



## Investigation of the ability to form biofilms *in-vitro* in sanitary-indicatory bacteria isolated from chickens

**Liliana Davydovska\***

Postgraduate Student

National University of Life and Environmental Sciences of Ukraine

03041, 15 Heroiv Oborony Str., Kyiv, Ukraine

<https://orcid.org/0000-0003-4385-4500>

**Artem Ushkalov**

PhD in Veterinary Sciences, Senior Researcher

National Scientific Centre "Institute of Experimental and Clinical Veterinary Medicine"

61023, 83 Hryhorii Skovoroda Str., Kharkiv, Ukraine

<https://orcid.org/0000-0001-8317-7909>

**Liliia Vyhovska**

Doctor of Veterinary Sciences, Senior Researcher

National University of Life and Environmental Sciences of Ukraine

03041, 15 Heroiv Oborony Str., Kyiv, Ukraine

<https://orcid.org/0000-0002-5631-9139>

**Valerii Ushkalov**

Doctor of Veterinary Sciences, Professor, Full Member  
of the National Academy of Agrarian Sciences of Ukraine

National University of Life and Environmental Sciences of Ukraine

03041, 15 Heroiv Oborony Str., Kyiv, Ukraine

<https://orcid.org/0000-0001-5694-632X>

**Yurii Vishovan**

PhD in Veterinary Medicine

Ukrainian Laboratory of Quality and Safety of Agricultural Products

08162, 7 Mashynobudivnykiv Str., Chabany, Ukraine

<https://orcid.org/0000-0003-1128-593X>

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\*Corresponding author



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**Abstract.** Biofilms provide resistance to antimicrobial agents and the body's immune response in microorganisms that colonise the digestive tract of animals, in particular poultry. The study of biofilm formation of indicator bacteria isolated from chickens under different keeping conditions allows assessing the impact of environmental factors on their phenotypic adaptation and potential risk to animal and human health. The purpose of the study was to determine the ability of *Escherichia coli* and *Enterococcus faecalis* bacteria isolated from chickens kept in a vivarium and on free range to form biofilms. Bacteriological, morphological, biochemical, and microscopic research methods were applied. The intensity of biofilm formation in indicator microorganisms was assessed by the adsorption/resorption index of a 0.1% solution of crystal violet using polystyrene Petri dishes. The optical density was measured spectrophotometrically at a wavelength of 570 nm. It was found that *E. coli*, *E. faecalis*, *Klebsiella spp.* and *Pseudomonas aeruginosa* were isolated in samples from chickens kept in a vivarium, while in free-range chickens, representatives of the genus *Klebsiella* and *Pseudomonas aeruginosa* were not detected, indicating a lower presence of potential pathogens in natural conditions. All the cultures under study formed low- or medium-density biofilms. For *E. coli* isolates obtained from free-range chickens, the average value  $\lambda = 0.264 \pm 0.09$ , while for vivarium isolates –  $\lambda = 0.187 \pm 0.07$ . Cultures of *E. faecalis* biofilms were formed with an intensity of  $\lambda = 0.217 \pm 0.04$  in free-range chickens and  $\lambda = 0.137 \pm 0.03$  in vivarium chickens. Consequently, isolates obtained from natural conditions were characterised by a higher intensity of biofilm formation – by 41.2% (*E. coli*) and 58.4% (*E. faecalis*) in comparison with the conditions of a controlled microclimate. This may indicate a stimulating effect of environmental factors on the expression of adhesion and biofilm formation genes. However, all cultures under study were isolated from clinically healthy chickens, which indicates a commensal nature of the microbiome. The results obtained are important for assessing the risks of horizontal transfer of resistance genes and the formation of stable microbial biofilms in poultry farming

**Keywords:** indicator bacteria; *Escherichia coli*; *Enterococcus faecalis*; poultry keeping conditions; phenotypic adaptation; sanitary and microbiological monitoring

## Introduction

With the development of poultry farming in the world, subclinical forms of infections pose a significant threat to poultry health and the stability of production processes. The high concentration of livestock, intensive rearing technologies, and constant pressure from bacterial and viral pathogens require strengthening comprehensive veterinary control measures. In Ukraine, poultry farming remains the leading branch of animal husbandry, but its further development is primarily determined by epizootic well-being, the level of biosafety, and the quality of preventive programmes. Key factors in the industry's sustainability include effective

monitoring of infectious agents, compliance with veterinary and sanitary requirements, optimisation of vaccination strategies, and timely detection of pathogens. It is veterinary support and control of zoonotic risks that determine the ability of poultry farms to resist infectious threats and ensure stable production.

J. Li *et al.* (2024) proved that an important component of maintaining the health and productivity of poultry is the state of the intestinal microbiome. It plays a key role in regulating digestion, converting nutrients, developing and maturing the immune system, maintaining intestinal barrier functions, and developing

resistance to pathogens. The gut microbiota is in dynamic balance with the host body, forming a complex system of interactions that determines the overall physiological stability of the body. It was established that the keeping conditions – in particular, stocking density, microclimate, stress factors, diet, and microbial pollution of the environment can significantly change the species composition of the microbiota and affect its functional activity.

