

Specific composition of indigenous microflora (*Lactobacillus* spp., *Bifidobacterium* spp., *Lactococcus* spp.) in farm animals

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To maintain a stable composition of the gastrointestinal tract microflora in farm animals it is necessary to use probiotic agents to ensure the full functioning of the digestive, hormonal, and immune systems of the body. Most modern probiotics include lactic acid bacteria and bifidobacteria, which are the most physiologically valuable components of a healthy organism's an indigenous microflora. The aim of this study was to provide indication and identification from the milk of healthy cows and gastric tract of healthy pigs and calves of the genus bacteria *Lactobacillus*, *Bifidobacterium*, and *Lactococcus*. The objects of research were cultures of microorganisms isolated from cows milk (82), the gastrointestinal tract of cattle (317), and piglets of different age groups (114). Bacteriological studies were carried out on the basis of the veterinary sanitation and parasitology laboratory of the National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" (Kharkiv) in accordance with current regulatory documents. According to the research of the gastrointestinal tract of clinically healthy calves and piglets isolated and typified to 317 and 114 cultures of microorganisms, the species composition of the microflora (82 bacterial cultures) of the cisternous and parenchymatous milk of clinically healthy cows was determined. A total of 513 isolates of microorganisms were isolated, including: *Enterobacter* spp. – 2 (0,39%), *Staphylococcus* spp. – 7 (1,37%), *Bacillus* spp. – 11 (2,14%), *Enterococcus* spp. – 33 (6,43%), *Lactococcus* spp. – 75 (14,62%), *Bifidobacterium* spp. – 170 (33,14%), and *Lactobacillus* spp. – 215 (41,91%). In the study of the biological properties of isolated microorganisms *Lactobacillus* spp. (215) established their species identity: *L. brevis* – 7 (3,26%), *L. delbrueckii* – 9 (4,19%), *L. acidophilus* – 21 (9,77%), *L. fermentum* – 23 (10,69%), *L. casei* – 57 (26,51%), and *L. plantarum* – 98 (45,58%). Cultures of *Bifidobacterium* spp. (170) belong to *B. suis* – 2 (1,18%), *B. breve* – 7 (4,12%), *B. lactentis* – 15 (8,82%), *B. bifidum* – 21 (12,35%), *B. longum* – 22 (12,94%), *B. infantis* – 25 (14,71%), and *B. adolescentis* – 78 (45,88%). From samples of biological material of farm animals, 75 cultures of the genus *Lactococcus* spp. were isolated (75) of which *Lactococcus lactis* is representative. Isolated bacteria *Lactobacillus* spp., *Bifidobacterium* spp. and *Lactococcus* spp. promising when creating innovative probiotic products for farm animals.

Key words: Calves; Piglets; Milk; Microorganisms; *Lactobacillus* spp; *Bifidobacterium* spp; *Lactococcus* spp

Introduction

In recent years, much attention has been paid to probiotic microorganisms in the scientific literature to maintain a balance of normal microflora (de Vrese & Schrezenmeier, 2008; Ravinder et al., 2012; Dash et al., 2018). The interest to probiotics in the scientific community, in the food and processing industries is growing. However, there is a need for comprehensive and consistent approaches to assess the characteristics and efficacy and safety of probiotics and the products containing them (Rijkers et al., 2010; Reid, 2012). Lactic acid bacteria and bifidobacteria are introduced into probiotics, food supplements and functional foods (Reid, 2006). Improvement and development of new preparations based on living cultures of microorganisms is one of the urgent tasks of modern biotechnology. The search for strains of microorganisms that have the properties of the vitamins synthesis, amino acids, enzymes, antibiotic substances, is crucial in the development of innovative probiotic drugs and requires methodologically correct research using modern methods (Toomy et al., 2010; Didari et al., 2014; Gujvinska et al., 2018). Isolation of lactobacilli was performed from the children's faeces. At the same time, 20 cultures were isolated, which were able to survive at low pH and in the presence of 0,25% bile salt for 2 hours. In addition, all strains of lactobacilli showed inhibitory activity against *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 (Tulumoglu et al., 2013). Five cultures of *Lactobacillus* spp. were isolated from human milk and their growth patterns, tolerance to acid and bile, antagonistic properties, etc. were studied (Tulumoglu et al., 2018).

