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Visualization of microbial biofilms in case of digestive disorders in lambs

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SUMMARY

The paper demonstrates morphometric and densitometric parameters of microbial biofilms recovered from lambs with digestive disorders. Changes of quantitative and species composition of the intestinal microbiocenoses in the lambs with digestive disorders were compared with the ones of the clinically healthy lambs. Light microscopy results demonstrated formation of three-dimensional biofilm structure in the form of dense grid consisting of gram-negative and gram-positive bacteria, yeast cells, hyphas and pseudohyphas surrounded with intracellular polymer matrix. Presence of blastospores aided to the increased number of cells attached to the substrate, and biofilm was formed, which consisted of rod and round cells attached to the microfungi cells. In the process of dispersion that occurred during the destruction of the intercellular matrix and bacterial and yeast cell detachment, branched structures separated from the microcolonies and colonized microorganism-free regions of the substrate. The intensity of biofilm formation by the microorganisms under study was evaluated by optic density measurement in 48 hours of cultivation. Fluorescence microscopy results demonstrated that the dynamics of changes of the viable microbial structures was specified by intermittent periods of increased or decreased biofilm formation intensity. Cells characterized by active growth and replication and forming alternating subpopulations were detected in the examined microbial cultures. When determining the viability of the microorganisms in the biofilms, the viable (green fluorescence) and non-viable (red fluorescence) cells were differentiated.

Keywords: Attachment, biofilms, bacteria, dispersion, microfungi, colonization resistance, intercellular matrix, fluorescence.

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Индикация биопленок микроорганизмов при болезнях органов пищеварения ягнят

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РЕЗЮМЕ

Приведены результаты морфометрических и денситометрических показателей биопленок микроорганизмов, выделенных при болезнях органов пищеварения ягнят. Установлены изменения количественного и видового состава микробиоценозов кишечника при болезнях органов пищеварения по сравнению с клинически здоровыми ягнятами. При проведении исследований методами оптической микроскопии выявили формирование трехмерной структуры биопленок в виде плотной сети, состоящей из грамотрицательных и грамположительных бактерий, дрожжевых клеток, гифальных и псевдогифальных форм, окруженных межклеточным полимерным матриксом. Наличие бластоспор обусловливало увеличение количества адгезированных к субстрату клеток, происходило формирование биологической пленки, состоящей из палочковидных и округлых клеток, удерживающихся на клетках микроскопических грибов. В процессе дисперсии при разрушении межклеточного матрикса и отделении бактериальных и дрожжевых клеток от микроколоний обособлялись разветвленные структуры и колонизировали свободные от микроорганизмов участки субстрата. Оценку интенсивности формирования биопленок изучаемыми микроорганизмами проводили, измеряя оптическую плотность через 48 ч культивирования. Результаты люминесцентных микроскопических исследований показали, что динамика изменений жизнеспособных структур микроорганизмов характеризовалась чередующимися периодами снижения и увеличения интенсивности формирования биопленки. В исследуемых культурах микроорганизмов выявляли клетки, характеризующиеся способностью к активному росту и размножению, образующие сменяющие друг друга субпопуляции. При детекции жизнеспособности микроорганизмов в составе биопленок дифференцировали жизнеспособные (зеленый спектр люминесценции) и нежизнеспособные клетки (красный спектр люминесценции).

Ключевые слова: Адгезия, биопленки, бактерии, дисперсия, микроскопические грибы, колонизационная резистентность, межклеточный матрикс, люминесценция.

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INTRODUCTION

The complex of the processes involving microbial circulation and reservation in the soil and water biocenoses is the ecological pillar of enzootic disease emergence as well as formation of the infectious disease agents' foci and their spread to the new territories and water areas [1–2].

Gastrointestinal and respiratory diseases amount up to 95.0% within the structure of the neonatal pathology, and *Salmonella* spp. и *Pasteurella* spp. are their etiological agents [3]. In case of pathologies clinically manifested with diarrhea, dehydration, toxaemia, specifically in case of colibacillosis, the lamb morbidity reaches 12.3–95.2% and lethality – 60.0–90.0% [4, 5]. In colostrum- and milk-suckling lambs decrease of bifid- and lactobacteria concentration and increase of the toxicogenic enterobacteria, enterococci and *Candida* microfungi were reported [6–11]. Small intestine colonization with pathogenic microorganisms, primarily with gram-negative enterobacteria, is associated with morphofunctional properties of the digestive organs of the animals in early postnatal life [12–14].

