P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article https://doi.org/10.47278/journal.ijvs/2024.183

Characteristics of Biofilms Formed by Pathogenic *Enterobacterales* Isolated from Infected Gastrointestinal Tracts of Rabbits

Ekaterina Lenchenko[1], Nadezhda Sachivkina[2]*, Arfenya Karamyan[3], Olga Volobueva[4], Ekaterina Neborak[5], Marina Avdonina[6], Oksana Nechet[7] and Maria Molchanova[8]

[1]Department of Veterinary Medicine, Russian Biotechnological University (BIOTECH University), 125080, Moscow, Russia
[2]Department of Microbiology V.S. Kiktenko, Institute of Medicine, Peoples Friendship University of Russia (RUDN University), 117198 Moscow, Russia
[3]Department of Veterinary Medicine, Agrarian Technological Institute, Peoples' Friendship University of Russia (RUDN University), 117198 Moscow, Russia
[4]Department of Microbiology and Immunology, Russian State Agrarian University, Moscow Timiryazev Agricultural Academy, 49 Timiryazevskaya Street, Moscow, Russia
[5]Department of Biochemistry T.T. Berezov, Institute of Medicine, Peoples Friendship University of Russia (RUDN University), 117198 Moscow, Russia
[6]Department of Linguistics and Intercultural Communication, Moscow State Linguistic University, 119034, Moscow, Russia
[7]Institute of Biochemical Technology and Nanotechnology, Peoples' Friendship University of Russia (RUDN University), 117198 Moscow, Russia
[8]Department of Foreign Languages, Institute of Medicine, Peoples' Friendship University of Russia (RUDN University), 117198 Moscow, Russia
***Corresponding author:** sachivkina@yandex.ru

Article History: 24-480 Received: 26-Apr-24 Revised: 22-May-24 Accepted: 24-May-24

ABSTRACT

The article presents the results of assessing the morphometric and densitometric parameters of biofilms of reference strains and clinical isolates of *Enterobacterales*. In a private rabbit farm, there was an outbreak of indigestion among young animals. Thirteen young rabbits with severe diarrhea were observed for over a week. Other animals did not have such symptoms. Sick rabbits were placed in a separate room. They took a stool test and blood from the ear (experience, n=13). For comparison purposes, the same samples were taken from healthy animals of the same age from the same farm (control, n=13). Among the 20 Gram-negative bacteria of *Enterobacterales* isolated from the fecal mass of ill rabbits, 14(70.0%) isolates *Escherichia coli* O78, O20, and O101; 4(20.0%) isolates *Klebsiella pneumoniae*; 2(10.0%) isolates *Salmonella* genus: one *S. enteritidis* and one *S. dublin* were identified. Morphometric and densitometric indices of biofilms of reference strains and fecal isolates from ill animals had common patterns of biofilm formation. However, the clinical isolates formed more robust biofilms than the museum strains. Many types of microorganisms can increase their pathogenicity when exposed to susceptible animals. Our study proved this fact in *Enterobacterales* by comparing it with museum strains at all stages described in detail, which formed one of the main pathogenic factors - robust biofilms.

Key words: Rabbits; Digestive system; Biofilms; *Salmonella; Escherichia; Klebsiella;* Phenotypic signs

INTRODUCTION

Adequate composition of the intestinal microbiota is necessary to preserve homeostasis, whereas modifying the quantitative and specific composition of the intestinal microbiota leads to the development of pathological processes, mainly inflammatory bowel diseases (Bagóné and Kovacs 2014, Bagóné et al. 2017). This can occur in humans, as well as animals such as rabbits. In a microbiological study performed on rabbits, the most representative microbiota taxa of the stomach of clinically healthy rabbits were the genera *Metanosphera* and the order *Rikenellaceae* and *Enterobacterales*, those of the duodenum, jejunum and ileum were the genera *Metanosphera*, *Candidatus*, and *Saccharimonas* and those of the colon were the *Rikenellaceae* family and

Cite This Article as: Lenchenko E, Sachivkina N, Karamyan A, Volobueva O, Neborak E, Avdonina M, Nechet O and Molchanova M, 2024. Characteristics of biofilms formed by pathogenic *Enterobacterales* isolated from infected gastrointestinal tracts of rabbits. International Journal of Veterinary Science x(x): xxxx. https://doi.org/10.47278/journal.ijvs/2024.183

Akkermansia genus (Cotozzolo et al. 2021). The digestive capacity of the intestinal flora contributes to the hydrolysis of complex polymers, for example, with the help of secreted hydrolases (polysaccharides, glycosidases, proteases, and peptidases). Subsequently, these compounds are decomposed into smaller ones - monosaccharides or amino acids with the help of hydrolytic species of microorganisms (Bagóné et al. 2017). When the composition of the evolutionarily formed microbiocenoses of the biotopes of the cavity organs of the respiratory, digestive, excretory, and reproductive systems is disturbed, an increase in the quantitative and persistent composition of pathogenic and potentially pathogenic microorganisms forming biofilms has been established (Lenchenko et al. 2019; Lenchenko et al. 2020; Gunardi et al. 2021). With a decrease in the colonization resistance of the intestine, as a rule, excessive growth of pathogenic *Enterobacterales* producing adhesive antigens, bacteriocins, hemolysins, thermolabile, and thermostable toxins is observed (Lenchenko et al. 2019; 2020). We revealed a multistage process of forming the three-dimensional structure of biofilms in the form of a dense network of bacterial cells surrounded by an intercellular polymer matrix. The retention of water molecules and the heterogeneity of the internal composition of biofilms, caused by the location of metabolically differentiated cells throughout the three-dimensional matrix, determine the protection of the population from the influence of various factors (Lenchenko et al. 2019; Gunardi et al. 2021). The mechanism of action of effective enzymatic preparations was achieved due to perforation and dehydration, as well as bacterial shrinkage, and there was a tendency toward the destruction of the bacterial cell in the areas of thinning of the biofilm (Thompson et al. 2006; Lenchenko et al. 2020). A positive correlation was established between the volume of the intercellular matrix of β-polysaccharides and the volume of viable cells. Bacteriocins and "Quorum sensing" (QS) molecules, which are types of protein metabolites characterized by nontoxicity, a lack of side-effects, and drug resistance, are also recognized as promising drugs (Boyen et al. 2009; Lenchenko et al. 2023). Such an organization ensures its physiological and functional stability, which is the key to competitive survival in the ecological niche. QS inhibition may be an attractive alternative to current methods of controlling multidrug-resistant microorganisms (Boyen et al. 2009). Resistance to adverse factors characterizes multicellular communities of cells adhered to the surface and united by the intercellular matrix of cells of various ecological niches (Lenchenko et al. 2023).