23 strains of lactic acid bacteria were experimentally isolated from cow's milk samples, 12 of which were coca strains and 11 were optional hetero-enzymatic lactobacilli. Based on the phenotypic characteristics of lactic acid bacteria were identified as *Lactococcus lactis*, *Enterococcus faecalis*, *E. faecium*, *E. durans*, *Lactobacillus paracasei* subsp. *paracasei*, *L. plantarum* and *L. rhamnosus* (Asmahan, 2011). The commensals of the gastrointestinal tract are bifidobacteria and some strains are considered probiotics because they have a positive effect on the composition and metabolic activity of the gut microbiota, as well as on the overall state of the macroorganism (Sanchez et al., 2008). *Bifidobacterium bifidum* CECT 4549 and *B. bifidum* M6 cultures were selected from the 19 strains tested because they had the highest resistance to bile acids (Margolles et al., 2003). Strains of *B. breve* and *B. longum* subsp. *longum* have been identified as potential probiotics for the treatment of intestinal disorders (Aloisio et al., 2012).

Biochemical properties of 40 strains of *Lactococcus lactis* isolated from indigenous Montenegrin dairy products have been characterized and studied. However, it was found that a large percentage (27,5%) of the tested strains showed safety for consumer health (Bojanic et al., 2017). Most probiotic bacteria on the market belong to the genera *Lactobacillus* and *Bifidobacterium* and are used to treat diarrhea and improve overall gastrointestinal discomfort (Ruiz et al., 2013; Dylag et al., 2014), reducing local and systemic allergic inflammation (Isolauri et al., 2012) exhibit immunomodulatory properties (Hor et al., 2019). The safety of the use of these microorganisms in various physiological states of the body has been proven (Chen et al., 2007; Dugoua et al., 2009).

Along with the most common cultures of *Lactobacillus* spp. and *Bifidobacterium* spp. used as a probiotic in recent years, the widespread acquisition of other microorganisms: *Escherichia coli*, *Streptococcus* spp., *Enterococcus* spp., *Bacteroides* spp., *Bacillus* spp., *Propionibacterium* spp. and various microscopic mushrooms. The properties of probiotic cultures have been shown to be specific for each strain (Rolfe, 2000). It should also be noted that in some cultures, such as *Bacillus cereus*, it is difficult to predict pathogenicity because it varies from strains used as probiotics to dangerous infectious isolates (Kamar et al., 2013; Zhu et al., 2016). To determine the widespread use of lactobacilli and bifidobacteria directly in production, a preliminary study of their properties *in vitro* systems is required, followed by the use of the test cultures on animals *in vivo* (Di Gioia et al., 2014).

Probiotics have shown promise for the treatment of certain diseases in both human and veterinary medicine, but understanding the molecular mechanisms behind the direct and indirect effects on the immune response of the intestine will contribute to better and possibly more effective treatment of diseases (Vieira et al., 2013). Although the vast majority of probiotics used today are generally considered safe and useful, a scientifically sound approach is needed in the application of these agents on a case-by-case basis (Boyle et al., 2006; Rijkers et al., 2011; Fijan, 2014). Considering the relevance of this field of research and development of innovative probiotic agent, we carried out the indication and identification from the milk of healthy cows and gastrointestinal tract of healthy pigs and calves of bacteria the genus *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, as the most physiologically.

Materials and Methods

The objects of study were cultures of micro-organisms isolated from cows' milk (82), gastrointestinal tract of cattle (317) and pigs of different age groups (114). Experimental studies were conducted in the laboratory of veterinary sanitation and parasitology of the National Science Center "Institute of Experimental and Clinical Veterinary Medicine" (Kharkiv).

Simple and selective nutrient media were used to isolate microorganisms from biological material and further determine their cultural properties (Basu et al., 2015). To detect and identify lactobacilli and bifidobacteria used MRS medium, Blaurok's medium, improved on their basis for 24-72 hours at 37°C (Süle et al., 2014; Yang et al., 2018; Gujvinska et al., 2018).

The biological properties of lactic acid bacteria were studied by tinctorial, cultural-morphological and biochemical characteristics. The morphology of the cells and colonies of the studied cultures was determined by microscopy of Gram stained smears, the growth pattern by sowing the cultures on liquid and solid nutrient media.

Identification of bacteria by species was performed on the spectrum of carbohydrate fermentation using the ARI 50 CH test systems and API 20Strep (Bio Mérieux, France). These systems determine the digestion possibility of 47 carbohydrates and their derivatives. The results were recorded after 24 and 48 hours. The results were interpreted using API-WEB (Professional). Species belonging to isolated microorganisms were determined by appropriate tests (van Teeseling et al., 2017). The data obtained were processed using a Microsoft Excel 7.0 software.