Incidence of drug resistance of the microorganisms, isolated from pathological material and contents of the purulent lesions from animal skin and muscles is reported [15–18].

Contamination with the microorganisms demonstrating high enzymatic activity, *inter alia*, at low temperatures, can occur at all stages of the food raw material handling and storage thus making the problem socially significant [12, 19, 20].

Pathogenesis of small bowel bacterial overgrowth syndrome following microorganism translocation and occurrence of microbial virulence factors is assured through the attachment properties, which are among the key factors of the biofilm architectonics formation specified by the optic density growth thus determining the length and retrospective properties of the diagnostic tests [21–23]. Biofilms of multiresistant strains of *Candida parapsilosis* demonstrated dissociation processes; densitometric values of the concentric phenotype 1.75 times increased optic density values of "crater" phenotype; optic density parameters of the smooth colonies were 20.0–60.0% lower as compared to other phenotypes [24]. The populations affected by the adverse factors were heterogenic, and they were not genetically different from the original population; they also preserved their genetic diversity necessary for colonization of the "cleansed" niche or new habitat [21, 23, 25, 27].

High performance of instrumental analytical methods was demonstrated for the *in vitro* and *in vivo* detection of viable microorganisms in the heterogenic population, as they allow for the detection of the polysaccharidic intercellular matrix and metabolically active cells through the interaction between the stains and cell wall polysaccharides [28–30].

Phase-contrast microscopy aids to the detection of aggregated motile cells, processes of the cell aggregation, evaluation of the degree of the biofilm biomass gain at different stages of their formation depending on the cultivation medium composition and oxygen content [31].

Electron microscopy of the biofilms of gram-negative, gram-positive bacteria and microfungi enables recovery of the mechanisms of the intercellular communication, sorption and aggregation of heterogenic biofilms as well as cyclic growth mode and phenotypic flexibility that precondition the persistence of nonculturable organisms in the bacteria carriers during the interepidemic periods [12, 21, 23, 32].

In order to reveal the mechanisms of the pathogen adaptation to parasitizing in the susceptible species, optimization of microbiological study design, development of anti-epidemic measures aimed at the animal disease prevention, approbation and selection of the microbial biofilm study tools and methods are of the highest priority thus defining the relevance of this research.

The research was aimed at the examination of morphometric and densitometric properties of microbial biofilms in case of gastrointestinal disorders in lambs.

MATERIALS AND METHODS

Strains. Isolates recovered from the gut contents of Agin breed-lambs at the age from 1 to 30 days old. Before testing, the animals were by analogy subdivided into two groups: Group I – lambs with digestive disorders (experimental, $n = 5$); Group II – clinically healthy lambs (control, $n = 5$).

Nutrient media: brain heart infusion broth, Chromocult® Coliform Agar, Cetrimide Agar, Yolk Salt Agar, HiCrome Candida Agar, Oxytetracycline Glucose Yeast Extract Agar, (HiMedia, India).

Test-kits: ENTERO-Rapid, NEFERM test 24 (Erba-Lachema, Czech Republic), API Staph (bioMérieux, France), Hi-Candida FGP Armavir Biofactory (Russia), RUP Institute of Experimental Veterinary Medicine named after S. N. Vyshelessky (Belarus).

Indication and identification of microorganisms. Morphological, cultural and biochemical properties of the microorganisms were examined using routine methods according to the Bergey's Manual of Systematic Bacteriology (1984–1989) and Guide to clinically significant fungi [33, 34]. The microorganisms were cultivated at 37 °C for 24 hours using liquid and solid nutrient media. *Escherichia* were serologically differentiated using diagnostic sera, and adhesive antigens K88, K99, 987P, F41, A20 were identified according to the "Guide on Use of Agglutinating O-coli sera" (Moscow, 1998).