L. Vygovska *et al.* (2025) found out that the international organisation FAO pointed out the existence of an intensive, subintensive, and extensive poultry rearing system, and household maintenance actually formed a separate group with the lowest level of biosafety. This is conditioned by the fact that owners of small farms, as a rule, are not aware of measures aimed at preventing the introduction and spread of infections in the herd. Under such conditions, the risk of circulation of bacterial pathogens that are dangerous to both poultry and humans increases. The most common among them are *Escherichia*, enterococci, and other conditionally pathogenic microorganisms. According to S.C. Pinto *et al.* (2022) and O. Bezpalko *et al.* (2024), the absence of systematic veterinary prevention contributes not only to the disease of poultry, but also creates conditions for the transmission of zoonotic infections to humans, especially in direct contact of owners with livestock. Thus, domestic poultry farming is a potential reservoir of pathogenic microorganisms and may be a risk factor for public health, which requires increased attention within the framework of the Ukrainian state strategy for ensuring biological safety and biological protection “One Health” (Order of the Cabinet of Ministers of Ukraine No. 1416-r, 2019).

In the context of contemporary approaches to biosafety and the concept of “One Health”, the study of biofilm formation in sanitary-indicator bacteria that are constantly present in the microbiota of farm animals is of particular

importance. It is such microorganisms that, under certain conditions, can become a reservoir of antibiotic resistance genes and a factor of horizontal transfer of pathogenicity determinants. The conditions of keeping poultry affect the level of phenotypic adaptation of these bacteria, including the ability to form biofilms. S. Sharma *et al.* (2023) proved that bacteria in biofilms have distinctive features from microbes in the planktonic state, showing increased antimicrobial resistance, avoidance of host immune factors, and increased resistance to adverse environmental factors. The presence of such signs in pathogenic bacteria contributes to a more severe course of the disease and longer treatment. As confirmed by M. Cordeiro *et al.* (2023), flagella, pili, and fimbriae are responsible for the beginning of the bacterial biofilm formation process, which provided primary contact between the microorganism and the epithelial cells of the macroorganism.

M. del Mar Cendra & E. Torrents (2021) noted that microorganisms aggregated on the surface synthesise extracellular polymer substances, extracellular matrix, extracellular DNA, and binding proteins to surrounding bacteria, including a number of regulatory factors (system of QS – quorum sensors), which affect the formation and destruction of biofilms. K. Xiaoxia *et al.* (2023) described the mechanisms of biofilm formation, their role in chronic, nosocomial, and medical infections, and the increased resistance of bacteria in biofilms to antibiotics. It was indicated that because of this resistance, conventional antibiotic therapy is often ineffective. The study highlighted that elimination of biofilm infections requires innovative technologies, not just classical antibiotics.

Thus, the analysed studies on the problems biofilm formation in microorganisms indicate that the ability of bacteria to form biofilms is one of the key mechanisms of their survival, colonisation, and pathogenicity. Biofilms provide microorganisms with protection against host immune

system factors and antimicrobial actions, contribute to the development of chronic infections, and can play a crucial role in the conservation and transmission of pathogens in poultry populations. Simultaneously, the level of biofilm formation is a variable characteristic that depends on the type of microorganism, environmental conditions, and factors of keeping poultry. This highlights the need to study the phenotypic features of biofilm formation in sanitary-indicatory bacteria isolated from poultry under different containment systems, since such microorganisms can act as a reservoir of resistance genes and potential determinants of pathogenicity. The data obtained are important for assessing epizootological risks in poultry farming and improving biosafety measures. Therefore, the purpose of this study was to compare the ability to form biofilms with sanitary-indicatory microorganisms isolated from chickens under different keeping conditions. The objectives of the study were: to isolate and identify sanitary-indicatory bacteria from cloacal flushes of chickens raised in various conditions; to assess the ability of isolates to form biofilms *in vitro*; to conduct a comparative analysis of biofilm formation between isolates and determine its possible relationship with the keeping conditions of chickens.

## Materials and Methods

Microbiological studies were conducted throughout 2024 in the scientific laboratory of the Department of Veterinary Epidemiology and

Animal Health, Faculty of Veterinary Medicine, National University of Life and Environmental Sciences of Ukraine, Kyiv, Ukraine. Biomaterial for research (faecal samples) was collected from clinically healthy chickens, 77 animals of 30-35-day-old Cobb 500 crossbreeds. In the vivarium of the Faculty of Veterinary Medicine of the National University of Life and Environmental Sciences of Ukraine (Group 1), chickens (n = 30) were kept in a standard KR 108 collapsible cage designed for laying hens and broilers. Chickens received a standard balanced diet and local optimal climatic conditions (temperature  $30 \pm 3^\circ\text{C}$ , humidity  $55 \pm 5\%$ ). On the household plot (Group 2) located within one of the amalgamated territorial communities of the Kyiv Oblast, chickens (n = 47) were kept free-range, fed a prepared feed mixture of crushed and steamed wheat and corn, kitchen waste, and given unlimited access to water.

Cloacal flush samples (10 samples per group) from clinically healthy chickens were delivered in a thermal container at  $2-8^\circ\text{C}$  in accordance with DSTU 8703-1:2017 (2017) and DSTU 8703-2:2017 (2017). Bacteriological studies to determine the morphological and biochemical characteristics of the isolated isolates and to establish the intensity of biofilm formation were conducted in the Research Laboratory of Epizootology and Infectious Diseases of the Faculty of Veterinary Medicine using certified culture media and equipment in accordance with current standards (Table 1).

