFarland, K. A., Breaker, J. D., & Suen, G. (2017). Microbial succession in the gastrointestinal tract of dairy cows from 2 weeks to first lactation. Scientific reports, 7(1), 40864.

Du, C., Deng, T., Zhou, Y., Ye, T., Zhou, Z., Zhang, S. ve diğ., 2019, Systematic analyses for candidate genes of milk production traits in water buffalo (Bubalus Bubalis). *Animal Genetics,* 50(3), 207-216.

Durso, L. M., Miller, D. N., Schmidt, T. B. ve Callaway, T., 2017, Tracking bacteria through the entire gastrointestinal tract of a beef steer. Agricultural & Environmental Letters, 2(1), 170016.

El-Salam, A., Mohamed, H. ve El-Shibiny, S., 2011, A comprehensive review on the composition and properties of buffalo milk. Dairy Science & Technology, 91(6), 663-699.

Fouhy, F., Ross, R. P., Fitzgerald, G. F., Stanton, C. ve Cotter, P. D., 2012, Composition of the early intestinal microbiota: knowledge, knowledge gaps and the use of high-throughput sequencing to address these gaps. *Gut Microbes*, 3(3), 203-220.

Garmendia, L., Hernandez, A., Sanchez, M. B. ve Martinez, J. L., 2012, Metagenomics and antibiotics. *Clinical Microbiology and Infection*, 18, 27–31.

Gupta, S., Allen-Vercoe, E. ve Petrof, E. O., 2016, Fecal microbiota transplantation: in perspective. *Therapeutic advances in gastroenterology*, 9(2), 229-239.

Gürsoy, N. C., ve Otlu B., 2017, Mikrobiyota çalışmalarında moleküler tanı yöntemleri. *Journal of Biotechnology and Strategic Health Research*, 1, 56-67.

Hashimoto-Hill, S. ve Alenghat, T., 2021, Inflammation-Associated Microbiota Composition Across Domestic Animals. Frontiers in Genetics, 12.

Hiergeist, A., Gläsner, J., Reischl, U. ve Gessner, A., 2015, Analyses of intestinal microbiota: culture versus sequencing. *Institute for Laboratory Animal Research Journal*, 56(2), 228-240.

Hong, M., Zhang, R., Liu, Y., Wu, Z. Ve Weng, P., 2021, The interaction effect between tea polyphenols and intestinal microbiota: Role in ameliorating neurological diseases. Journal of Food Biochemistry, e13870.

Jain, M., Olsen, H. E., Paten, B. ve Akeson, M., 2016, The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community. *Genome Biology*, 17(1), 1-11.

Jami, E., Israel, A., Kotser, A. ve Mizrahi, I., 2013, Exploring the bovine rumen bacterial community from birth to adulthood. The International Society for Microbial Ecology Journal, 7(6), 1069-1079.

Jiménez, E., Fernández, L., Maldonado, A., Martín, R., Olivares, M., Xaus, J. ve diğ., 2008, Oral administration of Lactobacillus strains isolated from breast milk as an alternative for the treatment of infectious mastitis during lactation. Applied and Environmental Microbiology, 75(15), 4650-4655.

Kekeç, I., Sipahi, N., ve İkiz, S. (2022). New generation genome sequencing methods. Journal of Surgery and Medicine, 6(4), 503-506.

Kızmaz, M. Z., Paylan, C. ve Erkan, S., 2017, DNA dizilemenin tarihsel gelişimi, *Gaziosmanpaşa Bilimsel Araştırma Dergisi,* 6(2), 47-53.

Koren, O., Goodrich, J. K., Cullender, T. C., Spor, A., Laitinen, K., Bäckhed, H. K. ve diğ., 2012, Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell, 150(3), 470-480.

Küllük, E. ve Dalgın, D., 2021, Veteriner sahada güncel mikrobiyota kavramı. Etlik Veteriner Mikrobiyoloji Dergisi, 32(1), 77-88.

Lee, H., Gurtowski, J., Yoo, S., Nattestad, M., Marcus, S., Goodwin, S. ve diğ., 2016, Third-generation sequencing and the future of genomics. *BioRxiv*, 048603.

Liu, X., 2016, Focus: Microbiome: Microbiome. *The Yale Journal of Biology and Medicine,* 89(3), 275.

Luczynski, P., Neufeld, K. M., Oriach, C. S., Clarke, G., Dinan, T. G. ve Cryan, J. F., 2016, Growing up in a Bubble : Using Germ-Free Animals to Assess the Influence of the Gut Microbiota on Brain and Behavior. International Journal of Neuropsychopharmacology, 19, 1–17.

Ley, R. E., Bäckhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D. ve Gordon, J. I., 2005, Obesity alters gut microbial ecology. Proceedings of the National Academy of Sciences, 102(31), 11070-11075.

