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FORMATION OF INTESTINAL BACTERIOCENOSIS IN CALVES ASSOCIATED WITH BACTERIAL INSEMINATION OF THE UTERINE CANAL IN HIGH-YIELDING COWS

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Summary. The article provides information on the microbiota composition in the vaginal secretions of cows during the dry period, specifically 30–15 days before calving. The article discusses how endometritis affects the qualitative and quantitative composition of the conditionally pathogenic microflora, as well as how dysbiotic microbiota disorders in the birth canal impact the development of gastrointestinal diseases in calves. The study results establish the quantitative limits of the conditionally pathogenic microbiota, which requires correction when exceeded. When cows have dysbiotic changes, there is an increase in the number of certain microorganisms in their vaginal swabs. Specifically, the number of *Escherichia coli*, sulfite-producing clostridia, and saprophytic microorganisms of the genus *Bacillus* goes beyond 6.0 ± 0.1 lg CFU/cm^3, while staphylococci and yeast-like fungi of the genus *Candida* exceed 5.9 ± 0.1 lg CFU/cm^3. Additionally, calves born to cows with dysbiotic disorders of vaginal secretion had dysbiotic disorders in the gastrointestinal tract in 86.1% of cases. In these cases, the number of lactobacilli was not higher than 4.8 ± 0.1 lg CFU/cm^3, bifidobacteria — 4.7 ± 0.1 lg CFU/cm^3. The number of *Escherichia coli* exceeded 4.6 ± 0.1 lg CFU/cm^3, which in 48.4% of cases resulted in the development of diarrhea of varying severity

Keywords: microbiota, dysbiosis, normal intestinal microflora, normal vaginal flora

Introduction. There is a lack of clear data regarding the development of intestinal bacteriocenosis in calves in relation to the bacterial contamination of the genital tract of highly productive cows (Bentsa, 2018; Bortnichuk, Sadovskyi and Sorokina, 1997; Basova et al., 2016; Maslianko et al., 2013; Kalinichenko, Korotkykh and Tishchenko, 2016; Petrova and Domracheva, 2012; Stravskyi and Stravska, 2014; Vlizlo, 2012; Yakubchak et al., 2005; Garrity et al., 2005; Hadzevych et al., 2021; Diao, Zhang and Fu, 2019; Liu et al., 2019; Bi et al., 2019; Alipour et al., 2018; Mayer et al., 2012; Uyeno, Sekiguchi and Kamagata, 2010; Baldwin et al., 2004).

Previous studies have shown that the number of microorganisms present in calves is minimal up to three days of age. The quantity of bifidobacteria and lactobacilli does not exceed 3 lg colony forming units per 1 cm^3 of large intestine content (Hadzevych et al., 2021).

After 15 days of age, calves develop a more stable gastrointestinal microflora that includes a protective barrier known as colonization resistance. The microflora in the intestinal mucosa displays antagonistic activity, preventing the penetration of pathogens. However, in newborn calves, the intestinal microflora's qualitative and quantitative composition is not yet strong enough to prevent the colonization of foreign microorganisms, including pathogens.

Numerous studies have shown that the microbiota of the birth canal plays a leading role in the formation of the microbiome of newborns (Bortnichuk, Sadovskyi and Sorokina, 1997; Basova et al., 2016 Maslianko et al., 2013; Kalinichenko, Korotkykh and Tishchenko, 2016; Petrova and Domracheva, 2012; Stravskyi and Stravska, 2014).

The aim of the study was to investigate the formation of calves' intestinal bacteriocenosis in relation to the bacterial colonization of the birth canal of highly productive cows.

Materials and methods. The study was carried out in 2022 in the Laboratory of Animal Bacterial Diseases of the NSC 'IECVM'. To determine the normocenosis, the vaginal secretion of cows in the dry period 15–30 days before calving and the swabs from the intestinal tract of calves at the age of 7–10 days were examined. All data were recorded, and at the end of the observation period, the data were analyzed and conclusions were drawn. A total of 120 cows and 114 calves were studied. The animal studies were conducted in accordance with the basic principles of bioethics.

