

MICROBIOTA FROM FECES AS AN INDICATOR OF THE HEALTH STATUS OF SOME WILD BIRDS IN THE CHIȘINĂU ZOO

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Abstract. The gut microbiota of birds, compared to other animals, is most dependent on living conditions, as they contact with different habitats and intermediate organisms during their lives. Wild birds, due to their lifestyle, diverse feeding habits, ability to migrate long distances, have specific physiological activity which in turn determines the complexity of their gut microbiota. The microbial community in captive birds differs significantly from wild species through differences in environment and diet. Analysis of the intestinal content of bifidobacteria, lactobacteria, enterococci and *Escherichia coli* in pheasant, black swan and mallard at the Chisinau Zoo, revealed the dominance of bifido- and lactobacteria. The numerical indices of intestinal enterococci, which were established in the gut content of investigated birds, indicate a possible symbiosis between them and bifidobacteria and lactobacteria. It can be assumed that this correlation between bifidobacteria and lactobacteria with enterococci keeps the numbers of *Escherichia coli* at a level that does not allow the development of dysbacteriosis in the wild captive birds. Also, no differences were found in the quantitative levels of bifidobacteria, lactobacteria, enterococci and *Escherichia coli* according to the phylogeny of the investigated birds and composition of feed. This indicates that the feed rations for wild birds in the Chisinau Zoo are balanced and represent the determining factor in establishing a favorable microbial profile in the gut, which in turn favors the good health of the digestive tract.

Keywords: gut bacteria, feed ration, birds.

Rezumat. Microbiota din materia fecală ca indicator al stării de sănătate a unor păsări sălbatice din Grădina Zoologică din Chișinău. Microbiota intestinală a păsărilor, comparativ cu alte animale, depinde cel mai mult de condițiile de viață, întrucât pe parcursul vieții contactează cu diferite habitate și organisme intermediare. Păsările sălbatice, datorită modului de viață, obiceiurilor alimentare diverse, capacității de migrare pe distanțe lungi, au activitate fiziologică specifică ce determină la rândul său complexitatea microbiotei lor intestinale. Comunitatea microbiană la păsările din captivitate diferă semnificativ de speciile sălbatice prin diferențele de mediu și de alimentație. Analiza conținutului intestinal al bifidobacteriilor, lactobacteriilor, enterococilor și al *Escherichia coli* la fazan, lebăda neagră și rața sălbatică de la Grădina Zoologică din Chișinău, a relevat dominanța efectivului de bifido- și lactobacterii. Indicii numerici ai enterococilor intestinali, care au fost constatați la păsările investigate, indică o posibilă simbioză între aceștia cu bifidobacteriile și lactobacteriile. Putem presupune că această corelație dintre bifido- și lactobacterii cu enterococii menține numericul de *Escherichia coli* la un nivel ce nu permite dezvoltarea disbacteriozei la păsările sălbatice din captivitate luate în studiu. De asemenea, nu au fost identificate diferențe în nivelul cantitativ al bifidobacteriilor, lactobacteriilor, enterococilor și *Escherichia coli* în funcție de filogenia păsărilor investigate și structura rației alimentare administrată. Aceasta denotă faptul că rațiile alimentare destinate păsărilor sălbatice din Grădina Zoologică din Chișinău sunt echilibrate și reprezintă factorul determinat în stabilirea unui profil microbian favorabil în intestin, ce favorizează la rândul său starea de sănătate bună a tubului digestiv.

Cuvinte cheie: bacterii intestinale, rație alimentară, păsări.

INTRODUCTION

The intestinal microbiota plays a crucial role in the health of host animals, which directly depend on such factors as geography, animal genetics, diet, which influence its nutrition and immunity, adaptation, and overall survival in the environment.