Biofilm densitometric parameters. Microbial biofilms were indicated by the degree of crystal violet binding (HiMedia, India) at 490 nm wavelength. The tested samples were added to the wells of 96-well plate (OAO "Medpolymer Company", Russia), cultivated in the constant aerobic environment at 37 °C for 48 hours. The liquid was discarded and the wells were washed with 200 µl of phosphate-buffered solution (PBS) for three times (pH 7.3). The plates were shaken for 5 min at each stage of washing. The samples were fixed with 150 µl of 96% ethanol for 15 min and dried out at 37 °C for 20 min. The microbial biofilms were stained by adding 0.5% stain solution in each well and subsequent cultivation at 37 °C for 5 min. The contents of the wells were discarded; the plates were washed with 200 µl of PBS three times (pH 7.3) and dried out. The bound stain was eluted from the attached cells with 200 µl of 96% ethanol for 30 min [35, 36].

Biofilm morphometric parameters. Before the light microscopy, the microorganisms were cultivated at 37 °C for 18–48 hours. The microorganisms were cultivated on the slides placed in the Petri dishes containing 20 ml of pepted meat broth (PMB) and 5 ml of 18-hour culture suspension at 10^5 CFU/ml [21]. Using Gram stain kit (BioVitrum, Russia), the preparations were fixed with ethanol and ether mixture (1:1) for 10 min, stained with 0.5% methylene blue solution and 1:2000 gentian violet water solution. 18.0 × 18.0 mm slides (Corning Inc., USA) were used for fluorescence microscopy. Before inoculation, the slides were rinsed with 70% ethanol / 30% deionized water and dried at 70 °C for 30 min. The slides were then placed in the wells of 12-well plates (OAO "Medpolymer", Russia), and microbial suspension ($OD_{600} = 0.08$) was pipetted at 5.0 ml/well and cultivated at 37 °C for 18–48 hours. The wells were washed with PBS (pH 7.2) twice and dried out. Hereafter, 15 µl of Live/Dead stain was added onto the slides at concentration of 1 mg/ml; the slides were covered with cover slip and stained for 10 min at 25 °C in the dark place [37]. The microscopy was carried out at representative sample with significant frequency ≥ 90.0% of Biomed MS-1 light microscope FOV (OOO "Biomed", Russia); fluorescence microscope Leica DMRB (Germany), equipped with 100× oil immersion objective with 510 nm dichroic filter and 515 nm long-wave pass filter.

The experimental results were statistically processed using Student's t-test and were deemed true at $p \leq 0.05$.

RESULTS AND DISCUSSION

Indication and identification of microorganisms. Lambs with gastrointestinal disorders demonstrated increased levels of bacteria and yeast-like fungi, which formed colonies on differential and diagnostic media: Chromocult® Coliform Agar, Cetrimide Agar, Yolk Salt Agar, HiCrome Candida Agar (Table 1).

By contrast to the clinically healthy animals (control), increased numbers of microorganisms isolated from lambs with gastrointestinal disorders (experimental) were observed on the above-mentioned media as compared with.

Due to sodium dodecyl sulfate present in Chromocult® Coliform Agar medium, growth of gram-positive bacteria was inhibited and growth of gram-positive bacteria increased: experimental – 7.15 ± 0.12 – 9.33 ± 0.26; control – 3.13 ± 0.12 – 7.13 ± 0.10; colonization index (CI) – 0.831%.

During the differentiation of the microorganisms, we bore in mind that in the above mentioned differentiation and diagnostic medium the *Escherichia* formed the colonies of violet color due to the presence of *β*-galactosidase and *β*-glucuronidase enzymes, which simultaneously degraded two chromogenic substrates. *Proteobacteria* and *Enterobacteriaceae* lack these enzymes and they formed colorless colonies on the medium. *Klebsiella* formed dark-pink colonies due to degradation of chromogenic substrate by *β*-galactosidase (Fig. 1a).

Presence of tryptophan in the media allowed testing indole formation by adding Kovack's reagent to the violet colonies. If the colonies changed their color to pink-red within 3–5 sec, the test deemed positive and *Escherichia* could be differentiated from the taxonomically similar *Enterobacteriaceae* spp. within 24 hours.