**Table 1.** Methods and standards for sample preparation, isolation and identification of microorganisms

Research stage	Microorganisms / indicators	Method / regulatory document
Preparation of samples and dilutions	Test samples, initial suspension, tenfold dilutions	ISO 6887-1:2017 (2017)
Selection and identification	<i>Salmonella spp.</i>	ISO 6579-1:2017 (2017)
Selection and identification	<i>Pseudomonas aeruginosa</i>	Classical bacteriological method
Selection and identification	<i>Listeria spp.</i> , <i>Listeria monocytogenes</i>	ISO 11290-1:2017 (2017)
Selection and identification	<i>Yersinia enterocolitica</i>	ISO 10273:2017 (2017)

Table 1. Continued

Research stage	Microorganisms / indicators	Method / regulatory document
Determination of MPN	Enterobacteria, <i>Escherichia coli</i> , <i>Klebsiella spp.</i>	ISO 21528-1:2017 (2017)
Determination of MPN	Enterococci	DSTU 8534:2015 (2015)

**Note:** MPN – most probable number. The method for determining the most probable number provided for the use of the MPN table with a 95% confidence interval and an appropriate formula for calculating the number of microorganisms

**Source:** compiled by the authors

To clarify morphological features, a generally accepted bacteriological approach was used, namely: cultivation on liquid and solid nutrient media. Bacteriological examination was performed by seeding cloacal flushes with a sterile swab on a nutritious broth. Cultivation was carried out at the optimal temperature for growth – 37°C, for 24 hours. Isolation and identification of a wide range of bacterial pathogens was performed for a comprehensive sanitary and microbiological assessment of cloacal flushes and confirmation of the absence of clinically significant pathogens. 9 isolates of *E. coli* and *E. faecalis* were selected for further study of the biofilm formation intensity as typical representatives of the intestinal microbiota of poultry and model objects of biofilm studies.

The cultures obtained on nutrient broth were transferred with a bacteriological loop using frequent broad strokes into separate Petri dishes with Endo agar and xylose-lysine-deoxycholate agar (XLD), covering the entire surface of the agar, and then incubated in a thermostat for 24 hours at 37°C. Subsequently, to obtain a pure culture, individual representative colonies were extracted from the agar surface using a bacteriological loop, transplanted into a nutrient broth with meat and peptones and slant nutrient agar with meat and peptones, and incubated at 37°C for 24 hours. Morphological properties were determined by microscopy of gram-stained smears, and typical growth was determined by inoculation of cultures on liquid and solid nutrient media.

To assess bacterial motility, isolated cultures were grown at 37°C in semi-liquid MPA (0.25-0.3%). Inoculation was performed by placing the sample in a column with semi-liquid agar. Mobility was also evaluated by microscopy of daily agar cultures using the “crushed drop” technique. The biochemical characteristics of the isolate were studied by inoculation with Hiss medium supplemented with various sugars (maltose, glucose, mannitol, sucrose, lactose, rhamnose, and raffinose). In addition, the ability to produce enzymes (ornithine decarboxylase, phenylalanine deaminase, lysine decarboxylase, arginine dehydrolyase), urea, and indole was evaluated, and the Foges-Proskauer reaction was performed.

Indirect estimation of bacterial biofilm biomass by crystal violet adsorption/resorption was used, following the method described by S. Stepanovic *et al.* (2000) and M. Kukhtyn & N. Krushelnytska (2014). Biofilms were stained with a 0.1% aqueous solution of crystalline violet at 30°C for 60 minutes. Sterile MPB was used as a control. To obtain reliable data, the experiments were repeated four times. The optical density was determined spectrophotometrically at a wavelength of 570 nm. With an optical density value of less than 0.1, it was assumed that the cultures under study did not form a biofilm; from 0.1 to 0.49, the ability to form a biofilm was considered low; an optical density value between 0.5 and 1.0 indicated the formation of a biofilm of medium density; an optical density value of 1.0 and above indicated the formation of a high-density biofilm.

The results of the biofilm formation study were analysed using the SPSS Statistics software suite. Before making comparisons, the normality of the distribution of quantitative indicators was checked using the Shapiro-Wilk test. To compare indicators between groups, the following methods were used: in the case of a normal distribution, a parametric t-test for independent samples; in case of deviation from normality – a nonparametric Mann-Whitney test. The results were presented as mean  $\pm$  standard deviation (SD). Differences at  $P < 0.05$  were considered statistically significant. During the research, the recommendations of ARRIVE (n.d.) and Directive 2010/63/EU (2010) on the ethical treatment of animals used for scientific research were followed. The research was approved by the Bioethics Expertise Commission of the National University of Life and Environmental Sciences of Ukraine of Ukraine on 26 November 2024, No. 022.2024.

## Results and Discussion

To assess the microbiological status of chickens kept in various conditions, bacteriological studies of cloacal flushes were conducted to identify zoonotically significant enterobacteria (*Salmonella spp.*, *Listeria spp.*, *Yersinia spp.*), which have potential epidemiological and veterinary sanitary significance. Based on the results of sowing followed by morphological, cultural, and biochemical identification, the pathogens of the listed genera were not detected in any of the samples studied in both experimental groups. Absence of *Salmonella spp.*, *Listeria spp.* and *Yersinia spp.* indicates a satisfactory sanitary and epizootic condition in the premises where chickens were kept, a low probability of participation of chickens as reservoirs of zoonotic pathogens in these conditions, and the effectiveness of hygienic and preventive measures. This result is important from a practical standpoint, since the presence of *Salmonella spp.*

or *Listeria spp.* in the number of young chickens is one of the key risk factors for food toxicoinfections and contamination of poultry products. In natural and small farms, the risk of introducing these pathogens remains traditionally higher due to the greater openness of the environment, but the data obtained do not confirm such a threat to the studied conditions.