Studies on the intestinal microflora in birds are less compared to mammals (CLEMENTE et al. 2012; WU et al., 2021), but they are relevant, as birds themselves are essential indicator organisms in several ecosystems, with a strong capacity for geographic dispersal and a wide range of distribution, which playing multiple ecological functions (WAITE & TAYLOR, 2015). All these characteristics have determined a complex gut microbial composition of birds, especially of the wild ones. The bird's gut microflora is unique and is influenced by the avian physiological characteristics, living conditions and lifestyle and the action of environmental factors (COLSTON & JACKSON, 2016; WANG et al., 2016; GARCIA-AMADO et al., 2018; WU et al., 2021). The research regarding the mechanisms of response and maintenance of the avian gut microbiota to environmental changes is increasingly linked to studies on survival of wild animal and the maintenance of their health. Birds are also a very suitable group to study host-microbiota interactions as there are over 10000 species and their diet and migration behaviour are very different along their life history (DONG et al., 2022).

Microbial community structures of the species in captivity may differ significantly from those of wild species through environmental and dietary differences. Wild birds have complex life cycle characteristics, diverse feeding habits, unique mating systems and the ability to migrate over long distances, making their physiological activities face more substantial selective pressure, thus contributing to the complexity of their gut microbiota (SUN et al., 2022). Therefore, studies in wild species are essential to understand the importance of multiple factors in modulating the gut microbiota diversity in natural ecological environments among avian species.

The microbiota community of captive birds, including zoo birds is less diverse and complex, compared to wild species, due to differences in environment and diet (SUN et al., 2022). Under captive conditions, food resources are the determining factor influencing avian microbiota. The simple composition of the gut microbiota of captive or domestic birds

who are fed a monotonous diet in turn causes insufficient stress response to external stimuli leading to an increase in the number of pathogenic microorganisms in the bacterial community of their gut (SUN et al., 2022). Thus, captive wild animals are highly susceptible to diseases and can act as reservoirs of pathogenic bacteria (AHMED et al, 2007; DAVIES et al., 2016).

The influence of feed ration on gut microbiota in captive birds has been studied by a group of researchers led by XIAO K. (XIAO et al., 2021). The research, conducted on a batch of 35 species of birds from a single zoo and 6 species of domesticated birds, showed that diets containing starch contribute to higher abundance of *Lactobacillus* in the gut microbiome, and those containing plant-derived fibres increase *Clostridium* levels. Carnivorous birds were found to have higher numbers of *Fusobacteria* and *Proteobacteria*, while birds fed on a corn- and soy-based diet were dominated by bacteria belonging to *Clostridia* and *Bacteroidia*. In addition, the functions of the microbial metagenome (such as lipid metabolism and amino acid synthesis) have been adapted to the different types of food for a host-beneficial state. In fact, the gut microbiome can be modulated by dietary diversity, which in turn helps to better understand the interactions between diet, microbiome and host, especially determining host health status (XIAO et al., 2021).

Thus, food, rather than the phylogeny of the host organism, is the main factor modulating the gut microbiome in birds, after minimizing the effect of other factors such as weather, season and geography. The adaptive evolution of microbes to food types formed a dietary-microbiome-host interaction reciprocal state (XIAO et al., 2021).

In the context of the mentioned above, a proposal was made to determine the health status of some wild zoo birds according to the quantitative level of bifidobacteria, lactobacteria, enterococci and *Escherichia coli* and the administered feed ration.

MATERIAL AND METHODS

The investigations were carried out on three (3) wild species: pheasant (*Phasianus colchicus* (Linnaeus, 1758)), black swan (*Cygnus atratus* (Latham, 1790)) and mallard (*Anas platyrhynchos* (Linnaeus, 1758)) from the Chisinau Zoo. The birds are kept in conditions characteristic of zoos. These species were selected because they are the most common in our area. The black swan represents the non-native species. The birds were kept outdoors in small cages with water and food available ad libitum.

The feed rations of the investigated birds were developed according to their nutrition particularity, in accordance with recommendations for bird feeding in zoos (EAZA, 2013) and are shown in the Table 1.