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S. ve diğ., 2008, Evolution of mammals and their gut microbes. Science, 320(5883), 1647-1651.

Magne, F., Gotteland, M., Gauthier, L., Zazueta, A., Pesoa, S., Navarrete, P. ve diğ., 2020, The firmicutes/bacteroidetes ratio: a relevant marker of gut dysbiosis in obese patients? Nutrients, 12(5), 1474.

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J. ve diğ., 2017, The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. Microbiology and Molecular Biology Reviews, 81(4), e00036-17.

Myer, P. R., Wells, J. E., Smith, T. P., Kuehn, L. A., Freetly, H. C., 2015, Microbial community profiles of the colon from steers differing in feed efficiency. Springerplus, 4(1), 1-13.

Myer, P. R., Freetly, H. C., Wells, J. E., Smith, T. P. L. ve Kuehn, L. A., 2017, Analysis of the gut bacterial communities in beef cattle and their association with feed intake, growth, and efficiency. Journal of Animal Science, 95(7), 3215-3224.

Nagaraja, T. G., Narayanan, S. K., Stewart, G. C. ve Chengappa, M. M., 2005, Fusobacterium necrophorum infections in animals: pathogenesis and pathogenic mechanisms. Anaerobe, 11(4), 239-246.

Nuriel-Ohayon, M., Neuman, H. ve Koren, O., 2016, Microbial changes during pregnancy, birth, and infancy. *Frontiers in Microbiology*, 1031.

O'Donnell, M. M., Harris, H. M., Ross, R. P. ve O'Toole, P. W., 2017, Core fecal microbiota of domesticated herbivorous ruminant, hindgut fermenters, and monogastric animals. Microbiologyopen, 6(5), e00509.

Park, Y. W. ve Haenlein, G. F., 2013, Milk and dairy products in human nutrition: production, composition and health. 1st Ed., John Wiley & Sons.

Pasha, T. N., 2013, Prospect of nutrition and feeding for sustainable buffalo production. *Buffalo Bulletin,* 32(Spec. Issue 1).

Ren, M., Li, H., Fu, Z., & Li, Q. (2021). Succession analysis of gut microbiota structure of participants from long-lived families in Hechi, Guangxi, China. Microorganisms, 9(12), 2524.

Sariozkan, S., 2011, Türkiye'de manda yetiştiriciliğinin önemi. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi*, 17(1), 163-166.

Schadt, E. E., Turner, S. ve Kasarskis, A., 2010, A window into third-generation sequencing. *Human Molecular Genetics*, 19(R2), R227-R240.

Shin, N. R., Whon, T. W. Ve Bae, J. W., 2015, Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends in Biotechnology, 33(9), 496-503.

Siddiky, M. N. A. ve Faruque, M. O., 2017, Buffaloes for dairying in South Asia: potential, challenges and way forward. SAARC *Journal of Agriculture*, 15(2), 227-239.

Smith, G. R. ve Thornton, E. A., 1993, The prevalence of Fusobacterium necrophorum biovar A in animal faeces. Epidemiology & Infection, 110(2), 327-331.

Susic, D., Bai, M. ve Henry, A., 2020, Microbiota and pregnancy. Microbiota in Health and Disease, 2(3), 1-11.

Tlaskalová-Hogenová, H., Štěpánková, R., Hudcovic, T., Tučková, L., Cukrowska, B., Lodinová-Žádnıková, R. ve diğ., 2004, Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. Immunology Letters, 93(2-3), 97-108.

Ademhan Tural, D., Kasikci, M., Eryilmaz Polat, S., Ozsezen, B., Hizal, M., Sunman, B., ... & Ozcelik, U. (2024). The airway microbiota in siblings with primary ciliary dyskinesia: Related factors and correlation with clinical characteristics. Pediatric Pulmonology, 59(3), 695-706.

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., ve Gordon, J. I., 2007, The Human Microbiome Project. *Nature*, 449(7164), 804–810.

Williams, J. E., Carrothers, J. M., Lackey, K. A., Beatty, N. F., Brooker, S. L., Peterson, H. K. ve diğ., 2019, Strong multivariate relations exist among milk, oral, and fecal microbiomes in mother-infant dyads during the first six months postpartum. *The Journal of Nutrition*, 149(6), 902-914

Yang, B., Le, J., Wu, P., Liu, J., Guan, L. L. ve Wang, J., 2018, Alfalfa intervention alters rumen microbial community development in Hu lambs during early life. Frontiers in Microbiology, 9, 574.

Zhang, J., Xu, C., Huo, D., Hu, Q. ve Peng, Q., 2017, Comparative study of the gut microbiome potentially related to milk protein in Murrah buffaloes (Bubalus bubalis) and Chinese Holstein cattle. Scientific Reports, 7(1), 1-11.