Thus, the basic microbiological status of both groups of chickens can be regarded as successful, which allowed further focusing on analysing the composition of commensal microflora and studying its functional properties, in particular, the ability to form biofilms. Such results are consistent with data from EFSA and ECDC (2024), according to which the risks of contamination with *Salmonella spp.* and other pathogenic enterobacteria in young poultry significantly depend on the sanitary quality of feed and water, and the control of sources of infection in the first weeks of life. In the present study, chickens of both groups were in proper sanitary and hygienic condition, which may explain the absence of pathogens.

However, differences in the composition of the commensal microbiota were established. In samples from chickens from household farms (Group 2), 10 cultures of *Enterococcus spp.* and 10 cultures of *E. coli* were isolated and identified, which corresponded to the typical spectrum of normal intestinal microflora of poultry. In samples from chickens kept in vivarium conditions (Group 1), the spectrum of isolated microorganisms was wider: in addition to 10 cultures of *E. coli* and 10 cultures of *Enterococcus spp.*, 2 cultures of representatives of the genus *Klebsiella spp.* and 1 culture of *Pseudomonas aeruginosa* were also highlighted. The presence of these species was more often associated with conditions with increased humidity levels and longer microbial persistence on the surfaces of equipment and maintenance materials. This may reflect a more stable microecological

environment of the vivarium, where external factors vary less than in vivo.

The isolation of *Klebsiella spp.* and *P. aeruginosa* in Group 1 is important in terms of the potential opportunistic pathogenicity of these bacteria. According to R.P. Sequeira et al. (2020) and N.B.V. Tran et al. (2023), *Klebsiella spp.* and *P. aeruginosa* can remain part of the commensal microflora for a long time, but under conditions of immune stress or dysbiosis, they can exhibit pathogenic properties, in particular in birds – septicemia, respiratory lesions, and omphalitis. On the other hand, in free-range chickens, microbial diversity was less broad but ecologically stable, which was consistent with data on the priority of dominance of basic symbiotic taxa under more natural environmental conditions. Free range provided a fairly diverse microbial contact with the environment (soil, vegetation), but without pronounced conditions for the persistence of opportunistic bacteria, which often accumulate in closed technological systems.

Thus, the results obtained suggest that the keeping conditions of poultry can affect the structure of the commensal microbiome, but do not necessarily determine the presence of pathogenic microflora. In vivarium conditions, more diverse microbial associations with the inclusion of opportunistic bacteria can be formed, whereas in free-range conditions, the microbial profile was more stable and physiologically typical, represented by *E. coli* and *Enterococcus spp.* The differences in the microflora of cloacal swabs between groups of chickens were that when kept “free-range”, no *Klebsiella spp.* and *P. aeruginosa* bacteria, which are potentially pathogenic to birds and humans, were detected, whereas in vivarium conditions they were isolated. This may indicate the influence of microbiological environment conditions on the development of the composition of commensal and opportunistic microbiota, and the possible

role of content density and microclimate in the selection of microorganisms.

Subsequently, the ability of indicator commensal bacteria was evaluated – *E. coli* and *E. faecalis* isolated from chickens under different keeping conditions, to form biofilms, which were considered as one of the key factors of potential pathogenicity, colonisation ability, and resistance of bacterial populations. Thus, determination of the intensity of biofilm formation allowed assessing not only the ecological adaptive properties of microbes, but also the risks of their possible transformation into pathogenic forms due to stress factors or microbiome imbalance. To determine the intensity of biofilm formation, five *E. coli* isolates obtained from chickens in Group 2 and four *E. coli* isolates from chickens in Group 1 were selected and purified to pure cultures. Similarly, *E. faecalis* biofilm formation was later evaluated, which allowed for a parallel comparative characterisation between species. Each experiment was performed in 4 repetitions ( $n = 4$ ), which ensured statistical reliability and reproducibility of the results. Results of estimation of optical density of *E. coli* biofilms at  $\lambda = 570$  nm, are shown in Table 2.

Culture *E. coli*, isolated from Group 2 chickens, they showed wide variability in the intensity of biofilm formation – from 0.061 to 0.704, which indicates the existence of populations with different adaptive strategies. Some isolates were characterised by a weak level of biofilm formation, while others formed moderately dense biofilm matrices. The average value for the group ( $n = 5$ ) was 0.264, which corresponds to a low or moderate level of biofilm formation.

Instead, *E. coli* isolates from Group 1 chickens had a smaller range of optical density fluctuations – 0.095–0.314, which may be due to the stability of microbiological and environmental influences, the absence of a wide range of external stressors, and limited contacts with natural bacterial associations. The average group value

was 0.187, which indicates mostly weak biofilm structures. The data obtained are consistent with the results of O. Bezpalko *et al.* (2024), where it was noted that free access to the natural microbial environment contributes to

phenotypic variability and increases the ecological plasticity of commensal microorganisms. While *E. coli* isolates obtained under controlled retention conditions were more often characterised by standardised functional activity profiles.