Results and Discussion

As a result of bacteriological examination of biological's samples material from the gastrointestinal tract of clinically healthy calves, 317 cultures of microorganisms were isolated and typed (Table 1).

Table 1. The ratio of isolated probiotic cultures.

Genus	Number	%
<i>Lactobacillus</i> spp.	127	40.06
<i>Bifidobacterium</i> spp.	104	32.81
<i>Lactococcus</i> spp.	42	13.25
<i>Enterococcus</i> spp.	33	10.41
<i>Bacillus</i> spp.	11	3.47
Total	317	100

From the materials of Table 1 shows that the largest number of isolated probiotic cultures (72,87%) are *Lactobacillus* spp. (40.06%) and *Bifidobacterium* spp. (32.81%), which in turn is confirmed by their popularity in the construction of probiotics for animals. The remaining selected crops (27.13%) are represented by *Lactococcus* spp., *Enterococcus* spp. and *Bacillus* spp. At detection of *Bacillus* spp. microorganisms in one case, *Bacillus subtilis* was isolated from the gastrointestinal tract of a newborn calf.

A total of 82 bacterial cultures were isolated in determining the species composition of the cisternal (16) and parenchymatous (21) milk microflora selected from clinically healthy cows (Table 2).

Table 2. The number of microorganisms isolated from the milk of clinically healthy cows.

Genus	Number		%
	Cistern milk	Parenchymal milk	
<i>Lactobacillus</i> spp.	16		35.57
<i>Lactococcus</i> spp.	11		24.44
<i>Bifidobacterium</i> spp.	11		24.44
<i>Staphylococcus</i> spp.	5		11.11
<i>Enterobacter</i> spp.	2		4.44
Total	45		100
	Parenchymal milk		
<i>Lactococcus</i> spp.	16		43.24
<i>Lactobacillus</i> spp.	10		27.03
<i>Bifidobacterium</i> spp.	9		24.32
<i>Staphylococcus</i> spp.	2		5.41
Total	37		100

As a result of the work (Table 2), 82 cultures of microorganisms were isolated from 37 milk samples, represented by lactic acid streptococci – 27 (32.93%), staphylococci – 7 (8.54%), enterobacteria – 2 (2.44%), lactobacilli – 26 (31.70%), and bifidobacteria – 20 (24.39%). Analysis of the results obtained in the study of cisternal and parenchymatous milk shows that there is no significant difference between the species composition of the microflora, except that enterobacteria in cisternal milk were isolated in 4.44% of cases, whereas they were absent in parenchymal milk. Based on the study of the cultural-morphological and biochemical properties of the bacteria isolated from milk, they were assigned to *Lactococcus lactis* (27), *Staphylococcus epidermidis* (7), *Escherichia coli* (1), *Proteus vulgaris* (1). The specificity of 153 cultures of *Lactobacillus* spp. and 124 cultures of *Bifidobacterium* spp. were determined using appropriate test systems (Table 3).

Table 3. Specific differentiation of lactobacilli and bifidobacteria isolated from cattle.

Genus	<i>Lactobacillus</i> spp.		Genus	<i>Bifidobacterium</i> spp.	
	Number	%		Number	%
<i>L. plantarum</i>	73	47.71	<i>B. adolescentis</i>	64	51.61
<i>L. casei</i>	30	19.61	<i>B. infantis</i>	19	15.32
<i>L. acidophilus</i>	21	13.72	<i>B. longum</i>	14	11.29
<i>L. fermentum</i>	17	11.11	<i>B. lactentis</i>	11	8.87
<i>L. delbrueckii</i>	7	4.58	<i>B. bifidum</i>	9	7.26
<i>L. brevis</i>	5	3.27	<i>B. breve</i>	7	5.65
Total	153	100	Total	124	100

The cultures of *Lactobacillus* spp. (153) were gram-positive, asparagenic long sticks and coccyx forms that were located in short chains. Lactobacilli grown on MRS-2 medium had a brushlike growth colonies in the middle and lower part of the tube, small transparent colonies were formed on MRS-4 agar medium and hydrolyzed milk agar. Colonies on agar medium had a diameter of 2-5 mm, convex, with entire edges, opaque, not pigmented.

Fermentation type metabolism was observed in cultures, nitrates were not recovered, gelatin was not liquefied, catalase-negative, cytochromes were not contained. As a result of the identification of lactobacilli, the test cultures are classified as *L. plantarum*, *L. casei*, *L. acidophilus*, *L. fermentum*, *L. delbrueckii*, *L. brevis*.