Control of the biosafety of food products of animal origin according to microbiological criteria is one of the most pressing problems due to the growing number of diseases in humans and animals. Toxigenic microorganisms especially deserve attention (Lenchenko et al. 2019; 2020). Dynamic and static methods of *in vitro, ex vivo,* and *in vivo* cultivation have been developed to determine the components of the intercellular matrix, the structure, and the level of gene expression during the formation of biofilms of microorganisms of various systematic groups (Lenchenko et al. 2019; 2020; Gunardi et al. 2021). The optimization of the scheme of microbiological diagnostics of diseases of the digestive system and the development of effective antibacterial drugs will be facilitated by further testing and finding effective ways to detect biofilms. The work aims to study the morphometric and densitometric indicators of biofilms and phenotypic signs of reference strains and clinical isolates of *Enterobacterales* from ill rabbits.

MATERIALS AND METHODS

Ethical approval

All animal experiments were performed by the Guide for the Care and Use of Laboratory Animals (Anonymous 2011) and were approved by the Ethics Committee for Animal Experimentation, Peoples' Friendship University of Russia, Moscow, Russia (protocol number 12a, date: 08 December 2022).

Animals

Samples were taken from New Zealand White Rabbits aged 25-30 days from different litters but located in the same room (n=26, both sexes). Animals were divided into two groups: I—clinically healthy rabbits (control, n=13); II—rabbits with clinical signs of digestive system diseases manifested by diarrhea (experience, n=13). Rabbits on the farm were kept in cages (length=50.0cm; width=40.0cm; height=30.0cm). The vivarium temperature was 20±2°C, relative humidity 45±1%. Two groups of rabbits (control and experimental) received the same granulated food. Its composition was described in detail by us in the previous article (Sachivkina et al. 2023). Water and food are provided freely. Infected animals were euthanized using sequential intramuscular injections of 1.5mg/kg xylazine and intravenous injections of 7.5mg/kg Anestofol® (Russia).

Hematological and biochemical parameters

Two mL of blood was collected from the marginal ear vein for hematological and biochemical analysis. We performed hematological parameters within 45min after blood sampling on the analyzer "Abacus juniorVet" (Austria). The following parameters such as the total number of erythrocytes (RBC), hemoglobin (HGB), hematocrit (HCT), and differential number of leukocytes/average number of leukocytes (LEU), including granulocytes (GR), monocytes (MONO) and lymphocytes (LYM), were taken into account ($P \leq 0.05$).

Biochemical analyses of blood serum were conducted within one hour after blood sampling on the analyzer "Chem Well 2902V" (USA). The following parameters such as alanine aminotransferase (ACT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and total bilirubin (TBIL), were taken into account ($P \leq 0.05$).

Microbial strains

We used isolates from the intestinal contents of rabbits and reference bacterial strains. After identifying clinical strains, we needed to compare strains *Salmonella dublin* ATCC 39184, *Salmonella enteritidis* ATCC 4931, *Escherichia coli* O6 ATCC 25922, and *Klebsiella pneumoniae* ATCC 1705 (Anonymous 2024), which were purchased with passports for these strains at the State

Research Institute for Standardization and Control of Medical Biological Preparations named after L.A. Tarasevich (Moscow). Microorganism cultures were stored in semi-solid 0.5% meat-peptone agar (MPA) at a temperature of 4±1°C.

Reagents

Microorganisms were cultured at 37±1°C for 24h or 48h on the following media: Blood Agar Base (Biomerieux, France), Nutrient Broth, and HiCrome Coliform Agar (Hi Media, India). The following test systems were used to differentiate microorganisms: ENTERO-Rapid and NEFERV test 24 (PLIVA-Lachema, Cech Republic). Serological identification of *Escherichia coli* bacteria was carried out with diagnostic serums of Armavir Biofactory, Russia.

Phenotypic signs of bacteria

To study the quantitative composition of rabbit intestines, 1.0g samples were homogenized; then, 9.0cm^3 of a sterile 0.85% NaCl solution was added before thoroughly mixing and maintaining for 10–15min at a temperature of 20±1°C to precipitate large particles. Lastly, a series of subsequent dilutions were established with a volume of 0.1mL.

The phenotypic signs of bacteria were studied using conventional methods in accordance with the classification system from Bergy's manual 1984–1989 (Bergey 1989). The study of various microorganism properties, morphological, cultural, and biochemical, was carried out using generally accepted methods using differential diagnostic nutrient media and commercial test systems.

Morphometric and densitometric indicators of bacterial biofilms

Morphometric and densitometric parameters of bacterial biofilms were studied using standard methods using flat-bottomed sterile 12-well culture plates with surface-treated lids for single-layer cell cultures. The volume of the wells was 6.8mL (Medpolymer, Russia); small glasses for microbiological studies (18.0×18.0mm) (Corning Inc., USA) were placed at the bottom of the wells. Next, 3.0mL of nutrient broth (HiMedia, India) and 1.0mL of bacterial suspension at a concentration of 0.5 units were added to the wells. (McFarland). Microorganisms were cultivated for 1 or 2 days at a thermostat temperature of 37°C. Biofilm preparations on the glass surface were poured with a mixture of alcohol and ether (1:1) for 10min for better fixation. Then, they were washed three times with 200.0µL of phosphate buffer solution (pH7.3) and dried. For microscopy, the preparations were stained with an aqueous solution of gentian violet at a dilution of 1:2000 (HiMedia, India) and a staining kit for Gram staining (BioVitrum, Russia).