Table 1. Daily feed ration administered to the investigated birds in Chisinau Zoo (g per bird).

Feed ration content (g per bird)	Pheasant	Black swan	Wild duck
Combined feed for birds, ПК-6, ПК-5	3,56	2,7	9,9
Oat	0,79		
Millet	3,16	0,76	3,96
Corn	0,79		
Oat flakes		0,78	0,99
Pea	0,79		
Grass flour	0,79	0,09	0,5
Bran	0,79		
Sunflower seeds	0,79		
Hemp seeds	0,395		
Flaxseed	0,158		
Different fruits, berries	2,37		
Carrot	2,37		0,99
Cabbage	2,37		2,5
Beet	0,79		
Onion	0,79		
Green salad		2,4	4,95
Fish			0,99
Gammarus	0,395		
Mealworms	0,395		
Cheese	0,79		
Eggs	0,79		
Yeast	0,079	0,066	0,297
Fish meal	0,079	0,063	0,099
Premix (commercial product)	0,079	0,060	0,139

For analysis of the quantitative level of bifidobacteria, lactobacteria, enterococci and *Escherichia coli* in the intestinal contents, faecal sample were taken. Based on several studies, faecal matter is recommended for the study of the gut microbiota in birds, as this sample is a useful alternative for the analysis of gut microbes and provides an appropriate evaluation thereof (VIDEVALL et al., 2018; BERLOW et al., 2019; GARCIA-MAZCORRO et al., 2021). Avian faeces comprise both faeces and urine, and the cloaca contains a mixed population of microbes from the digestive, reproductive and urinary systems (LEE et al., 2020; GARCIA-MAZCORRO et al., 2021).

All faeces' samples were collected from the Chisinau Zoo at the same period (January 2023). The samples were collected immediately after defecation. The collection of samples from certain individuals was not pursued, as the birds are kept in a common space and are fed the same food ration. From the place of collection to the laboratory, the samples were kept in a portable refrigerator, in order to avoid the denaturation of the microbiological material. The microbiological analysis was performed on the day of sampling.

The content of microorganisms was determined using classical microbiological methods (GARMASHEVA & KOVALENKO, 2010). Their inoculation of microorganisms was performed on a culture medium specially prepared for each group of bacteria – bifidobacteria on Bifidobacterium Agar (produced and marketed by the company “Himedia”), lactobacteria on MRS Agar (“Himedia”), enterococci on Bile Esculine Azide Agar ISO 7899-2 (“Himedia”) and *Escherichia coli* on Endo Agar (“Himedia”). Each group of microorganisms were studied and counted separately.

In order to establish the number of viable microbial cells for every group of investigated microorganisms, the initial material (faecal sample), in the amount of 1 g, was taken for each determination. After a series of dilutions were performed – diluting the intestinal content samples from 10⁻¹ to 10⁻⁹, then inoculating and incubating them on nutrient medium, elective to each microorganism species. After incubation at (37±1) °C for 48-72 hours under aerobic or anaerobic conditions, depending on the microorganism species, typical colonies grown on Petri dishes with the last dilutions were counted. The number of viable bacteria in 1 g of intestinal contents is determined by the formula:

$$X = a \cdot 10^n, \text{ where}$$

X - is the number of viable individuals in 1 g of intestinal contents;

a - the number of colonies in the last Petri dish in which growth is recorded;

n - is the degree of dilution.

Bacterial growth activity was determined by counting colony-forming units (CFU) on Petri dishes after successive dilutions. The number of CFU is expressed in decimal logarithms (log₁₀). The viability of strains on experimental medium is expressed as % relative to control. Assays were performed in 3 replicates (SM EN ISO 4833-:2014).

The experiments were carried out in accordance with Directive 86/609 /EEC of 24 November 1986 on the Protection of Animals Used for Experimental and Other Scientific Purposes and were approved by the Methodical and Ethics Committees of the Institute of Physiology and Sanocreatology, Moldova State University.