The microorganisms of *Bifidobacterium* spp. (124) were gram-positive sticks, variable in shape and arrangement. Usually they were curved, club-shaped and often branched at the ends, not movable. After 48 hours of culturing, the cells did not stain evenly. All tested microorganisms grew only under anaerobic conditions. On the surface of the solid medium MRS they formed cream-white, convex, round, with an equal colony margin, with a diameter of 1-2 mm, with a paste-like consistency.

In the semi-liquid environment, the studied strains formed colonies in the form of "comets", "nail" etc. In the liquid medium, without anaerobic conditions, bottom growth was observed in a high column of medium and in anaerobic conditions, there was a uniform growth throughout the volume of the medium. The strain had no activity of catalase and nitrate reductase, did not form glucose gas. As a result of studies on the identification of bifidobacteria, they are classified as *B. adolescentis*, *B. infantis*, *B. bifidum*, *B. breve*, *B. lactentis*, and *B. longum*.

In determining the species belonging to lactobacilli, the largest number of cultures was attributed to *L. plantarum* (73), and *L. casei* (30) species, accounting for 67.32% of all tested microorganisms. Among bifidobacterias, *B. adolescentis* (64), and *B. infantis* (19) were the most commonly identified, accounting for 66.93% of the isolates tested. The results of studies show that biological material selected from clinically healthy cattle is dominated by *Lactobacillus plantarum* (47.71%) and *Bifidobacterium adolescentis* (51.61%).

Based on the study of the cultural and morphological properties of the selected cultures, 69 isolates of the genus *Lactococcus* were isolated. The test bacteria had cells of spherical or oval shape, were gram-positive, non-motile, had no capsules, grew in pairs and short circuits in a liquid medium and did not form endospores. The strains were optional anaerobes, catalase and oxidase negative, chemorotrophs, had fermentation-type metabolism, fermented carbohydrates with lactic acid, and did not produce gas. According to the established characteristics, the culture is classified as *Lactococcus lactis*.

The next step was to study species belonging to 28 cultures of lactic acid bacteria and 26 cultures of bifidobacteria isolated from healthy pigs aged 1-15 days, as well as 40 cultures of lactic acid bacteria and 20 cultures of bifidobacteria from pigs aged 30-120 days (Table 4).

Table 4. Microorganisms isolated from clinically healthy piglets.

Age of animals	Genus	Number	%
Piglets 1-15 days old		Lactic acid bacteria	
	<i>L. casei var. rhamnosus</i>	11	39.29
	<i>L. fermentum</i>	6	21.43
	<i>L. plantarum</i>	5	17.86
	<i>L. brevis</i>	2	7.14
	<i>L. delbrueckii</i>	2	7.14
	<i>Lactococcus lactis</i>	2	7.14
	Total	28	100
		Bifidobacteria	
	<i>B. bifidum</i>	12	46.15
<i>B. adolescentis</i>	6	23.08	
<i>B. infantis</i>	6	23.08	
<i>B. suis</i>	2	7.69	
Total	26	100	
Piglets 30-120 days old		Lactic acid bacteria	
	<i>L. plantarum</i>	20	50.0
	<i>L. casei var. rhamnosus</i>	16	40.0
	<i>Lactococcus lactis</i>	4	10.0
	Total	40	100
		Bifidobacteria	
	<i>B. adolescentis</i>	8	40.0
	<i>B. longum</i>	8	40.0
	<i>B. lactentis</i>	4	20.0
	Total	20	100

The species composition of microorganisms of two different age groups of piglets revealed some differences. Thus, hetero-enzymatic species of lactobacilli (*L. fermentum*, *L. brevis*, *L. delbrueckii*) were found only in 1-15 daily piglets, accounting for 35.71% (10) of the total number of identified microorganisms. *L. casei var. rhamnosus*, and *L. plantarum*, as well as *Lactococcus lactis* were isolated from pigs of both groups.

Significant differences in the species composition of microorganisms isolated from pigs of different ages were found among bifidobacteria. Among *Bifidobacterium* spp. only piglets 1-15 days old are characterized by the presence of *B. bifidum*, *B. infantis*, and *B. suis*, accounting for 76.92% (20) of the total number of detected bifidobacteria, and for animals 30-120 days old – *B. lactentis* and *B. longum*, which is 60.0%, respectively (12). *B. adolescentis* was a common culture found in piglets of both age groups. Thus, among the lactic acid bacteria, the most common and versatile species for all age groups of piglets was *L. casei var. rhamnosus*, and among bifidobacteria - *B. adolescentis*.