**Table 2.** Indicators of optical density of biofilms formed by *E. coli*

Culture	Variant	<i>E. coli</i> , free-range chickens					<i>E. coli</i> , chickens kept in a vivarium			
		1	2	3	4	5	1	2	3	4
Cultivation medium, T°C		TSB, 37 ± 1°C								
λ 570, result with control	control	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003
	1	0.089	0.131	0.574	0.269	0.276	0.175	0.098	0.149	0.133
	2	0.074	0.218	0.492	0.147	0.160	0.205	0.126	0.202	0.118
	3	0.064	0.156	0.707	0.399	0.283	0.241	0.204	0.272	0.185
	4	0.129	0.230	0.655	0.159	0.117	0.209	0.172	0.317	0.237
	Range of values λ, Min-Max	0.064-0.129	0.131-0.230	0.492-0.707	0.147-0.399	0.117-0.283	0.175-0.241	0.098-0.204	0.149-0.317	0.118-0.237
	Average value, n=4	0.089	0.184	0.607	0.244	0.209	0.208	0.150	0.235	0.168
	Range of average values λ, Min-Max	0.089-0.607					0.150-0.235			
	M (mean)	0.267 (n=5)					0.1903 (n=4)			
	M +/- m	0.27 ± 0.09					0.19 ± 0.02			
	1	0.086	0.128	0.571	0.266	0.273	0.172	0.095	0.146	0.130
	2	0.071	0.215	0.489	0.144	0.157	0.202	0.123	0.199	0.115
	3	0.061	0.153	0.704	0.396	0.280	0.238	0.201	0.269	0.182
4	0.126	0.227	0.652	0.156	0.114	0.206	0.169	0.314	0.234	
Actual value (λ 570 – control)	Range of values λ, Min-Max	0.061-0.126	0.128-0.227	0.489-0.704	0.144-0.396	0.114-0.280	0.172-0.238	0.095-0.201	0.146-0.314	0.115-0.234
	Average value, n=4	0.086	0.181	0.604	0.241	0.206	0.205	0.147	0.232	0.165
	Range of average values λ, Min-Max	0.086-0.604					0.147-0.232			
	Average value of λ in the group	0.264 (n=5)					0.187 (n=4)			
	M +/- m	0.26 ± 0.09					0.19 ± 0.02			

**Note:** interpretation of optical density (OD) values: TSB – trypton-soy broth; OD < 0.1 – the culture does not form biofilms; OD from 0.1 to 0.49 – the culture forms low-density biofilms; OD from 0.5 to 1.0 – the culture forms medium-density biofilms; OD > 1.0 – the culture forms high-density biofilms

**Source:** developed by the authors based on own research

It is important to note that even moderate levels of biofilm formation in commensal isolates are important for the stability and balance of intestinal microbial communities, since biofilms act as a kind of ecological reservoir that ensures the colonisation stability of the population and its protection from competitive microbes and external stress influences. Thus, it was

found that the keeping conditions of chickens play a significant role in the phenotypic manifestation of the ability of *E. coli* to the formation of biofilms. The revealed variability in the intensity of biofilm formation may indicate that this indicator is not a fixed species characteristic, but is formed as a response to the influence of external environmental factors. Free-range

conditions involving a wider range of microecological stimuli and microbial associations probably contributed to the activation of adaptive mechanisms associated with increased colonisation resistance and the formation of protective biofilm structures. In contrast, in the controlled microbiological environment of vivarium, where the influence of external factors and microbial competition was minimised, a decrease in the intensity of biofilm formation was observed, which can be considered as a result of a reduced need for structured forms of population survival.

The data obtained are consistent with current ideas about biofilm as a phenotypic manifestation of bacterial ecological adaptation, which is formed in response to changes in environmental parameters, such as the presence of competitive microorganisms, fluctuations in pH and temperature, and the availability of nutrients and substrates for adhesion (Gupta et al., 2016; Sonderholm et al., 2017). Thus, *E. coli* as part of the chicken microbiome, it not only acts as a commensal resident, but also demonstrates flexibility in choosing a survival strategy that is of important environmental and veterinary sanitary importance.

Analysis of the intensity of biofilm formation showed that among the *E. coli* isolates selected from Group 2 chickens, three cultures

out of five formed low-density biofilms, one culture (*E. coli* No. 3) was characterised by the formation of medium-density biofilms, and the culture *E. coli* No. 1 did not form biofilms in three repetitions, whereas in one repetition it showed the formation of low-density biofilms (Table 2). In contrast, all *E. coli* isolates obtained from Group 1 chickens stably formed low-density biofilms in all study variants (n=4), which indicated more uniform adaptive properties of the bacterial population under conditions of limited contact with natural microbiocenoses.

Further studies were aimed at evaluating the biofilm-forming ability of *E. faecalis* isolates and comparison of the results obtained with the data on *E. coli*, which allowed tracing interspecific differences in adaptive mechanisms associated with the development of biofilm structures in the composition of the resident gut microbiota of chickens. When evaluating the ability to form biofilms with isolates of *Enterococcus faecalis*, the study included 6 cultures isolated from Group 2 chickens and 2 cultures obtained from Group 1 chickens. As in the case of *E. coli*, each measurement was performed in 4 repetitions (n=4), which ensured the reliability of the obtained comparative indicators. The results of determining the density of biofilms in these cultures are shown in Table 3.