RESULTS

Data on the quantitative level of certain intestinal bacteria reveal the health status of the digestive tract and the whole body. Among the bacteria of the intestinal microflora, it was proposed to study the quantitative level of bifidobacteria, lactobacteria, enterococci and *Escherichia coli*, since representatives of these groups of bacteria they are the subject of several investigations that report the significance of the microbiota for the health of the host organism.

Bifidobacteria and lactobacteria in the gut microbiota are associated with health-promoting benefits and reduced abundance of this genus is linked to several diseases; for this reason, Bifidobacterium spp. and Lactobacillus ssp, as lactic acid bacteria, are categorized by several researchers to probiotic microbial groups living in the digestive tract to improve its condition (WONG et al, 2020).

Enterococci (namely *E. faecalis* and *E. faecium*) are found in a range of different animals and, although they are considered as the main predisposing factor for severe infections, they have been shown to contribute to colonic homeostasis and can reduce the severity of infectious diarrhoea in humans and animals (DUBIN & PAMER, 2014).

The most prevalent opportunistic enterobacterium in captive animals may be considered *Escherichia coli* that is associated with systemic disease in birds, but is also a natural inhabitant of the gut in poultry and most other animals. Normally, it is kept in check by other bacteria in the gut, but if large colonies form it can cause severe discomfort, illness, and mortality (SANCHES et al., 2017).

The quantitative level of mentioned bacteria was determined in three wild species kept in captivity in the Chisinau Zoo: pheasant, black swan and mallard.

The obtained results regarding the quantitative level of bifidobacteria, lactobacteria, enterococci and *Escherichia coli* in the intestinal contents (faecal matter) of pheasant are shown in Fig. 1.

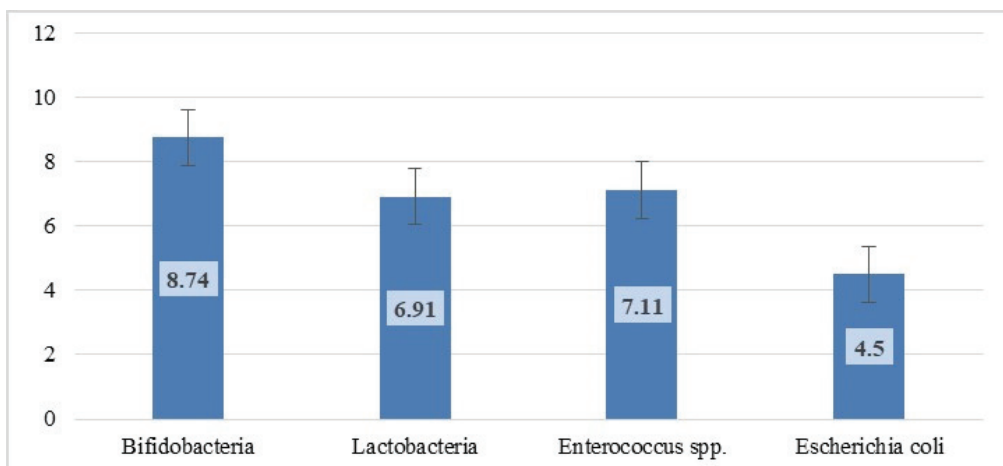


Figure 1. Quantitative levels of bifidobacteria, lactobacteria, enterococci and *Escherichia coli* in the intestinal content (faecal matter) of pheasant (log/g bacterial cells).