Preparations for optical and scanning electron microscopy were prepared according to standard methods (Lenchenko et al. 2019; 2020; 2023). Biofilm studies were carried out using a Trinocular Unico optical microscope (Unico, USA), scanning electron microscopy was performed using a Hitachi TM3030 Plus (Japan) with sputtering with gold ions Q150T ES (Quorum Technologies, UK). The biofilms' optical density (OD) was measured in a microplate photometric analyzer, Immunochem-2100 (HTI, USA), at a wavelength of 490nm.

For weak biofilm producers, the OD of the sample culture (sample density, ODs) is <2 times (ODs≤0.196) higher than the control OD (control density, ODc=0.088) (i.e., nutrient medium without inoculum). ODs exceed ODc by 2–4 times (0.196–0.392) for moderate biofilm producers. Finally, for strong biofilm producers, ODs exceed ODc by more than four times (ODs≥0.392).

Statistical analysis

The results were analyzed using SPSS 20.0 (IBM Corp., Armonk, NY, USA). The significance of the results was determined using Student's *t*-test, and the results were considered significant when P<0.05.

RESULTS

Hematological and biochemical parameters of rabbit blood with diseases of the digestive system

The hematological and biochemical parameters of the examined blood of rabbits with clinical signs of diseases of the digestive system, manifested by diarrhea, differed from those in relation to clinically healthy rabbits. In the group of studied animals, a decrease in the total number of leukocytes by 23.09% and lymphocytes - by 27.19% was observed compared with control animals, which indicated a deterioration in the condition of the studied animals and a decrease in the reactivity of the organism. The level of erythrocytes in the group of animals under study is lower by 14.86% and hematocrit - by 21.79% compared with the indicators in the control group of animals. Such changes indicate the diagnosable ill-being of animals, a decrease in tissue oxygenation, and a violation of metabolic processes in tissues and organs in the studied animals. Along with the recorded changes, indicating a clinically determined pathology, there are changes in the clinical and biochemical parameters of the blood, detected in the dysfunction of the gastrointestinal tract. The level of transferases and bile pigments was increased in the group of animals under study: alanine aminotransferase - by 20.51%, aspartate aminotransferase - by 37.54%, lactate dehydrogenase - by 28.09%, total bilirubin - by 23.27%. There are significant changes in the integral indicators in the group of animals under study compared with the control animals that did not show symptoms of diarrhea and dyspeptic disorder. The results of hematological and biochemical analysis are presented in Table 1.

Phenotypic signs of enterobacterales isolated from rabbits with diseases of the digestive system

In samples from ill rabbits, the number of microorganisms grown on the medium Chromocult Coliform agar was 6.98±0.14lg/g. similar indicators of clinically healthy animals (control) were 3.11±0.12lg/g. When determining the colonization resistance of the intestine, the colonization index was taken into account (%), i.e., the ratio of the number of microorganisms to 1.0g of intestinal contents of clinically healthy rabbits (control) and the number of microorganisms to 1.0g of intestinal contents in animals with diseases (experience). The colonization index (%) was 0.446±0.26.

Table 1: Hematological and biochemical parameters of healthy (control) and experimental rabbits

Parameters	Units	Experimental (n=13)	Control (n=13)
Erythrocytes	10^{12}/L	4.71±0.19a	5.41±0.18b
Hemoglobin	g/L	95.13±2.74a	107.35±2.52b
Hematocrit	%	28.64±0.71a	38.88±1.31b
Leukocytes	Thousand/μL	7.28±0.38a	5.73±0.46b
Granulocytes	Thousand/μL	4.72±0.35a	2.17±0.42b
Monocytes	Thousand/μL	0.18±0.03a	0.20±0.03a
Lymphocytes	μL	62.11±0.14a	44.21±0.15b
ALT	U/L	57.50±0.46a	45.24±0.34b
AST	U/L	29.31±3.35a	21.31±2.89b
LDH	U/L	352.7±25.91a	251.8±21.35b
Bilirubin total	μmol/L	10.14±1.59a	6.50±1.89b

Control - clinically healthy rabbits. Experimental - rabbits with clinical signs of diseases of the digestive system, manifested by diarrhea. Mean+SD bearing different alphabets in a row differ significantly (P<0.05).

On the chromogenic Chromocult Coliform agar medium, the growth of gram-positive bacteria was inhibited due to sodium dodecyl sulfate in the composition. *Escherichia* formed purple-colored colonies due to the presence of the enzymes β-galactosidase and β-glucuronidase, splitting two chromogenic substrates simultaneously. *Salmonella* lacking these enzymes formed colorless colonies on the solid medium. *Klebsiella*, due to the cleavage of the chromogenic substrate by the enzyme β-galactosidase, formed colonies of pink color. The presence of tryptophan in the medium made it possible to conduct a test for indole formation; for this, a drop of Kovacs reagent was applied to purple colonies. When the color of the colonies changed to pink red within 3–5s, the test was considered positive, which made it possible to differentiate *Escherichia* from taxonomically similar species within 24h. Among the 20 Gram-negative bacteria of *Enterobacterales* isolated from the intestinal contents of ill rabbits, 14(70.0%) isolates *Escherichia coli* O78, O20, and O101; 4(20.0%) isolates *Klebsiella pneumoniae*; 2(10.0%) isolates *Salmonella* genus: one *S. enteritidis* and one *S. dublin* were identified.

It should be noted that both reference strains and isolates from the contents of the intestines of rabbits formed large convex mucous and white colonies at 37±1°C after 24 and 48h on MPA (d=3.0–6.0mm). As the microorganisms grew, a uniform turbidity of the meat-peptone broth medium was observed. The growth of these bacteria was observed both under aerobic and anaerobic conditions in an aerostat with GasPak. The microorganism cultures examined were gram-negative, the catalase test was positive, and the oxidase test was negative. They fermented D-glucose to produce lactic acid and carbon dioxide. *Salmonella* fermented glucose and mannitol with acid and gas, did not ferment sucrose, did not produce indole and ammonia, but did produce hydrogen sulfide. *Escherichia* formed indole, utilized sodium acetate, did not produce hydrogen sulfide, did not utilize citrate or sodium malonate, and did not produce urease or phenylalanine deaminase. *Klebsiella* fermented glucose and sodium citrate, produced acetylmethylcarbinol, fermented inositol, hydrolyzed urea, and did not form indole or hydrogen sulfide. Phenotypic and biochemical characteristics of isolates from the feces of diseased rabbits corresponded to those of the reference strains. There were no differences, so we combined these properties at one table. Differential diagnostic signs of the studied cultures of microorganisms are given in Table 2.