**Table 3.** Indicators of optical density of biofilms formed by *E. faecalis*

Culture	Variant	<i>E. faecalis</i> , free-range chickens						<i>E. faecalis</i> , chickens kept in a vivarium	
		1	2	4	5	7	8	1	2
Cultivation medium, T°C		TSB, 37 ± 1°C							
λ 570, result with control	control	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003
	1	0.268	0.213	0.137	0.194	0.208	0.203	0.155	0.139
	2	0.209	0.246	0.151	0.217	0.201	0.209	0.166	0.103
	3	0.269	0.227	0.235	0.297	0.210	0.208	0.153	0.137
	4	0.207	0.242	0.254	0.215	0.250	0.204	0.168	0.105
	Range of values λ, Min-Max	0.207-0.269	0.213-0.246	0.137-0.254	0.194-0.297	0.201-0.250	0.203-0.209	0.152-0.168	0.103-0.139
	Average value, n = 4	0.238	0.232	0.194	0.230	0.217	0.206	0.160	0.121
	Range of average values λ, Min-Max	0.194-0.238						0.121-0.160	
	M (mean)	0.165						0.141	
	M +/- m	0.22 ± 0.01						0.14 ± 0.02	

Table 3. Continued

Culture	Variant	<i>E. faecalis</i> , free-range chickens						<i>E. faecalis</i> , chickens kept in a vivarium	
		1	2	4	5	7	8	1	2
Actual value of $\lambda$ 570 ( $\lambda$ 570 - control)	1	0.265	0.210	0.134	0.191	0.205	0.200	0.152	0.136
	2	0.206	0.243	0.148	0.214	0.199	0.206	0.163	0.100
	3	0.267	0.224	0.232	0.294	0.207	0.205	0.156	0.132
	4	0.204	0.239	0.251	0.212	0.247	0.201	0.159	0.104
	Average value, n=4	0.235	0.229	0.191	0.227	0.215	0.203	0.157	0.118
	Average value by keeping conditions	0.162						0.137	
	Range of average values $\lambda$ , Min-Max	0.191-0.235						0.118-0.157	
	M (mean)	0.217 (n=6)						0.137 (n=2)	
	M +/- m	0.22 ± 0.01						0.14 ± 0.02	

**Note:** interpretation of optical density values (OD): TSB – trypton-soy broth; OSH < 0.1 – the culture does not form biofilms; OD from 0.1 to 0.49 – the culture forms low-density biofilms; OD from 0.5 to 1.0 – the culture forms medium-density biofilms; OD > 1.0 – the culture forms high-density biofilms

**Source:** developed by the authors based on own research

These results are consistent with data from studies by F. Lebreton *et al.* (2014) and C. Geraldes *et al.* (2022), who indicated that *E. faecalis*, despite their commensal status, can switch to an opportunistic phenotype under conditions of increased environmental pressure and inter-microbial competition. In particular, it was found that in natural and farm ecosystems *E. faecalis* more actively expresses surface attachment proteins (MSCRAMMs), genes of intercellular signalling systems, and factors involved in the synthesis of the polysaccharide matrix of biofilms. On the contrary, in a vivarium environment where a controlled microclimate, standardised feeding, and the influence of foreign microbial factors are minimised, the need for bacteria to form complex survival structures decreases. Therefore, isolates obtained from chickens kept in a vivarium were more likely to show low-intensity biofilm formation, reflecting the state of microbiological stability of the environment in which the host macroorganism is located. Thus, the identified differences characterise the biofilm not as a static feature of the species, but as a dynamic adaptation phenotype formed in response to environmental challenges.

This confirms the concept of ecological plasticity of the microbiome and emphasises that the keeping conditions are an important factor that can modulate the pathobiological properties of commensal bacteria.

Analysis of the obtained indicators of optical density of biofilms shows that all eight studied cultures of *E. faecalis* formed low-density biofilms. This uniformity of biofilm formation intensity indicates a relatively uniform potential of commensal enterococci for adhesion and formation of microbial consortia on surfaces. This is consistent with their physiological role as permanent components of the avian gut microbiota.

In previous studies, O. Bezpalko *et al.* (2024) found differences in the composition of indicator bacteria in chicken droppings depending on the conditions of poultry rearing. Thus, zoonotic pathogenic bacteria were not detected in free-range chickens, while such bacteria were present in poultry raised in simulated conditions of an industrial poultry house with controlled microclimate parameters and a standard diet. This determined the need for further study not only of the species structure of the microbiota, but also of the biological properties

of commensal bacteria, in particular, the ability to form biofilms as an important factor in their resistance and potential pathogenicity. The current study focused on comparing properties of *E. coli* and *E. faecalis* isolated from chickens raised in different keeping conditions.

Biofilm formation, according to the data by S. Sharma *et al.* (2023), is a key mechanism for bacterial survival and the development of their antibiotic resistance. That is why the study of the intensity and features of biofilm formation by indicator bacteria in the poultry intestine is important for understanding the mechanisms of their potential pathogenicity and role in the development of dysbiosis. *E. coli* cultures isolated from Group 2 chickens formed low-density biofilms with an average optical density value  $\lambda=0.264$ . Similarly, *E. coli* isolates obtained from Group 1 chickens also formed low-density biofilms, but the average value for this group was lower and was 0.187 (which is 29.2% less than the average optical density of the *E. coli* isolate from free-range chickens kept on a private plot). Culture of *E. faecalis* isolated from free-range chickens formed low-density biofilms with an average value of  $\lambda=0.217$ , while enterococci isolated from chickens kept in a vivarium were characterised by a lower average biofilm density – 0.137.

A comparative analysis of the results obtained shows that the ability of microorganisms to form biofilms can vary depending on the external conditions of the host macroorganism. In particular, *E. coli* and *E. faecalis* isolates from free-range chickens were characterised by higher values of optical density of biofilms compared to isolates from poultry in standardised vivarium conditions. Thus, the average level of biofilm formation in the group *E. coli* from Group 2 was 41.17% higher, and *E. faecalis* – 58.4% higher compared to the corresponding Group 1 chicken cultures.