The data on the content of investigated bacteria show a good health status in pheasant, as a higher level of “good” bacteria (bifidobacteria, lactobacteria) and a lower level of *Escherichia coli* were found. The content of lactobacteria is almost identical to that of enterococci, indicating a possible symbiosis between them and bifidobacteria and lactobacteria, which keeps the *E. coli* count at a level that does not allow the development of dysbacteriosis in pheasant. This situation regarding the number of studied bacteria may be due to the varied structure of the feeding ration for pheasant in the Chisinau Zoo (Table 1). As mentioned, the ration was elaborated according to the specific diet of the investigated birds. Thus, the ration for pheasant is quite well structured according to the main nutrients, contains rich sources of vegetable fibres, raw vegetable and berries, and proteins (Table 1).

Data on the content (numerical value) of investigated bacteria in the black swan are presented in Fig. 2.

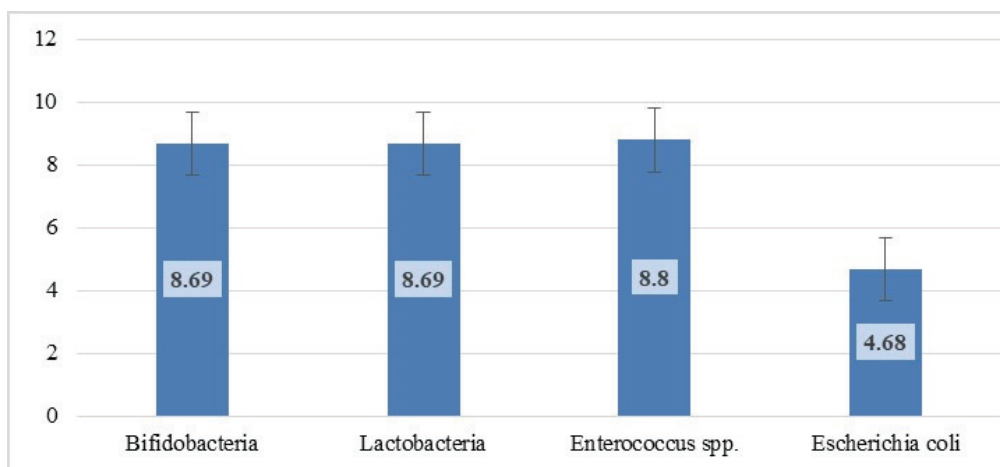


Figure 2. Quantitative levels of bifidobacteria, lactobacteria, enterococci and *Escherichia coli* in the intestinal content (faecal matter) of the black swan (log/g bacterial cells).

Analysis of the content of bifidobacteria, lactobacteria, enterococci and *Escherichia coli* in the intestinal content of the black swan showed the same quantitative level of bifidobacteria and lactobacteria, which is slightly exceeded (with 0.11 units) by that of enterococci. However, this correlation of bifidobacteria and lactobacteria with enterococci keeps the *Escherichia coli* content under control (Fig. 2). Note that the *E. coli* content in black swan does not differ significantly from that in pheasant. We can assume that the gut content of the investigated bacteria correlates with the structure of the diet. We observe that the food ration of the swan differs from that of pheasant, is less varied, but is elaborated according to the specific nutrition of these wild birds and contains natural sources of vegetable fibres and animal protein.

Following the same model, the microbiological analysis of mallard faecal matter was performed and the data are shown in Fig. 3.