Table 2: Differential diagnostic signs of *Enterobacterales* isolates

Signs	*Enterobacterales*			
	Salmonella dublin	*Salmonella enteritidis*	*Escherichia coli*	*Klebsiella pneumoniae*
Oxidase	–	–	–	–
Catalase	+	+	+	+
Lactose	–	–	+	+
Indole	–	–	+	–
Sorbitol	+	+	+	+
Hydrogen sulfide	+	+	–	–
Citrate	+	+	–	+
Urea	–	–	–	+
Gelatin	–	–	–	–
Voges-Proskauer reaction	–	–	–	+

Note: +, positive test; –, negative test.

Morphometric and densitometric indicators of biofilms reference strains and isolates from diseases of the digestive system

After 1-2 days of cultivation on a liquid nutrient medium, standard stages of forming a heterogeneous structure of biofilms of the studied species were revealed. Morphometric indicators of reference strains and isolates from the intestinal contents of rabbits with clinical signs of digestive system diseases did not differ significantly. Optical and scanning electron microscopy revealed the main stages of population development: adhesion, fixation, microcolony, growth, and dispersion.

Adhesion is the deposition and primary attachment of planktonic forms of *Enterobacterales* to the surface of the substrate (in this case, glass). The bacteria in the area in direct contact with the substrate had a flattened shape. Due to the presence of bacterial adhesins, primary attachment took place. The initial stages of attachment of planktonic forms of bacteria can be reversible due to insufficient interaction between microorganisms and the substrate. An irreversible process of adhesion of microorganisms, called fixation, is observed during the secretion of intercellular polymers, mainly polysaccharides. Fixation is microorganisms' final (irreversible) attachment due to intercellular substances that provide strong adhesion. At this stage, densely located Gram-negative rod-shaped bacteria can be observed (Fig. 1).

The development stage of a microcolony is called maturation I, where microorganisms attached to the surface contribute to the attachment of subsequent cells. At the stage of coaggregation, binary cell division is observed, and colonization of individual sections of the substrate is observed due to intercellular adhesion. At the boundaries of the formed biofilm clusters, round pores, and tubules were found, containing liquid and surrounded by membrane structures. Due to the localization of metabolically differentiated cells throughout the three-dimensional matrix, the hydration and heterogeneity of the internal environment of biofilms determine the protection of the population from the effects of various factors.

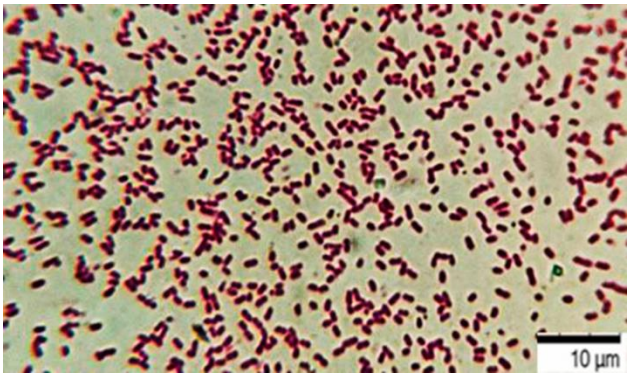

Fig. 1: Culture of *S. enteritidis* ATCC 4931, Nutrient Both, 37±1°C, 24h: Gram-negative rod-shaped bacteria. Gram staining. Magnification: 10×100, immersion (H604 Trinocular Unico, USA).

During the growth stage (maturation II), microcolonies increase in size while gradually changing shape. The mechanical stability of the population is provided by the intercellular matrix containing polysaccharides, which are markers of biofilm formation. Due to the gradual unification of a standard intercellular matrix, heteromorphic structures of various sizes are formed (Fig. 2).

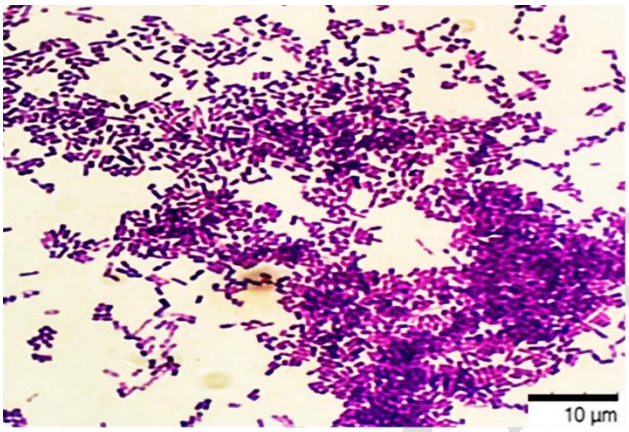

Fig. 2: *K. pneumoniae* culture (isolate of rabbit intestinal contents), Nutrient Both, 37±1°C, 48h: rod-shaped bacteria. Gentian violet staining. Magnification: 10×100, immersion (H604 Trinocular Unico, USA).

Two types of cell-cell interactions have been observed during biofilm formation: direct contact followed by cluster formation and matrix-mediated interaction. This indicated the active production by bacteria of a substance of varying optical density, an intercellular matrix that filled the space between cells. Various enzymes - exopolysaccharides produced by bacteria - have a protective effect, which leads to a change in phenotypic characteristics and a decrease in the metabolic rate of the population. Due to the cells attached to the substrate and the intercellular matrix, a double diffuse layer was formed on the surface of the abiotic substrate, and the cells themselves were arranged in groups in the form of a single layer (Fig. 3).