Animals were housed, maintained, and fed according to standards and rations. The quantitative and qualitative diets of animals in different housing systems did not differ. The material was delivered to the laboratory and examined within 2 hours of collection. Serial 10-fold dilutions of homogenized material in a sterile isotonic sodium chloride solution from 10^{-1} to 10^{-10} were performed to determine the quantitative value of microorganisms.

From each tube of the titration series, 1 cm^3 of homogenate was inoculated onto the optimal culture media for each microbial species and incubated under optimal temperature conditions and hours.

Simple and selective culture media produced by Farmaktiv LLC (Ukraine) and HiMedia Laboratories Prv. Limited (India) were used. For the isolation of enterobacteria, Endo, Ploskirev, Levin, MacConkey media, bismuth sulfite agar, Olkenitsky medium, selenite broth (for the accumulation of *Salmonella*), Simons medium were used; for staphylococci — egg yolk salt agar, Chistovich's medium; for streptococci — media containing glucose (1%), blood (5–10%) and serum (10–20%); for fungi and yeasts — Sabouraud's and Wort's agar; for anaerobes — Kitt-Tarozzi, Wilson-Blair agar, L. D. agar with esculin (for anaerobes), Voget-Fredette agar. For the isolation of bifidobacteria, Blaurock's medium was used, and for the isolation of lactobacilli, LactoBacAgar.

To determine the hemolytic activity of microorganisms, 5% blood agar was used, and coagulase activity was determined using dry rabbit citrate plasma produced by Pharmstandard-Biolik PJSC (Ukraine).

After incubation of the cultures on the media under optimal conditions, the colonies grown from each dilution were counted. The population level of microorganisms was expressed in the decimal logarithm of the indicator — lg CFU/cm^3 (colony forming units in 1 cm^3 of material).

To determine the number of microorganisms, the degree of dilution, the number of growing colonies and the inoculation dose were taken into account.

The number of colonies of forming units in 1 cm^3 of material was calculated using the formula (1):

$$M = \frac{N}{V} \times 10^{n+1} \quad (1)$$

where: M is the number of microorganisms in 1 cm^3; N is the average number of colonies in 1 bacteriological dish; V is the volume of suspension applied to the surface of the agar during seeding; 10^{n+1} is the dilution from which seeding was performed (Vlizlo, 2012; Yakubchak et al., 2005). Bacteria were identified according to Bergey's Manual of Systematics Bacteriology (Garrity et al., 2005).

Statistical processing of numerical data was carried out by the method of alternative analysis using the application Microsoft Excel (Microsoft Office). The relative share of the characteristic in the statistical population (in percent) (M) and its error (m) were determined, and the level of reliability (p) was determined.

Results. The results of the determination of the composition of the microflora of the vaginal secretion of cows in the dry period are shown in Table 1.

Table 1 — Microbiota of vaginal secretion of cows in the dry period 15–30 days before calving

Indicators	Frequency of isolation of microorganisms from cows that							
	had no clinical manifestations of diseases of the reproductive system (n = 80)				had clinical manifestations of diseases of the reproductive system (n = 40)			
	n	%	colonization density, lg	amount, in 1 cm^3	n	%	colonization density, lg	amount, in 1 cm^3
Lactobacilli	80	100	5.3 ± 0.1	989 ± 238×10^4	32	80	2.4 ± 0.1	15 ± 4.9×10^2
Escherichia coli without hemolytic properties	58	72.5	2.4 ± 0.1	212 ± 51.2×10^2	40	100	6.0 ± 0.1	68 ± 17.4×10^5
Escherichia coli with hemolytic properties	2	2.5	1 ± 0	4 ± 1.5×10^1	25	62.5	4.3 ± 0.1	179 ± 51×10^3
Sulfite-reducing clostridia	2	2.5	1 ± 0	35.8 ± 23×10^1	22	55	6.0 ± 0.1	100 ± 26×10^5
Representatives of the family Enterobacteriaceae	80	100	4.0 ± 0.1	86 ± 39×10^3	40	100	7.3 ± 0.1	184 ± 43×10^6
Enterococci	35	43.7	2.4 ± 0.1	91 ± 31×10^2	34	85	4.8 ± 0.1	78 ± 25×10^4
Staphylococci coagulase-negative	80	100	2.3 ± 0.1	126 ± 28×10^3	40	100	5.9 ± 0.1	105 ± 40.4×10^5
Staphylococci coagulase-positive	0	0	0	0	28	70	5.1 ± 0.1	176 ± 52.7×10^4
Streptococci	80	100	3.5 ± 0.1	35 ± 11.2×10^3	40	100	6.3 ± 0.1	411 ± 115×10^5
Yeast-like fungi of the genus *Candida*	56	70	4.0 ± 0.1	104 ± 39.3×10^3	23	57.5	5.9 ± 0.1	152 ± 54.7×10^5
Saprophytic microorganisms of the genus *Bacillus*	16	20	3.7 ± 0.1	22 ± 3.8×10^3	32	80	6.8 ± 0.1	152 ± 44.7×10^6