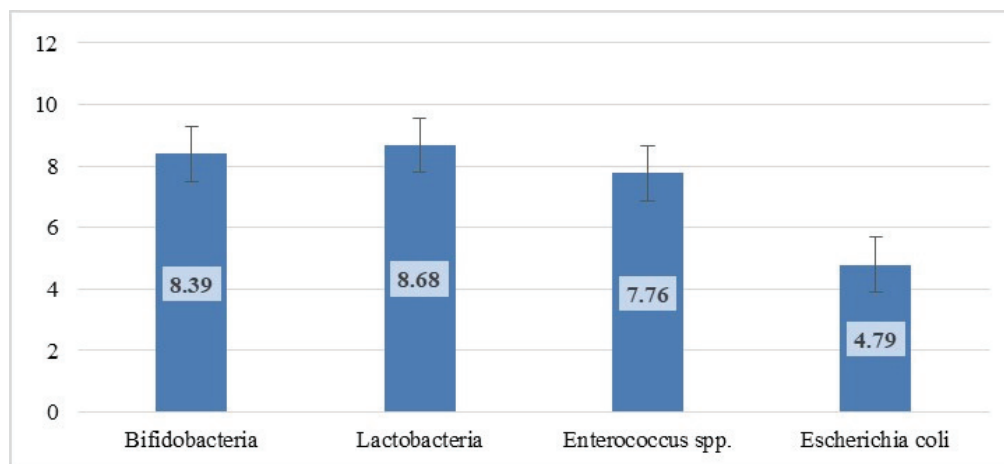


Figure 3. Quantitative levels of bifidobacteria, lactobacteria, enterococci and *Escherichia coli* in the intestinal content (faecal matter) of mallard (log/g bacterial cells).

The content of the studied bacteria in the faecal sample from mallard did not differ significantly from that of the other two species (pheasant and black swan). In fact, in mallard, a net numerical dominance of “good” bacteria – bifidobacteria and especially lactobacteria – was established compared to enterococci and especially *Escherichia coli* (Fig. 3). The feed ration destined for mallard was developed according to the same principle as for the other two wild bird species and contains sources of fibres and proteins identical to those in the wild environment (Table 1). It should be noted that the feed ration for mallard is more diverse than that for swan, which may explain the higher content of bifido- and lactobacteria.

The correlation between the investigated bacteria in the gut content (faecal matter) of the three wild bird species is reflected in Fig. 4.

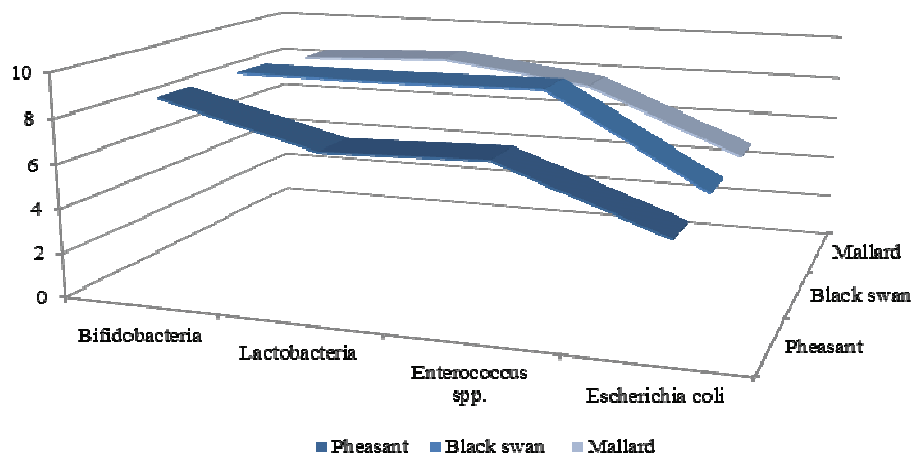


Figure 4. Variation of bifidobacteria, lacticobacteria, enterococcus and *Escherichia coli* intestinal content (log/g bacterial cells) at pheasant, black swan and mallard.

Thus, in the gut content of pheasant, the highest level of bifidobacteria was determined. The quantitative value of bifidobacteria is almost identical to the content of bifidobacteria and lactobacteria in black swan and to that of lactobacteria in mallard. We can assume that the higher level of bifidobacteria in the intestinal content of pheasant compensates for the lower level of lactobacteria in their co-report with pathogenic bacteria. The numerical indices of enterococci are higher in black swan compared to mallard and pheasant. In general, a dominance of “good” bacteria is observed, which keeps *Escherichia coli* at an admissible “safe” level. It can be assumed that a favourable interrelationship is formed between bifidobacteria, lactobacteria and enterococci contributing to the maintenance of colon homeostasis.