Enterobacteriaceae were catalase-positive, oxidase-negative and they fermented D-glucose and polyatomic alcohols forming acid and gas. *Escherichia* formed indole, utilized sodium acetate; they did not form hydrogen sulfide or utilize citrate and sodium malonate; they did not produce urease, phenylalanyldeaminase but fermented

Table 1
Quantitative composition of microorganisms in case of digestive disorders

Таблица 1
Количественный состав микроорганизмов при болезнях органов пищеварения

Media	Amount of microorganisms (CFU, lg/g)		Colonization index*, %
	Control	Experiment	
Chromocult® Coliform Agar ($n = 20$)	3.13 ± 0.12 – 7.13 ± 0.10	7.15 ± 0.12 – 9.33 ± 0.26	0.831
Cetrimide Agar ($n = 6$)	1.01 ± 0.12 – 2.01 ± 0.10	2.84 ± 0.11 – 3.03 ± 0.16	0.356
Yolk Salt Agar ($n = 6$)	0.21 ± 0.11 – 0.82 ± 0.10	0.83 ± 0.07 – 1.36 ± 0.09	0.253
HiCrome Candida Agar ($n = 6$)	1.74 ± 0.13 – 2.18 ± 0.03	4.14± 0.12 – 5.01 ± 0.08	0.420

* Proportion of microorganisms (CFU, lg/g) in 1.0 g of the tested sample collected from clinically healthy animals (control) and microorganisms (CFU, lg/g) in 1 g of the tested sample collected from the animals with digestive disorders (experimental).

* Отношение количества микроорганизмов (КОЕ, lg/г) в 1,0 г исследуемого материала клинически здоровых животных (контроль) и количества микроорганизмов (КОЕ, lg/г) в 1,0 г исследуемого материала при болезнях органов пищеварения (опыт).

sucrose and dulcite. *Klebsiella* utilized glucose, sodium citrate and produced acetylmethylcarbinol; they fermented inose and hydrolyzed urea, but did not form indole and hydrogen sulfide. *Proteobacteria* formed hydrogen sulfide and urease; they reduced nitrates, hydrolyzed gelatin, fermented glucose and demonstrated positive reaction with methyl red; they also deamidated phenylalanine, failed to decarboxylize lysine and differed in their ability to utilize sodium citrate. *Enterobacteriaceae* did not form indole and hydrogen sulfide; they fermented glucose, lactose, rhamnose, xylose, maltose, sorbite, arabinose, raffinose; they utilized citrate, sodium malonate; hydrolyzed gelatin and differed in their ability to utilize inose, dulcite, salicin and adonitol (Table 2).

During the recording of differentiating features the following bacteria were identified among the twenty isolated gram-negative pure microbial cultures: 19 *Enterobacteriaceae* isolates (95.0%), of which 13 (65.0%) were *Escherichia coli*, 4 (20.0%) – *Klebsiella pneumonia*, 2 (10.0%) – *Proteus vulgaris* and 1 (5.0%) – *Enterobacter cloacae*.

Serological tests of 13 (65.0%) identified *E. coli* isolates demonstrated that three isolates were positive for polyvalent Group 1 serum (serogroups O2, O78, O33); four (20.0%) – for Group 2 serum (serogroups O9, O15, O26, O111), two (10.0%) – for Group 3 serum. Four microbial cultures produced adhesive antigens: O33:F41 – 1 (5.0%), O111:A20 – 1 (5.0%), O2:A20 – 1 (5.0%), O9:A20 – 1 (5.0%).