In cows with endometritis, a violation of the qualitative ratio of microflora was observed due to the appearance and increase in the vaginal secretion of *Escherichia coli*, staphylococci and sulfite-reducing clostridia, the appearance of hemolytic strains of *Escherichia coli* and coagulase-positive staphylococci, and a decrease in lactobacilli:

(1) the amount of lactobacilli with endometritis was $15 \pm 4{,}9\times10^2$ CFU/cm^3, in clinically healthy animals — $989 \pm 238\times10^4$ CFU/cm^3;

(2) the amount of *Escherichia coli* — $68 \pm 17{,}4\times10^5$ CFU/cm^3, in clinically healthy animals — $212 \pm 51{,}2\times10^2$ CFU/cm^3;

(3) the amount of sulfite-reducing clostridia — $100 \pm 26\times10^5$ CFU/cm^3; in clinically healthy animals — $35{,}8 \pm 23\times10^1$ CFU/cm^3;

(4) the amount of bacteria from the family Enterobacteriaceae to $184 \pm 43\times10^6$ (in clinically healthy animals — $86 \pm 39\times10^3$ CFU/cm^3;

(5) the amount of yeast-like fungi of the genus *Candida* to $152 \pm 54{,}7\times10^5$ CFU/cm^3; in clinically healthy animals — $104 \pm 39{,}3\times10^3$ CFU/cm^3;

(6) the amount of saprophytic microorganisms of the genus *Bacillus* to $152 \pm 44{,}7\times10^6$ CFU/cm^3; in clinically healthy animals — $22 \pm 3{,}8\times10^3$ CFU/cm^3;

(7) the amount of coagulase-positive staphylococci — $176 \pm 52{,}7\times10^4$ CFU/cm^3, in clinically healthy animals — $126 \pm 28\times10^3$ CFU/cm^3.

Calves obtained from cows with endometritis in 86.1% of cases had dysbiotic disorders in the gastrointestinal tract (Table 2), namely the number of lactobacilli was not higher than 4.8 ± 0.1 lg CFU/cm^3, bifidobacteria — 4.7 ± 0.1 lg CFU/cm^3. The number of *Escherichia coli* was 6.2 ± 0.1 lg CFU/cm^3 (Table 3), saprophytic microorganisms of the genus *Bacillus* — 6.0 ± 0.1 lg CFU/cm^3, which in 48.4% of cases (15 heads) resulted in the development of diarrhea of varying severity.

Table 2 — Presence of dysbiotic changes in the gastrointestinal tract of calves from sick and healthy cows

Calves from healthy cows (n = 78)				Calves obtained from cows with endometritis (n = 36)			
no dysbiotic changes in the gastrointestinal tract		had dysbiotic changes in the gastrointestinal tract		no dysbiotic changes in the gastrointestinal tract		had dysbiotic changes in the gastrointestinal tract	
n	%	n	%	n	%	n	%
68	87.1	10	12.9	5	13.9	31	86.1