Dispersion is the scattering of heteromorphic cells of a population separating from the peripheral part of a mature biofilm. Destructive processes are detected in some areas, which are accompanied by a violation of the integrity of the

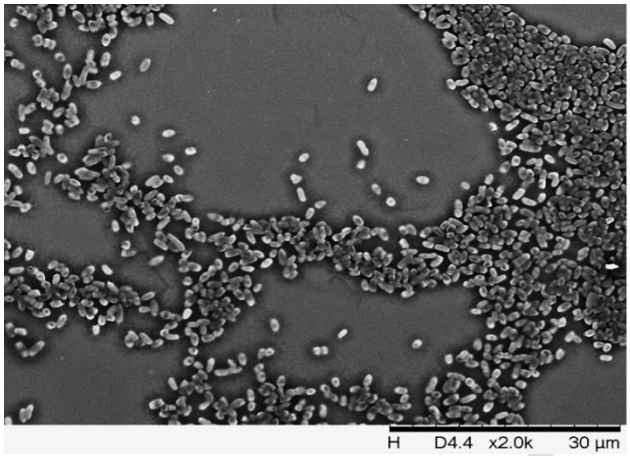

Fig. 3: *E. coli* culture (isolate of rabbit intestinal contents), Nutrient Both medium, 37±1°C, 24h: densely located bacteria. Scanning electron microscopy. Magnification: 2000× Hitachi TM3030 Plus (Japan).

intercellular matrix. Heteromorphic cells and partially lysed cells are detected at such sites. At the same time, a significant population of cells are viable, retaining the ability to adhere and participate in the formation of new microcolonies. As a rule, a decrease in the optical density of both the intercellular matrix and the bacterial cells is detected. As a result of cell division, individual cells are periodically torn off, which are able to attach to free areas of the studied surface after a while to form a new microcolony.

Analyzing the OD of the studied bacterial species, it was found that the ODs exceeded the ODc by more than four times. This means that both reference strains and isolates *S. enteritidis*, *S. dublin*, *E. coli*, and *K. pneumoniae* from ill rabbits were strong producers of biofilms (Table 3). But if we look closely at the average OD values of the strains, we will notice that the clinical strains of absolutely all the microorganisms studied formed more robust biofilms compared to the museum cultures.

Table 3: Optical density of the studied bacteria

Bacteria	OD, Clinical strains	OD, Reference strains
S. enteritidis	0.470±0.039a	0.399±0.018b
S. dublin	0.480±0.041a	0.418±0.023a
E. coli	0.554±0.029a	0.437±0.021b
K. pneumoniae	0.503±0.034a	0.401±0.024b

ODs or nutrient medium without inoculum=0.088. Mean±SD bearing different alphabets in a row differ significantly (P<0.05).

This fact should be considered for the development of new drugs against microbial biofilms. In the experience, it is better to take clinical strains from sick animals than museum cultures of microorganisms. All other phenotypic characteristics will be the same.

DISCUSSION

The results of our own research and literature data allow us to state that a biofilm is a community of cells attached to the surface and united by an intercellular matrix, differing from single (planktonic) bacteria by growth parameters and the expression of specific genes (Costerton et al. 1995, Fernandes et al. 2018; Lenchenko et

al. 2019; 2020; 2023). Like other authors, we identified five main stages in the formation of biofilms: adhesion, fixation, microcolony, growth, and dispersion; these stages are considered reversible (Donlan 2002; Cotter et al. 2013; Lenchenko et al. 2020; Lee et al. 2021).

The pathogenesis of overgrowth syndrome with the persistence of microorganisms and the degree of spread in environmental objects are due to the presence of dissociative variants, with the dispersion of uncultivated cells of microorganisms that benefit from hyper-aggregating the architectonics of heterogeneous biofilms (Lenchenko et al. 2019; 2020; Sachivkina et al. 2022). Adhesion, i.e., attachment of bacteria to the surface, depends on the structure of the substrate; accordingly, there is convincing evidence that attachment increases with an increase in the roughness or wrinkling of the surface of the carrier (O' Shea et al. 2013; Gunardi et al. 2021; Maru et al. 2021). The degree of adhesion of *Enterobacterales* is due to the presence of flagella and fimbriae, a feature of the structure of the cell wall producing an intercellular matrix, mainly of polysaccharides (Lenchenko et al. 2019; 2023). Depending on the surface area and roughness, the degree of adhesion of *Enterobacter* spH1 and *Citrobacter freundii* H3 bacteria to the substrate increased in the following order: activated carbon, silica gel, iron/activated carbon, iron/silica gel, aluminum oxide, and maghemite (Maru et al. 2021).

In the light of modern data, the mechanism of action of effective drugs is due to perforation, dehydration, cell shrinkage, as well as a tendency to thin, destruction of the biofilm matrix (Sachivkina et al. 2022; Lenchenko et al. 2023). Bacteriocins are promising, representing a variety of protein metabolites characterized by drug resistance, nontoxicity, and absence of side effects (Fernandes et al. 2018; Maru et al. 2021). The actual type of inhibition of biofilms is considered to be a decrease in the adhesive properties of microorganisms; for example, bacteriocin *L. lactis* UQ2 reduces the adhesive properties of *L. monocytogenes* in relation to the stainless-steel substrate (García-Almendárez et al. 2008). In particular, plantaricin Q7 can not only inhibit the growth of *Listeria* but also inhibit the formation of biofilm and reduce the mature biofilm of *Listeria monocytogenes* (Liu et al. 2022a, b). The inhibitory effect of drugs based on ferulic and gallic acid is due to a mechanism that prevents the adhesion of bacteria *E. coli*, *L. monocytogenes*, *P. aeruginosa*, and *S. aureus* (Borges et al. 2012). *Dellaglioa algida* can inhibit the formation or damage the integrity of the biofilm of pseudomonads, and it can also reduce the mobility, including swarming and swimming, of *P. fragi* and restrain the swarming of *P. fluorescens* (Sun et al. 2022). Fermentation strategies based on *Escherichia coli* biofilm for the production of L-threonine allowed increasing production from 10.5 to 14.1g/L during periodic fermentation and further to 17.5g/L during continuous (repetitive) fermentation with increased productivity (Chen et al. 2019). When exposed to creatine kinase, with concentrations of 0.5–1.0MIC, inhibition of biofilms was achieved by reducing the optical density and shape of microorganisms; at a higher concentration (2.0MIC), the inhibition effect was achieved due to significant perforation of biofilms (Srinivasan et al. 2021). The *Enterococcus faecium* s6 strain is able to use the hemicellulose fraction, the main component of which is xylose, for homo fermentation of lactic acid and minimal yield of byproducts at a concentration of xylose in biomass of 50.0–75.0g/L (Abdel-Rahman 2022). The processes of biofilm formation are mediated by the velocity of the hydrodynamic flow adjacent to the substrate and the coating of the substrate with various biomolecules, e.g., polysaccharides and proteins (Mishra et al. 2015).