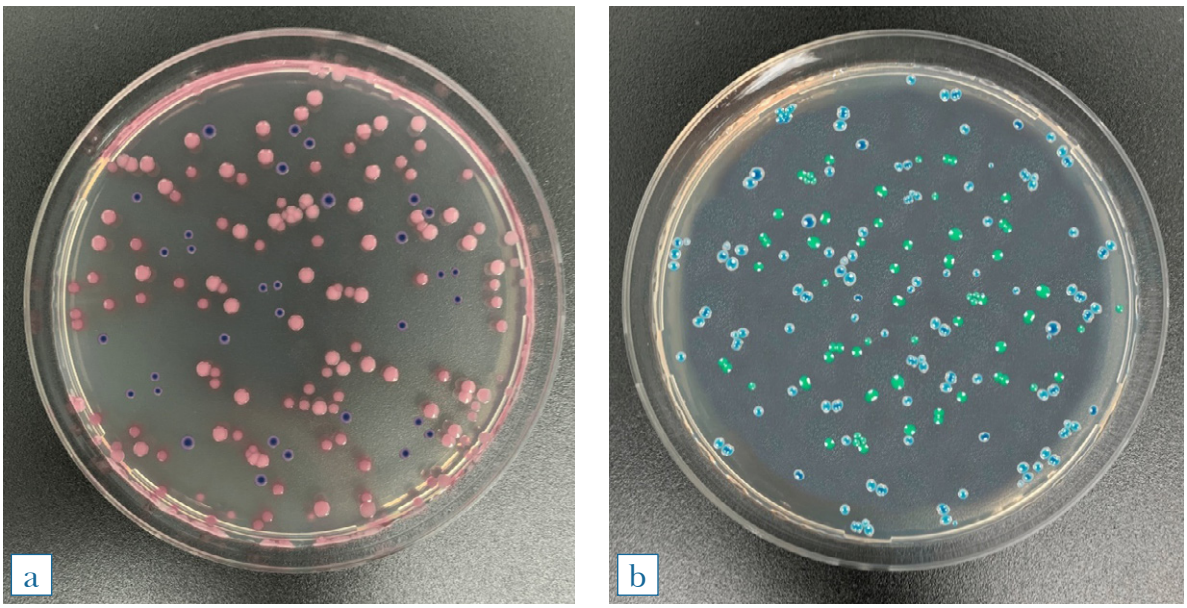

Fig. 1. Morphology of microbial colonies in intestinal microbiocinoses of lambs with digestive disorders (growth at 37 °C, 48 hours):
a – Chromocult® Coliform Agar, 76 × 10^{-9} CFU; b – HiCrome Candida Agar, 68 × 10^{-9} CFU

Рис. 1. Морфология колоний микроорганизмов микробиоценозов кишечника ягнят при болезнях органов пищеварения (рост при 37°С, 48 ч):
a – Chromocult® Coliform Agar, 76 × 10^{-9} КОЕ; b – HiCrome Candida Agar, 68 × 10^{-9} КОЕ

Increased amounts of microorganisms in case of digestive disorders were also observed on Cetrimide Agar: experimental – 2.84 ± 0.11 –3.03 ± 0.16; control – 1.01 ± 0.12 – 2.01 ± 0.10; colonization index – 0.356%. Out of six recovered microorganisms, one isolate (16.7%) was identified as gram-negative aerobic *Pseudomonas aeruginosa*. The above-mentioned bacteria converted nitrites to nitrates, they possessed proteolytic properties (dissolved gelatin and coagulated blood sera, hydrolyzed casein), coagulated litmus milk and broke down clots; they did not ferment maltose or form indole and hydrogen sulfide.

The number of microorganisms also increased on Yolk Salt Agar that contained 10.0% of sodium chloride: experimental – 0.83 ± 0.07 – 1.36 ± 0.09; control – 0.21 ± 0.11 – 0.82 ± 0.10; colonization index – 0.253%. Out of six recovered microorganisms, two isolates (33.3%) were identified as gram-positive *Staphylococcus* spp.: *S. aureus* – 1 (16.7%), *S. epidermidis* – 1 (16.7 %). These bacteria formed dome-shaped nontransparent colonies of white, golden, orange or yellow color and 2.0–2.5 mm in diameter. Coagulation of blood serum plasma was reported, which contained 1.0–4.0% of sodium citrate and clotted on the slide. Growth was observed with 15.0% of sodium chloride or 40% of bile. Under anaerobic conditions, the microorganisms fermented glucose and mannitol; they produced ammonia, coagulated and peptonized milk, and they did not ferment dulcite, salicin and inulin.

The number of microbial colonies on HiCrome Candida Agar also increased: experimental – 4.14 ± 0.12 – 5.01 ± 0.08; control – 1.74 ± 0.13 – 2.18 ± 0.03; colonization index – 0.420%. Out of six recovered microorganisms, three isolates (50.0%) were identified as yeast-like fungi: *Candida albicans* – 2 (33.3%), *Candida parapsilosis* – 1 (16.7%). The microorganisms grown on Sabouraud glucose agar formed smooth, dome-shaped colonies of white color and soft, even consistency (S-shape). Due to the degradation of chromogenic hexoaminidase substrate with β-N-acetylgalactominidase, *C. albicans* microfungi formed the colonies of pale green color. *C. parapsilosis* colonies, lacking the above-specified enzyme, formed blue colonies on the medium (Fig. 1b).