Table 3 — Results of studies of rectal swabs from calves of 5–7 days of age

Indicators	Qualitative and quantitative changes in the microbiota in the rectum of calves							
	without dysbiotic changes (n = 73)				with dysbiotic changes (n=41)			
	n	%	colonization density, lg	amount, in 1 cm^3	n	%	colonization density, lg	amount, in 1 cm^3
Lactobacilli	73	100	8.0 ± 0.1	$143 \pm 40\times10^7$	41	100	4.8 ± 0.1	$45 \pm 9.8\times10^4$
Bifidobacteria	73	100	6.8 ± 0.1	$96 \pm 38\times10^6$	41	100	4.7 ± 0.1	$48 \pm 14\times10^4$
Escherichia coli	73	100	4.6 ± 0.1	$83 \pm 20\times10^4$	41	100	6.2 ± 0.1	$182 \pm 38\times10^5$
Sulfite-reducing clostridia	15	20.5	2.7 ± 0.1	$44 \pm 6\times10^2$	22	53.6	4.8 ± 0.1	$65 \pm 15\times10^4$
Conditionally pathogenic microorganisms from the family Enterobacteriaceae	73	100	2.8 ± 0.1	$114 \pm 50\times10^2$	41	100	6.1 ± 0.1	$116 \pm 32\times10^5$
Enterococci	73	100	3.8 ± 0.1	$230 \pm 67\times10^3$	41	100	5.8 ± 0.1	$49 \pm 16\times10^5$
Staphylococci	60	82.1	3.5 ± 0.1	$135 \pm 54\times10^3$	23	56.1	4.6 ± 0.1	$113 \pm 82\times10^4$
Saprophytes of the genus *Bacillus*	73	100	2.9 ± 0.1	$223 \pm 45\times10^2$	41	100	6.0 ± 0.1	$137 \pm 46\times10^5$

Discussions. The results of the research once again confirmed the data obtained in previous years that in calves, in order to exclude dysbiotic disorders, it is necessary to maintain the optimal composition and quantitative level of the main microflora of the intestinal tract, in particular, the number of lactobacilli should not be lower than 6 lg CFU/cm^3, bifidobacteria — 7 lg CFU/cm^3, the number of *Escherichia coli* not higher than 7 lg CFU/cm^3 (Hadzevych et al., 2021). In addition, it has been proved that the presence of a violation of the qualitative and quantitative composition of the microflora in the birth canal in cows contributes to the occurrence of dysbiotic changes in the microflora of the gastrointestinal tract of calves in 86.1% of cases and in 48.4% of cases of diarrhea of varying severity. Based on the analysis of literature data, no unified normative indicators for the composition of the gastrointestinal tract normoflora in calves and vaginal secretions in cows

have been established (Bortnichuk, Sadovskyi and Sorokina, 1997 Basova et al., 2016 Maslianko et al., 2013; Kalinichenko, Korotkykh and Tishchenko, 2016; Petrova and Domracheva, 2012; Stravskyi and Stravska, 2014; Vlizlo, 2012; Yakubchak et al., 2005; Garrity et al., 2005; Hadzevych et al., 2021; Diao, Zhang and Fu, 2019; Liu et al., 2019; Bi et al., 2019; Alipour et al., 2018; Mayer et al., 2012; Uyeno, Sekiguchi and Kamagata, 2010). However, there is undeniable evidence that dysbiosis and opportunistic microbiota in the birth canal play a significant role in the etiology and development of endometritis, along with specific infections (Bortnichuk, Sadovskyi and Sorokina, 1997). The microbiota of the gastrointestinal tract of calves after birth is formed within seven days (Hadzevych et al., 2021; Diao, Zhang and Fu, 2019), according to the results of some authors — twelve days (Uyeno, Sekiguchi and Kamagata, 2010). There are reports that microbial colonization of the mammalian intestine can begin even before birth, but these observations are controversial due to problems with reliable sampling and analysis of the rarely distributed microbiota. The mammalian gastrointestinal tract is populated with a diverse microbiota before birth, which changes rapidly in early postnatal life (Alipour et al., 2018). The method and regimens of feeding also influenced the direct transmission of bacteria from the mother and the environment to newborns. In bottle-fed animals, bacteria from the mother's vagina (46%), ambient air (31%), and floor (12%) predominated (Bi et al., 2019). In addition, there are studies indicating that certain microflora may be genetically or epigenetically influenced. Understanding that there are several correspondences between the intestinal microbiota of healthy calves and that there may be a genetic influence on the fecal flora may help prevent diarrheal diseases in the future (Mayer et al., 2012). In diseases of the reproductive organs of cows, potentially pathogenic bacteria contaminate calves at birth and contribute to the development of dysbiotic states — persistent qualitative and quantitative changes in the microbiota and gastrointestinal diseases (Yakubchak et al., 2005; Garrity et al., 2005; Hadzevych et al., 2021).