Promising antimicrobial peptides that inhibit and destroy biofilms have a compositional type of action (Lee et al. 2021; Sachivkina et al. 2022). Composite preparations are designed to inhibit the growth of multispecies biofilms of both bacteria and yeast-like fungi (Mishra et al. 2015; Abdel-Rahman, 2022). Insect extracts black soldier fly and yellow mealworm fats, which have an inhibitory effect on the microbiota of the cecum of rabbits, are considered potential antibacterial ingredients of the feed (Dabbou et al. 2020; Puón-Peláez et al. 2022).

Monitoring studies of the etiological significance of pathogenic *Enterobacterales* in the development of diarrhea, dehydration and toxemia are an urgent problem due to the increasing trend of statistically significant increase in epizootic rabbit enteropathy (Dewree et al. 2007; Al-Eqabi et al. 2022). When keeping animals in limited areas, completing farms with animals of different types and ages, a decrease in the immune status, and an increase in the number and spectrum of pathogenic ubiquitous microorganisms contribute to the spread of infectious pathology of various origins (Abbas et al. 2022; Potekhina et al. 2023; Jamil et al. 2023; Sadat et al. 2023; Almulhim et al. 2024). The results of studies of the heterogeneous structure of biofilms, phenotypic signs of adaptive strategies of uncultivated L-forms and virulence factors can be used in the comparative study of the biological properties of epizootic strains of *Enterobacterales* to optimize the scheme of bacteriological diagnostics, as well as in the development of antibacterial drugs (Lenchenko et al. 2019; 2020; 2023). Of the studied isolates of *Escherichia coli*, 100.0% were resistant to clindamycin and erythromycin, and 67.0% were resistant to amoxi/clavulinic acid and cefoxitin (Nadi et al. 2024). Specific phages, in particular, «BacWash TM» and «Ecolicicid PX,» are promising for the rotation of antiseptics and disinfectants (Abbas et al. 2022; Aziz et al. 2023). To develop new drugs against microbial biofilms in 2023, scientists from India have applied exciting strategies (Ramasubbu et al. 2023 Shanmugam et al. 2023; Tharani et al. 2023). This confirms the fact that the development of new antibacterial drugs is of interest worldwide and the emphasis on the use of clinical strains, as in our study. Knowledge of the physiological microbiota of the gastrointestinal tract and disease-causing bacteria is critical to ensure the growth and health of young rabbits. It is important to emphasize that knowledge about the families and genera of microbiota in the various tracts of the rabbit digestive system is very limited, except for the cecum. A detailed step-by-step analysis of the ability of pathogenic bacteria to produce biofilms will help in the search for good methods of controlling them. In the future, it will be useful to study the multi-species biofilms of the gastrointestinal tract, since many microorganisms in symbiosis are known to be more resistant to antibiotics.

We also plan to study the sensitivity of isolated strains from rabbits to antibiotics.

Conclusion

In this study, the results of morphometric and densitometric indicators of biofilms of reference bacterial strains and fecal isolates from ill rabbits are presented. We identified 20 Gram-negative bacteria of *Enterobacterales* isolated from the fecal mass of ill rabbits, 14(70.0%) isolates *of Escherichia coli* O78, O20, and O101; 4(20.0%) isolates *of Klebsiella pneumoniae*; 2(10.0%) isolates *Salmonella* genus: one *S. enteritidis* and one *S. dublin* were identified. The studied cultures of microorganisms were Gram-negative, catalase-positive, and oxidase-negative, and they fermented D-glucose and polyatomic alcohols with the formation of acid and gas. Morphometric and densitometric indicators of biofilms of reference strains and isolates from ill animals had common patterns of biofilm formation. Both reference strains and clinical isolates were strong producers of biofilms. However, the clinical strains of all the studied microorganisms, *S. enteritidis*, *S. dublin*, *E. coli*, and *K. pneumoniae*, formed more robust biofilms than the museum cultures. This fact should be considered when developing new drugs against microbial biofilms.

Authors' contributions

All authors were actively involved with different responsibilities. Ekaterina Lenchenko, Nadezhda Sachivkina, Arfenya Karamyan and Olga Volobueva: preparing research proposal, sample collection and conducted laboratory work. Ekaterina Neborak, Marina Avdonina, Oksana Nechet and Maria Molchanova: statistical analyses and write manuscript. All authors edited, read, and approved the final manuscript.

Acknowledgment

This study has been funded by the RUDN University Strategic Academic Leadership Program.

Conflict of interest

The authors declare no conflicts of interest related to this study.

REFERENCES

Abbas RZ, Alsayeqh AF and Aqib AI, 2022. Role of bacteriophages for optimized health and production of poultry. Animals (Basel) 12(23): 3378. https://doi.org/10.3390/ani12233378

Abdel-Rahman MA, 2022. *Enterococcus faecium* s6 enabled efficient homofermentative lactic acid production from xylan-derived sugars. Fermentation 8(3): 134. https://doi.org/10.3390/fermentation8030134

Al-Eqabi SRS, Ismail Ibrahim Z and Jamal Mohammed Jawad Z, 2022. Immunopathological changes post-infection with *Enterobacter cloacae* in rabbits. Archives of Razi Institute 77(1): 179-186. https://doi.org/10.22092/ARI.2022.357468.2044

Almulhim FS, Zaki AKA, Albarrak SM and Abo-Aziza FAM, 2024. Impact of age, parity and milking frequency on dromedary camels' susceptibility to subclinical mastitis. International Journal of Veterinary Science 13(5): 565-573. https://doi.org/10.47278/journal.ijvs/2024.132

Anonymous, 2011. Committee for the update of the guide for the care and use of laboratory animals of the institute for laboratory animal research, division on earth and life studies, national research council. *Guide for the Care and Use of Laboratory Animals*; National Academy Press: Washington, DC, USA. https://www.nebrwesleyan.edu/sites/default/files/inline-files/care-use-laboratory-animals-guide-8-ed.pdf. (accessed on 22 May 2024).