These data are consistent with the idea of biofilm as a form of phenotypic adaptation

of bacteria to environmental factors. M. Sönderholm *et al.* (2017) emphasised that biofilm organisation is an evolutionarily formed mechanism of resistance to changes in the physical and chemical parameters of the environment. Similar conclusions were given by C. de la Fuente-Núñez *et al.* (2013), who considered biofilm formation as a form of collective bacterial behaviour activated by external stress. P. Gupta *et al.* (2016) proved the dependence of the biofilm structure on environmental conditions and the metabolic state of the population.

According to the results obtained, the absolute values of biofilm formation by *E. coli* and *E. faecalis* isolates corresponded to a low level of intensity. This is probably conditioned by the clinically healthy condition of the bird. Similar observations were described by C. Chiang-Ni *et al.* (2024), who established that non-clinical isolates of *Clostridium innocuum* most of often show a low ability to form biofilms, while pathogenic strains are characterised by a higher intensity.

*E. coli* of commensal origin have a significantly lower biofilm formation capacity compared to isolates obtained from animals or people with clinical pathology. In particular, R.P. Mahale *et al.* (2025) showed that biofilms formed only 16.6% of commensal isolates, while among clinical isolates – 77.2%. The study by D. Kalantar-Neyestanaki *et al.* (2023) demonstrated that in clinical strains, biofilm formation was accompanied by the presence of adhesion genes (*icaADBC*, *mecA*, *fbe*, etc.), whereas in non-clinical isolates, these genes were rare or showed low expression levels. However, even commensal isolates were able to form basic biofilm structures that ensure environmental sustainability and competitiveness in the microbial community. This is consistent with the data by K. Otokunefor *et al.* (2020), who showed the spread of signs of biofilm formation among natural populations of *E. coli*, not associated with pathology.

According to the results of bacteriological studies, it was found that chickens of both groups with different content were not found to have *Salmonella spp.*, *Listeria spp.*, and *Yersinia spp.*, which indicates that the bird was not contaminated with zoonotic pathogens at the time of the study. Moreover, differences in the structure of the commensal microbiota were found: in samples from free-range chickens, the microbial spectrum was mainly represented by *E. coli* and *Enterococcus spp.*, while in the vivarium conditions, *Klebsiella spp.* and *Pseudomonas aeruginosa* were additionally isolated, which may indicate the influence of a stable artificially controlled microecological environment on the preservation of opportunistic bacteria.

Comparison of the results obtained with the literature data shows that commensal isolates of *E. coli* and *E. faecalis* obtained from clinically healthy chickens, expectedly formed mainly low-density biofilms. This is consistent with the data by S. Ramos *et al.* (2020) and B.A. Lindstedt *et al.* (2018), who emphasised that most non-clinical strains of *E. coli* show only a basic level of adhesiveness and do not show the intense biofilm formation characteristic of pathogenic variants. Similarly, data by B. Krawczyk *et al.* (2021) and C. Gerales *et al.* (2022) confirmed that *E. faecalis* commensal origin usually forms weak biofilm structures, while clinically significant strains have significantly higher adhesive activity.

In the current study, the average optical density values for *E. coli* and *E. faecalis* (0.264 and 0.217, respectively, in the free-range group) did not exceed the low-intensity limits, which is consistent with the characteristics of non-pathogenic isolates described by the above researchers. However, cultures obtained from free-range chickens had higher rates of biofilm formation compared to vivarium isolates. This is partially consistent with the data by T.T.M. Manders *et al.* (2025), who indicated that under more

variable environmental conditions, bacteria are more active in implementing adaptive mechanisms, in particular biofilm formation. However, in the presented experiment, none of the isolates reached the levels of biofilm formation described in pathological strains, where the optical density values often exceed the threshold of medium or high intensity. This indicates that, despite certain differences between the keeping conditions, all isolates retained the commensal phenotype and did not exhibit properties characteristic of clinically significant variants of *E. coli* or *E. faecalis*. Thus, the data obtained in the current study are consistent with the literature reports on low levels of biofilm formation in non-clinical strains and simultaneously complement them, demonstrating that the intensity of this process can vary depending on the conditions of poultry keeping, but not to the level characteristic of pathogenic isolates.

Analysis of the intensity of biofilm formation showed that *E. coli*, so and *E. faecalis* formed mainly low-density biofilms, which correlates with the clinically healthy state of poultry and confirms the commensal status of isolates. However, bacteria isolated from free-range chickens had higher average levels of biofilm formation compared to vivarium isolates (by 41.17% for *E. coli* and by 58.4% for *E. faecalis*). These differences indicate the influence of environmental factors on the phenotypic realisation of biofilm properties, this is consistent with the data of leading international studies on the ecological adaptation of microorganisms (de la Fuente-Núñez *et al.*, 2013; Gupta *et al.*, 2016; Sonderholm *et al.*, 2017). Thus, it was found that the conditions of keeping poultry can modulate the biological properties of indicator components of the microbiome, in particular, their ability to form biofilms. The results showed the importance of taking into consideration environmental factors, depending on the conditions of keeping chickens.

## Conclusions

In the conducted studies, the intensity of biofilm formation in isolates of indicator bacteria isolated from chickens kept in various conditions was determined. *E. coli* and *E. faecalis* were selected as indicator microorganisms, given the available data on their possible synergistic interaction in the intestinal microbiome and their impact on the survival, development, and overall physiological condition of young poultry. In addition, it was noted that the coexistence of *E. coli* and *E. faecalis* can enhance the colonisation potential of both species and create conditions for chronic dysbiotic conditions.