The yeast-like fungi *Candida* spp. could grow with cycloheximide; they fermented sucrose, maltose, xylose and had no urease activity. *C. albicans* fermented galactose and *C. parapsilosis* did not ferment trehalose (Table 3).

Densitometric parameters of microbial biofilms. The tested microorganisms were distributed by the intensity of biofilm formation according to optic density measurements in 48 hours of cultivation.

The obtained results demonstrated that absolute values of optic density (OD_s) of *P. aeruginosa*, *S. aureus* and *C. albicans* amounted to 0.454 ± 0.09 – 0.526 ± 0.08, biofilm formation intensity – $I \geq 0.3–0.4$; hence, these are powerful biofilm-producing microorganisms.

Tests for biofilm formation capacities of *E. coli*, *K. pneumonia*, *C. parapsilosis* demonstrated that their optic density (OD_s) ranged from 0.391 ± 0.07 to 0.571 ± 0.05, and biofilm formation intensity was $I \geq 0.2–0.3$. Therefore, these microorganisms were categorized as moderate biofilm-producing microorganisms.

It was determined that *P. vulgaris* and *E. cloacae* had poor capacities of biofilm formation: their optic density (OD_s) ranged from 0.246 ± 0.03 to 0.284 ± 0.08 and biofilm formation intensity was $I \geq 0.1–0.2$ (Table 4).

Morphometric parameters of microbial biofilms. In 18–48 hours of cultivation at 37 °C, microscopy of gentian violet-,

Table 2
Differentiation of enterobacteria by their biochemical properties

Таблица 2
Дифференциация энтеробактерий по биохимическим свойствам

Biochemical parameters	Bacterium species			
	Escherichia coli	*Klebsiella pneumoniae*	*Proteus vulgaris*	*Enterobacter cloacae*
Oxidase	–	–	–	–
Catalase	+	+	+	+
Lactose	+	+	–	+
Indole	+	–	+	–
Sorbitol	+	+	–	+
Hydrogen sulfide	–	–	+	–
Citrate	–	+	+	+
Urea	–	+	+	–
Gelatin	–	–	+	+
Voges-Proskauer reaction	–	+	+	+

"+" – positive test result (положительный тест);
"–" – negative test result (отрицательный тест).

Table 3
Differentiation of yeast-like fungi *Candida* spp. by their biochemical properties

Таблица 3
Дифференциация дрожжеподобных грибов *Candida* spp. по биохимическим свойствам

Carbohydrate	Microorganism	
	C. albicans	*C. parapsilosis*
Urease	–	–
Melibiose	–	–
Lactose	–	–
Maltose	+	+
Sucrose	+	+
Galactose	+	–
Cellobiose	–	–
Inose	–	–
Xylose	+	+
Dulcite	–	–
Raffinose	–	–
Trehalose	+	–

"+" – positive test result (положительный тест);
"–" – negative test result (отрицательный тест).

Table 4
Determination of the bacterial biofilm formation intensity by optic density

Таблица 4
Оценка интенсивности формирования биопленок бактериями по оптической плотности

Microbial cultures	Cell size, nm	Optic density				
		OD_s			OD_c	t_d
		1	2	3		
E. coli	(1.4–3.8) × (0.5–0.8)	0.542	0.571	0.550	0.098	4.2
K. pneumoniae	(0.6–6.0) × (0.3–1.0)	0.514	0.493	0.502	0.099	4.4
P. vulgaris	(1.0–3.0) × (0.4–0.8)	0.284	0.279	0.275	0.097	4.2
E. cloacae	(0.6–1.0) × (1.2–3.0)	0.246	0.256	0.267	0.099	3.6
P. aeruginosa	(1.5–5.0) × (0.5–1.4)	0.458	0.462	0.454	0.098	3.9
S. aureus	(1.5–1.6)	0.481	0.485	0.477	0.098	4.4
C. albicans	(2.0–3.0) × (3.0–5.0)	0.520	0.532	0.526	0.099	4.5
C. parapsilosis	(1.7–2.0) × (3.0–4.0)	0.391	0.397	0.403	0.097	4.2

OD_s – tested sample (исследуемый образец); OD_c – control (контроль); t_d – confidence factor (коэффициент достоверности).

methylene blue- and Gram-stained preparations demonstrated heterogeneous structures of the biofilms formed by the tested isolates of the gram-negative and gram-positive bacteria and yeast-like fungi *Candida* spp. (Fig. 2).