Anonymous, 2024. The Global Bioresource Center. ATCC at https://www.atcc.org/ (accessed on 22 May 2024).

Aziz S, Ameer T, Younus M, Nisa Qu, Naeem I, Ali U, Akhtar T, Hanif AM and Munir B, 2023. Salmonellosis: food-borne plague. In: Abbas RZ, Saeed NM, Younus M, Aguilar-Marcelino L and Khan A (eds), One Health Triad, Unique Scientific Publishers, Faisalabad, Pakistan, Vol. 2, pp: 18-24. https://doi.org/10.47278/book.oht/2023.36

Bergey DH, 1989. Bergey's manual of systematic bacteriology, Baltimore, MD: Williams & Wilkins, vol. 4, pp: 2648.

Bagóné VV and Kovacs MZ, 2014. The rabbit caecal microbiota: development, composition and its role in the prevention of digestive diseases – a review on recent literature in the light of molecular genetic methods. Acta Agraria Kaposváriensis 18: 55-65.

Bagóné VV, Dalle ZA, Cullere M, Bónai A, Bosco A, Szendrő Z, Tornyos G, Pósa R, Bóta B, Kovács M and Zsolnai A, 2017. Quantitative PCR with 16S rRNA-gene-targeted specific primers for analysis of caecal microbial community in growing rabbits after dietary supplementation of thyme (Thymus vulgaris) and spirulina (Arthrospira platensis). Italian Journal of Animal Science 17: 1-9. https://doi.org/10.1080/1828051X.2017.1400413

Borges A, Saavedra MJ and Simões M, 2012. The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. Biofouling 28(7): 755–767. https://doi.org/10.1080/08927014.2012.706751

Boyen F, Eeckhaut V and Van Immerseel F, 2009. Quorum sensing in veterinary pathogens: mechanisms, clinical importance and future perspectives. Veterinary Microbiology 135(3-4): 187–195. https://doi.org/10.1016/j.vetmic.2008.12.025

Chen T, Liu N, Ren P, Xi X, Yang L and Sun W, 2019. Efficient biofilm-based fermentation strategies for l-threonine production by *Escherichia coli*. Frontiers in Microbiology 10: 1773. https://doi.org/10.3389/fmicb.2019.01773

Costerton JW, Lewandowski Z and Caldwell DE, 1995. Microbial biofilms. Annual Review of Microbiology 49: 711–745. https://doi.org/10.1146/annurev.mi.49.100195.003431

Cotozzolo E, Cremonesi P, Curone G, Menchetti L, Riva F, Biscarini F, Marongiu ML, Castrica M, Castiglioni B and Miraglia D, 2021. Characterization of bacterial microbiota composition along the gastrointestinal tract in rabbits. Animals 11: 31. https://dx.doi.org/10.3390/ani11010031

Cotter PD, Ross RP and Hill C, 2013. Bacteriocins - a viable alternative to antibiotics? Nature reviews. Microbiology 11(2): 95–105. https://doi.org/10.1038/nrmicro2937

Dabbou S, Ferrocino I, Gasco L, Schiavone A, Trocino A, Xiccato G, Barroeta AC, Maione S, Soglia D and Biasato I, 2020. Antimicrobial effects of black soldier fly and yellow mealworm fats and their impact on gut microbiota of growing rabbits. Animals 10(8): 1292. https://doi.org/10.3390/ani10081292

Donlan RM, 2002. Biofilms: microbial life on surfaces. Emerging Infectious Diseases 8(9): 881–890. https://doi.org/10.3201/eid0809.020063

Dewree R, Meulemans L, Lassence C, Desmecht D and Ducatelle R, 2007. Experimentally induced epizootic rabbit enteropathy: clinical, histopathologicaI, ultrastructural, bacteriological and haematological findings. World Rabbit Science 15: 91-102. https://doi.org/10.4995/wrs.2007.602

Fernandes RA, Monteiro DR, Arias LS, Fernandes GL, Delbem ACB and Barbosa DB, 2018. Virulence Factors in *Candida*

and *Streptococcus mutans* Biofilms Mediated by Farnesol. Indian Journal of Microbiology 58(2): 138–145. https://doi.org/10.1007/s12088-018-0714-4

García-Almendárez BE, Cann IKO, Martin SE, Guerrero-Legarreta I and Regalado C, 2008. Effect of *Lactococcus lactis UQ2* and its bacteriocin on *Listeria monocytogenes* biofilms. Food Control 19(7): 670-680. https://doi.org/10.1016/j.foodcont.2007.07.015

Gunardi WD, Karuniawati A, Umbas R, Bardosono S, Lydia A, Soebandrio A and Safari D, 2021. Biofilm-producing bacteria and risk factors (gender and duration of catheterization) characterized as catheter-associated biofilm formation. International Journal of Microbiology 2021: 8869275. https://doi.org/10.1155/2021/8869275

Jamil T, Mehmood A, Farhan MHR, Kalim F, Hadi F, Younas K, Iqbal Q, Asrar R and Nawaz A, 2023. Bacteriophage therapy: Effective antibiotic replacer against emerging ghost of antimicrobial resistant bacteria. In: Khan A, Abbas RZ, Aguilar-Marcelino L, Saeed NM and Younus M (eds), One Health Triad, Unique Scientific Publishers, Faisalabad, Pakistan, Vol. I, pp: 158-167. https://doi.org/10.47278/book.oht/2023.24

Lee JH, Kim YG, Khadke SK and Lee J, 2021. Antibiofilm and antifungal activities of medium-chain fatty acids against *Candida albicans* via mimicking of the quorum-sensing molecule farnesol. Microbial Biotechnology 14(4): 1353–1366. https://doi.org/10.1111/1751-7915.13710

Lenchenko E, Lozovoy D, Strizhakov A, Vatnikov Y, Byakhova V, Kulikov E, Sturov N, Kuznetsov V, Avdotin V and Grishin V, 2019. Features of formation of *Yersinia enterocolitica* biofilms. Veterinary World 12(1): 136–140. https://doi.org/10.14202/vetworld.2019.136-140

Lenchenko E, Blumenkrants D, Sachivkina N, Shadrova N and Ibragimova A, 2020. Morphological and adhesive properties of *Klebsiella pneumonia* biofilms. Veterinary World 13(1): 197–200. https://doi.org/10.14202/vetworld.2020.197-200