Our view is in line with the opinion of other researchers (Asmahan, 2011; Aloisio et al., 2012), which indicate that most often probiotics include microorganisms of the genus *Lactobacillus* and *Bifidobacterium*, which have variability in antagonistic activity. Therefore, it was among these microorganisms that the most effective probiotic strains of microorganisms were searched.

According to the literature, lactobacilli of the genus *Lactobacillus* belong to the group of gram-positive asparagine sticks of the usual form (Felis & Dellaglio, 2007; Gujvinska & Paliy, 2018). Lactobacilli grown on MRS-2 medium had a brushlike growth colonies in the middle and lower part of the tube, small transparent colonies were formed on MRS-4 agar medium and hydrolyzed milk agar. Colonies on agar medium convex, with entire edges, opaque, not pigmented (Hor et al., 2019). According to cultural and morphological characteristics, the cultures of lactobacilli selected from samples of biological material from farm animals were assigned to *L. plantarum*, *L. casei*, *L. acidophilus*, *L. fermentum*, *L. delbrueckii*, *L. brevis*.

Microorganisms *Bifidobacterium* spp. belong to the group of gram-positive non-spore-forming sticks of irregular shape (Cheikhyoussef et al., 2008). Bifidobacteria grown on Blaurok medium have stem growth in the middle of the tube. In smears, the sticks are very variable in shape, 0.5-1.3 x 1.5-8 microns in size, slightly curved. Cells arranged individually, in pairs, in a V-shape, sometimes by a chain, palisade or slingshot, also have swollen coccyx forms (Margolles et al., 2003). According to the cultural and morphological features, the cultures of bifidobacteria we have allocated were attributed to *B. adolescentis*, *B. infantis*, *B. bifidum*, *B. breve*, *B. lactentis*, *B. longum*, and *B. suis*. According to existing data, lactococci belong to the group of gram-positive cocci (Neville et al., 2012; Prabhurajeshwar & Chandrakanth, 2017). Bacteria have cells of spherical or oval shape, 0.5-1.2 x 0.5-1.5 microns in size, grow in pairs and short chains in liquid medium, do not form endospores, gram-positive, no motile, have no capsules. These microorganisms are optional anaerobes, chemoorganotrophs, have fermentation-type metabolism, ferment carbohydrates with the formation of lactic acid, do not form gas, catalase- and oxidase-negative. According to cultural and morphological features we have identified 75 cultures of the genus *Lactococcus*. Biological studies have allowed to isolate a large number of bacteria *Lactobacillus* spp., *Bifidobacterium* spp. and *Lactococcus* spp., which can be used to create probiotic products for farm animals.

Prospects for further studies include the selection of lactic acid bacteria to create probiotic drugs for the prevention and treatment of dysbiosis in farm animals.

Conclusion

In bacteriological examination of biological material samples from the gastrointestinal tract of calves, pigs and milk of clinically healthy cows 513 isolates of microorganisms were indicated, including *Enterobacter* spp. – 2 (0.39%), *Staphylococcus* spp. – 7 (1.37%), *Bacillus* spp. – 11 (2.14%), *Enterococcus* spp. – 33 (6.43%), *Lactococcus* spp. – 75 (14.62%), *Bifidobacterium* spp. – 170 (33.14%), and *Lactobacillus* spp. – 215 (41.91%). In the study of biological properties of isolated microorganisms *Lactobacillus* spp. (215) referred to *L. brevis* – 7 (3.26%), *L. delbrueckii* – 9 (4.19%), *L. acidophilus* – 21 (9.77%), *L. fermentum* – 23 (10.69%), *L. casei* – 57 (26.51%), and *L. plantarum* – 98 (45.58%). According to cultural and morphological features were isolated cultures of *Bifidobacterium* spp. (170) are classified as *B. suis* – 2 (1.18%), *B. breve* – 7 (4.12%), *B. lactentis* – 15 (8.82%), *B. bifidum* – 21

(12.35%), *B. longum* – 22 (12.94%), *B. infantis* – 25 (14.71%), and *B. adolescentis* – 78 (45.88%). From the samples of biological material from farm animals, 75 cultures of the genus *Lactococcus* spp. were isolated (75), represented by *Lactococcus lactis*.

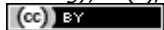
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