Intercellular communication processes were implemented stepwise: sedimentation; fixation (primary attachment); formation of monolayer and intercellular connections (co-aggregation), growth of microcolonies; formation of clusters and architectonics of the mature biofilms; dispersion.

During the destruction of intercellular matrix and detachment of the bacterial and yeast cells from the microcolonies, the dispersion was detected as separate arm-like structures, which colonized those areas of the substrate that were free from the microorganisms.

Through the synthesis of the intercellular polymer matrix, the 3D-structure of biofilms is formed as a dense grid of gram-negative and gram-positive bacteria, yeast cells, hyphas and pseudohyphas. Multi-species biofilms were generally characterized by the attachment of

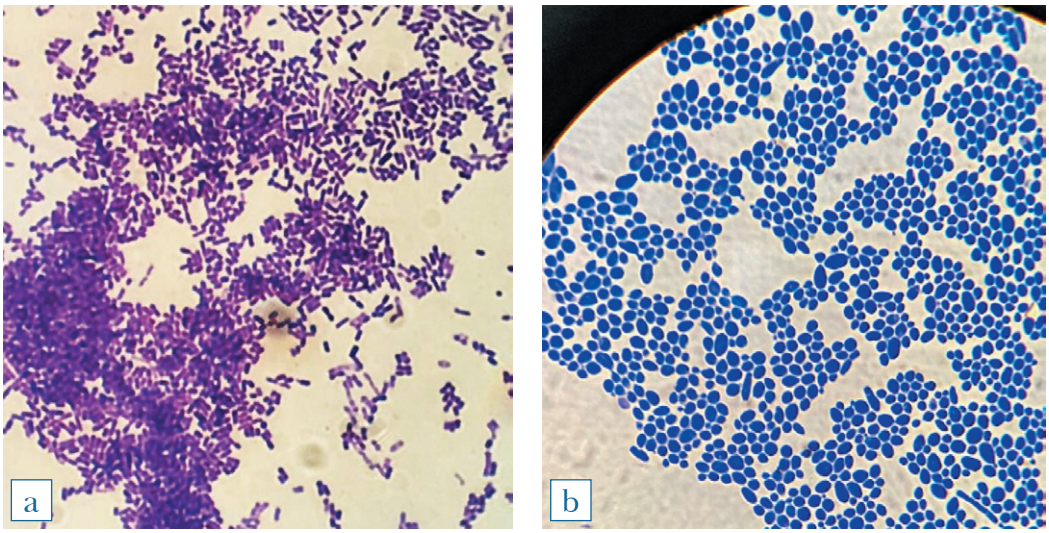

Fig. 2. Morphology of microbial biofilms (growth at 37 °C, 48 hours, MPB):
a – K. pneumoniae bacteria. Staining with methylene blue, oc. 10, obj. 100, immersion;
b – yeast-like fungi C. parapsilosis. Staining with methylene blue, oc. 10, obj. 100, immersion

Рис. 2. Морфология биопленок микроорганизмов (рост при 37 °С, 48 ч, МПБ):
a – бактерии K. pneumoniae. Окрашивание метиленовым синим, ок. 10, об. 100, иммерсия;
b – дрожжеподобные грибы C. parapsilosis. Окрашивание метиленовым синим, ок. 10, об. 100, иммерсия

gram-negative and gram-positive bacteria to the yeast-like fungi. Presence of microfungial blastospores aided to the increased number of cells attached to the cellular substrate, and biofilm was formed, which consisted of bacterial cells attached to the yeast cells (Fig. 3).