Lenchenko E, Sachivkina N, Lobaeva T, Zhabo N and Avdonina M, 2023. Bird immunobiological parameters in the dissemination of the biofilm-forming bacteria *Escherichia coli*. Veterinary World 16(5): 1052–1060. https://doi.org/10.14202/vetworld.2023.1052-1060

Liu Y, Bu Y, Li J, Liu Y, Ao L and Gong P, 2022a. Inhibition Activity of plantaricin Q7 produced by *Lactobacillus plantarum Q7* against *Listeria monocytogenes* and its biofilm. Fermentation 8: 75. https://doi.org/10.3390/fermentation8020075

Liu B, Cui Y and Ali Q, 2022b. Gut microbiota modulate rabbit meat quality in response to dietary fiber. Frontiers in Nutrition 9: 849429. https://doi.org/10.3389/fnut.2022.849429

Maru B, López F, Medina F and Constanti M, 2021. Improvement of biohydrogen and usable chemical products from glycerol by co-culture of *Enterobacter spH1* and *Citrobacter freundii* H3 using different supports as surface immobilization. Fermentation 7: 154. https://doi.org/10.3390/fermentation7030154

Mishra B, Golla RM, Lau K, Lushnikova T and Wang G, 2015. Anti-*Staphylococcal* biofilm effects of human cathelicidin peptides. ACS Medicinal Chemistry Letters 7(1): 117-121. https://doi.org/10.1021/acsmedchemlett.5b00433

Nadi WG, Ahmed LI, Awad AAN and Taher EM, 2024 Occurrence, antimicrobial resistance, and virulence of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* isolated from dairy products. International Journal of Veterinary Science 13(2): 218-225. https://doi.org/10.47278/journal.ijvs/2023.079

O' Shea EF, Cotter PD, Ross RP and Hill C, 2013. Strategies to improve the bacteriocin protection provided by lactic acid bacteria. Current Opinion in Biotechnology 24(2): 130–134. https://doi.org/10.1016/j.copbio.2012.12.003

Potekhina RM, Tarasova EY, Matrosova LE, Khammadov NI, Saifutdinov AM, Ermolaeva OK, Tanaseva SA, Mishina NN, Nigmatulin GN, Mukharlyamova AZ, Smolentsev SY and Semenov EI, 2023. A case of laying hens mycosis caused by *Fusarium proliferatum*. Veterinary Medicine International 2023: 5281260. https://doi.org/10.1155/2023/5281260

Puón-Peláez X-HD, McEwan NR, Álvarez-Martínez RC, Mariscal-Landín G, Nava-Morales GM, Mosqueda J and Olvera-Ramírez AM, 2022. Effect of feeding insoluble fiber on the microbiota and metabolites of the caecum and feces of rabbits recovering from epizootic rabbit enteropathy relative to non-infected rabbits. Pathogens 11(5): 571. https://doi.org/10.3390/pathogens11050571

Ramasubbu K, Padmanabhan S, Al-Ghanim KA, Nicoletti M, Govindarajan M, Sachivkina N and Rajeswari VD, 2023. Green synthesis of copper oxide nanoparticles using *Sesbania grandiflora* leaf extract and their evaluation of anti-diabetic, cytotoxic, anti-microbial, and anti-inflammatory properties in an in-vitro approach. Fermentation 9(4): 332. https://doi.org/10.3390/fermentation9040332

Sadat A, Farag AM, Elhanafi D, Awad A, Elmahallawy EK, Alsowayeh N, El-khadragy MF and Elshopakey GE, 2023. Immunological and oxidative biomarkers in bovine serum from healthy, clinical, and sub-clinical mastitis caused by *Escherichia coli* and *Staphylococcus aureus* infection. Animals 13(5): 892. https://doi.org/10.3390/ani13050892

Sachivkina N, Karamyan A, Petrukhina O, Kuznetsova O, Neborak E and Ibragimova A, 2023. A rabbit model of ear otitis established using the *Malassezia pachydermatis* strain C23 from dogs. Veterinary World 16(11): 2192–2199. https://doi.org/10.14202/vetworld.2023.2192-2199

Sachivkina N, Vasilieva E, Lenchenko E, Kuznetsova O, Karamyan A, Ibragimova A, Zhabo N and Molchanova M, 2022. Reduction in pathogenicity in yeast-like fungi by farnesol in quail model. Animals 12: 489. https://doi.org/10.3390/ani12040489

Srinivasan R, Santhakumari S, Poonguzhali P, Geetha M, Dyavaiah M and Xiangmin L, 2021. Bacterial biofilm inhibition: a focused review on recent therapeutic strategies for combating the biofilm mediated infections. Frontiers in Microbiology 12: 676458. https://doi.org/10.3389/fmicb.2021.676458

Sun Y, Liu S, Zhan Z, Wei T, Ma T, Sun J and Song J, 2022. Antibacterial mechanism of *Dellaglioa algida* against *Pseudomonas fluorescens* and *Pseudomonas fragi*. Fermentation 8 (7): 298. https://doi.org/10.3390/fermentation8070298

Shanmugam R, Munusamy T, Jayakodi S, Al-Ghanim KA, Nicoletti M, Sachivkina N and Govindarajan M, 2023. Probiotic-bacteria (*Lactobacillus fermentum*)-wrapped zinc oxide nanoparticles: biosynthesis, characterization, and antibacterial activity. Fermentation 9(5): 413. https://doi.org/10.3390/fermentation9050413

Tharani M, Rajeshkumar S, Al-Ghanim KA, Nicoletti M, Sachivkina N and Govindarajan M, 2023. *Terminalia chebula*-assisted silver nanoparticles: biological potential, synthesis, characterization, and ecotoxicity. Biomedicines 11(5): 1472. https://doi.org/10.3390/biomedicines11051472

Thompson LJ, Gray V, Lindsay D and von Holy A, 2006. Carbon: nitrogen: phosphorus ratios influence biofilm formation by *Enterobacter cloacae* and *Citrobacter freundii*. Journal of Applied Microbiology 101(5): 1105–1113. https://doi.org/10.1111/j.1365-2672.2006.03003.x