DISCUSSIONS

The relevant literature generally classifies the microbial flora of the digestive tract into: 1) indigenous, beneficial microorganisms that have come to have a close relationship with the host through a long period of evolution and 2) potentially pathogenic microorganisms. The health of the digestive tract and the whole body depends on the co-existence of these two types of micro-organisms. Many factors influence the interactions between intestinal micro-organisms and their hosts. Stressful conditions experienced by birds in their natural habitat can cause a large imbalance

of different types of gut microorganisms, inducing various digestive problems. However, under natural conditions in wild birds, due to their lifestyle, interaction with different living environments, which are new sources of microorganisms, a diverse gut microbiota is established, which determined significant resistance to the action of stress factors (SUN et al., 2022).

Under captive conditions, in both domestic and wild birds, gut microflora diversity is lower and mostly induced by the structure of the feed ration. Furthermore, captive wildlife animals are very susceptible to opportunistic diseases and they may act as a reservoir of pathogenic bacteria (AHMED et al., 2007; DAVIES et al., 2016). In cases of inappropriate husbandry and inadequate nutrition, pathogenic bacteria can develop, suppressing beneficial microflora and causing severe discomfort, illness and mortality. Among gut bacteria, *Escherichia coli* may be considered the most prevalent opportunistic enterobacterium in captive animals and has been associated with systemic disease in birds (DHO-MOULIN & FAIRBROTHER, 1999).

Furthermore, upon the analysis of the feed ration structure and the variation of bifidobacteria, lactobacteria, enterococci and *Escherichia coli* in the intestinal contents of the investigated birds, no specific correlation between these two indices (feed ration and microbiota) was established. That is, a higher or lower relationship between certain components of the feed ration and the quantitative indices of the analysed microorganisms was not determined.

In addition, practically no differences in the numbers of bifidobacteria, lactobacteria, enterococci and *Escherichia coli* were found according to the species (phylogenetic) characteristics of the studied birds.

This denotes that food rations are balanced and represent the determining factor in establishing a favourable microbial profile in the gut that is ensured by a numerical dominance of bifidobacteria and lactobacteria in the intestinal contents. It is known that the presence of lactobacteria and bifidobacteria in the gastrointestinal tract of animals is often associated with health benefits, including the production of metabolites such as short-chain fatty acids and vitamins, immune system development and prevention of intestinal disorders (O'CALLAGHAN & van SINDEREN, 2016). In the intestinal lumen, Lactobacillus and Bifidobacterium strains, by producing lactic acid, H₂O₂, bacteriocins and other inhibitory agents, directly inhibit some viruses, preserve epithelial integrity and compete with pathogens for intestinal epithelial cell receptors (SERVIN, 2004). Enterococci are one of the most controversial groups of bacteria but it was proved that these bacteria participate in the modulation of the immune system in humans and animals. Enterococci produce small peptides called enterocins that exhibit broad antimicrobial activity, inhibiting the multiplication of bacteria including *Escherichia coli* (KRAWCZYK et al., 2021).

Based on the obtained data, we can assume that the bacteria from the Bifidobacterium, Lactobacillus and Enterococcus groups form a consortium that contributes to the maintenance of bacterial homeostasis in the intestines called "eubiosis".

CONCLUSIONS

The analysis of the intestinal microbiota of wild birds in captivity at the Chisinau Zoo revealed a good health status of the digestive tract, determined by the numerical dominance of bifidobacteria, lactobacteria and enterococci and a low level of *Escherichia coli*.

The correlation of the enterococci content with the bifidobacteria and lactobacteria content points to a possible symbiosis of these bacteria, contributing to the maintenance of the homeostasis of the intestinal microbiota and inhibition of the pathogenic action of *Escherichia coli*.

The feed rations for wild birds in the Chisinau Zoo are balanced and are the determining factor in establishing a favourable microbial profile in the gut, that contribute to health benefits for the host.

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