According to the literature, the reproductive system of cows is considered infected in the absence of bifidobacteria and lactobacilli in the cervical canal and the presence of opportunistic microflora, especially with pronounced hemolytic properties in the amount of 2 lg CFU/cm^3, or without hemolytic properties in the amount of 4 lg CFU/cm^3, or the presence of opportunistic microflora in associations consisting of 2 or more species, especially if they have pronounced virulent and antibiotic-resistant properties (Petrova and Domracheva, 2012). It has been reported that the microflora of the genital tract gradually increases during gestation regardless of breed, especially the number of *Escherichia coli* (Liu et al., 2019). Our studies show that the maximum amount of saprophytic microorganisms of the genus *Bacillus* in vaginal swabs 15–30 days before calving should not exceed 3.7 ± 0.1 lg CFU/cm^3, staphylococci — 2.3 ± 0.1 lg CFU/cm^3, yeast-like fungi of the genus *Candida* — 4.0 ± 0.1 lg CFU/cm^3, *Escherichia coli* without hemolytic properties — 2.4 ± 0.1 CFU/cm^3, sulfite-producing clostridia — 1.0 ± 0.1 lg CFU/cm^3, representatives of the family Enterobacteriaceae — 4.0 ± 0.1 lg CFU/cm^3. Other qualitative and quantitative indicators of the microbiota need to be analyzed and, if necessary, corrected with probiotic, antibacterial drugs or improved animal housing conditions.

Calves born from cows with dysbiotic disorders of vaginal secretion in 86.1% of cases had dysbiotic disorders in the gastrointestinal tract, namely the number of lactobacilli 4.8 ± 0.1 lg CFU/cm^3, bifidobacteria — 4.7 ± 0.1 lg CFU/cm^3, *Escherichia coli* — 6.2 ± 0.1 lg CFU/cm^3, saprophytic microorganisms of the genus *Bacillus* — 6.0 ± 0.1 lg CFU/cm^3, which in 48.4% of cases resulted in the development of diarrhea of varying severity.

Conclusions. 1. Excessive accumulation of opportunistic microbiota in the birth canal leads to the development of dysbiosis and endometritis in cows and contributes to the occurrence of gastrointestinal disorders in calves at birth.

2. Qualitative and quantitative changes in the microbiota of vaginal secretions were observed in cows with endometritis. Hemolytic forms of *Escherichia coli* were found in 62.5% of cases, sulfite-producing clostridia in 55%, and coagulase-positive staphylococci in 70%. No particularly dangerous specific pathogens were found.

3. Quantitative limits of conditionally pathogenic microbiota have been established, the exceeding of which requires correction. In the development of endometritis in cows, the number of *Escherichia coli* and sulfite-producing clostridia in vaginal swabs is at the level of 6.0 ± 0.1 lg CFU/cm^3, staphylococci — 5.9 ± 0.1 lg CFU/cm^3, saprophytic microorganisms of the genus *Bacillus* — 6.8 ± 0.1 lg CFU/cm^3, yeast-like fungi of the genus *Candida* — 5.9 ± 0.1 lg CFU/cm^3.

4. Calves born from cows with dysbiotic disorders of vaginal secretion in 86.1% of cases had dysbiotic disorders of the gastrointestinal tract, namely the number of lactobacilli was at the level of 4.8 ± 0.1 lg CFU/cm^3, bifidobacteria — 4.7 ± 0.1 lg CFU/cm^3. *Escherichia coli* — 6.2 ± 0.1 lg CFU/cm^3, saprophytic microorganisms of the genus Bacillus — 6.0 ± 0.1 lg CFU/cm^3, which in 48.4% of cases led to the development of diarrhea of varying severity.

5. Thus, by studying the qualitative and quantitative composition of the microbiota of vaginal secretions in cows before calving, it is possible to reasonably predict the development of gastrointestinal diseases in calves and endometritis in cows, which makes it possible to develop and take appropriate measures in advance to prevent, treat and prevent recurrence of disease.

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