In 18–72 hours of cultivation, the fluorescence microscopy demonstrated that the dynamics of changes of the viable microbial structures was specified by intermittent periods of increased or decreased biofilm formation intensity. Cells characterized by active growth and replication and forming alternating subpopulations were detected in the examined microbial cultures. When determining the viability of the microorganisms within the biofilms, the viable (green fluorescence) and non-viable (red fluorescence) cells were differentiated. Alternations of the microbial growth intensity are driven by the presence of various dissociative variants advantaging during the formation of the biofilm architectonics. Owing to the reaction of the fluorescent stains with the cell wall polysaccharides, structures of the metabolically active cells were differentiated within the biofilms: green fluorescence – viable cells and red fluorescence – non-viable cells (Fig. 4).

CONCLUSION

Twenty-six isolates were recovered from 38 lambs with gastrointestinal disorders, including: *Escherichia coli* – 13, *Klebsiella pneumoniae* – 4, *Proteus vulgaris* – 2, *Enterobacter cloacae* – 1, *Pseudomonas aeruginosa* – 1, *Staphylococcus aureus* – 1, *Staphylococcus epidermidis* – 1, *Candida albicans* – 2, *Candida parapsilosis* – 1.

In 18–48 hours of cultivation at 37 °C, the light microscopy demonstrated heterogeneous structure of the biofilms formed by the tested isolates of the gram-negative and gram-positive bacteria and yeast-like fungi *Candida* spp.

Owing to the reaction of the fluorescent stains with the cell wall polysaccharides, structures of the metabolically active cells were differentiated within the biofilms: green fluorescence - viable cells and red fluorescence – non-viable cells.

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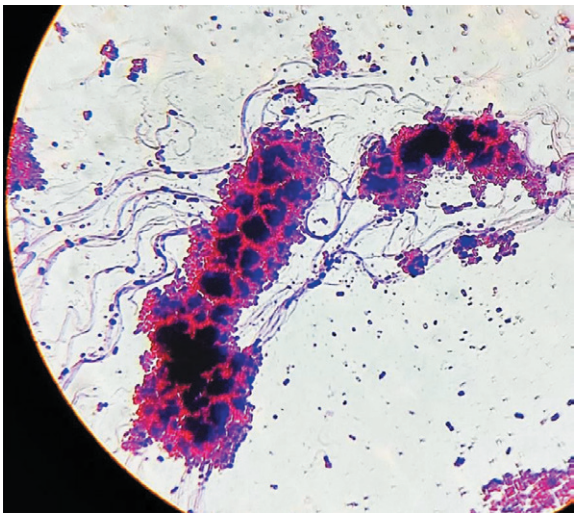

Fig. 3. Morphology of microbial biofilms (growth at 37 °C, 48 hours, MPB): P. aeruginosa bacteria and yeast-like fungi C. albicans. Gram staining, oc. 10, obj. 100, immersion

Рис. 3. Морфология биопленок микроорганизмов (рост при 37 °С, 48 ч, МПБ): бактерии P. aeruginosa и дрожжеподобные грибы C. albicans. Окрашивание по Граму, ок. 10, об. 100, иммерсия

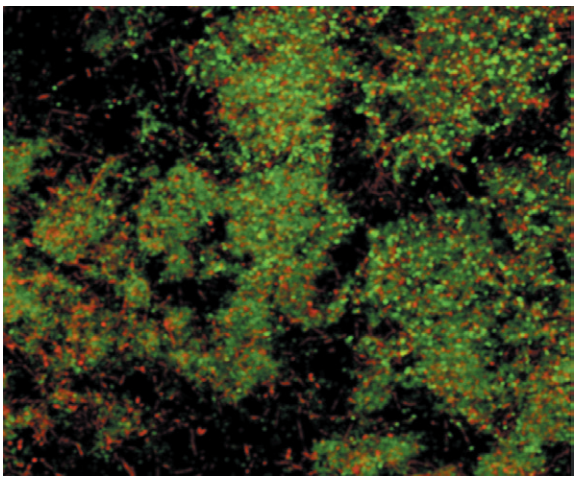

Fig. 4. Morphology of K. pneumonia biofilms (growth at 37 °C, 72 hours, MPB). Live/Dead stains, oc.10, obj. 200, immersion

Рис. 4. Морфология биопленок бактерий K. pneumonia (рост при 37 °С, 72 ч, МПБ). Комплекс красителей Live/Dead, ок. 10, об. 200, иммерсия

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