It was found that isolates obtained from clinically healthy chickens generally formed low-density biofilms. This is consistent with the commensal status of the studied strains and confirms that high levels of biofilm formation are more often characteristic of pathogenic or colonising active bacterial variants. However, cultures isolated from free-range chickens showed a higher biofilm formation intensity compared to vivarium isolates: the average optical density for *E. coli* was higher by 41.17%, and for *E. faecalis* – by 58.4%. This may indicate that more variable environmental conditions (microbial diversity, diet differences, exposure to natural substrates) stimulate the activation

of adhesion systems and the formation of biofilm structures as a survival mechanism for resident microorganisms.

These results support the hypothesis of ecological regulation of biofilm formation and indicate phenotypic plasticity of commensal isolates. However, to establish the exact mechanisms of adaptation, it is necessary to conduct molecular genetic analysis (assessment of the expression of genes encoding adhesion factors and matrix components), and transcriptomic and proteomic studies. Prospects for further research consist in expanding the range of microbiome species studied, analysing the relationship between biofilm formation and antibiotic resistance, and assessing potential risks to poultry systems in the context of the “One Health” concept.

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## Conflict of Interest

None.

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## Дослідження здатності до формування біоплівок *in-vitro* у санітарно-показових бактерій, виділених від курчат

**Ліліана Давидовська**

Аспірант

Національний університет біоресурсів і природокористування України  
03041, вул. Героїв Оборони, 15, м. Київ, Україна  
<https://orcid.org/0000-0003-4385-4500>

**Артем Ушкалов**

Кандидат ветеринарних наук, старший науковий співробітник  
Національний науковий центр «Інститут експериментальної і клінічної ветеринарної  
медицини»  
61023, вул. Григорія Сковороди, 83, м. Харків, Україна  
<https://orcid.org/0000-0001-8317-7909>

**Лілія Виговська**

Доктор ветеринарних наук, старший науковий співробітник  
Національний університет біоресурсів і природокористування України  
03041, вул. Героїв Оборони, 15, м. Київ, Україна  
<https://orcid.org/0000-0002-5631-9139>

**Валерій Ушкалов**

Доктор ветеринарних наук, професор, дійсний член НААН України  
Національний університет біоресурсів і природокористування України  
03041, вул. Героїв Оборони, 15, м. Київ, Україна  
<https://orcid.org/0000-0001-5694-632X>

**Юрій Вішован**

Доктор філософії в галузі ветеринарної медицини  
Українська лабораторія якості та безпеки продукції сільського господарства  
08162, вул. Машинобудівників, 7, смт. Чабани, Україна  
<https://orcid.org/0000-0003-1128-593X>

**Анотація.** У мікроорганізмів, що колонізують травний тракт тварин, зокрема домашньої птиці, біоплівки забезпечують стійкість до антимікробних засобів та імунної відповіді організму. Дослідження біоплівкоутворення індикаторних бактерій, ізольованих від курчат за різних умов утримання, дає можливість оцінити вплив екологічних факторів на їх фенотипову адаптацію та потенційний ризик для здоров'я тварин і людей. Мета роботи полягала у визначенні здатності до утворення біоплівок у бактерій *Escherichia coli* та *Enterococcus faecalis*, виділених від курчат, утримуваних у віварії та на вільному вигулі. Застосовано бактеріологічні, морфологічні, біохімічні та мікроскопічні методи досліджень. Інтенсивність біоплівкоутворення в індикаторних мікроорганізмів оцінювали за показником адсорбції/резорбції 0,1 % розчину кристалічного фіолетового з використанням полістиролових чашок Петрі. Оптичну щільність вимірювали спектрофотометрично при довжині хвилі 570 нм. Встановлено, що у зразках від курчат, що утримувались у віварії, виділено *E. coli*, *E. faecalis*, *Klebsiella spp.* та *Pseudomonas aeruginosa*, тоді як у курчат на вільному вигулі представників роду клебсієл та синьогнійної палички не виявлено, що

вказує на нижчу присутність потенційних патогенів у природних умовах утримання. Всі досліджені культури формували біоплівки низької або середньої щільності. Для ізолятів *E. coli*, отриманих від курчат вільного вихулу, середнє значення  $\lambda = 0,264 \pm 0,09$ , тоді як для ізолятів із віварію –  $\lambda = 0,187 \pm 0,07$ . Культури *E. faecalis* утворювали біоплівки з інтенсивністю  $\lambda = 0,217 \pm 0,04$  у курчат на вільному вихулі та  $\lambda = 0,137 \pm 0,03$  у курчат із віварію. Отже, ізоляти, отримані з природних умов, відрізнялися вищою інтенсивністю формування біоплівок – на 41,2 % (*E. coli*) та 58,4 % (*E. faecalis*) порівняно з умовами контрольованого мікроклімату. Це може свідчити про стимулюючий вплив факторів довкілля на експресію генів адгезії та біоплівкоутворення. Водночас усі досліджені культури були ізольовані від клінічно здорових птахів, що вказує на комєнсальний характер мікробіому. Отримані результати є важливими для оцінки ризиків горизонтального перенесення генів стійкості та формування стабільних мікробних біоплівок у птахівництві

**Ключові слова:** індикаторні бактерії; *Escherichia coli*; *Enterococcus faecalis*; умови утримання птиці; фенотипова адаптація; санітарно-мікробіологічний моніторинг