Study of morphological, biological and probiotic properties of autochthonous lactobacilli from the intestines of pheasants

Yuri Lysenko[1*], *Albina* Luneva[1], *Margarita* Yakovets[2], *Evgeniy* Marchenko[1], and *Maria* Lifentsova[2]

[1]Federal State Budgetary Educational Institution of Higher Education "Russian State Agrarian University – Moscow Timiryazev Agricultural Academy", Moscow, 127434, Russian Federation
[2]Federal State Budgetary Educational Institution of Higher Education "Kuban State Agrarian University named after I. T. Trubilin", Krasnodar, 350044, Russian Federation

Abstract. The work studied the morphological, biological and probiotic properties of representatives of the genus Lactobacillus (*Loigolactobacillus coryniformis, Lactobacillus johnsonii, Lactobacillus reuteri*), which were isolated from the contents of the caecum of domesticated pheasants using microbiological methods and identified to the species level using modern molecular genetic methods. It was found that when analyzing the morphological properties of the microorganisms, they had all the signs confirming their belonging to representatives of lactobacilli. It was revealed that *Loigolactobacillus coryniformis* and *Lactobacillus johnsonii* are capable of utilizing their entire range presented in the studies when studying the fermentability of carbohydrates. It was determined that the probiotic strains *Lactobacillus johnsonii* and *Loigolactobacillus coryniformis* were capable of exhibiting high acid-forming activity, the value of which was 271.1 ° and 231.2 °T, and also turned out to be highly resistant in reactions with aggressive nutrient media similar to biological fluids of the gastrointestinal tract. The sensitivity of lactobacilli to antibiotics was studied, demonstrating that the studied lactobacilli strains belong to intermediate-resistant microorganisms. It was established that *Lactobacillus johnsonii* and *Loigolactobacillus coryniformis* are promising as components for probiotic compositions, since they exhibit high antagonistic and anti-adhesive properties in relation to opportunistic microorganisms, and also have high adhesion in relation to enterocytes of the intestine of birds.

1 Introduction

Intensive use of antibiotics in poultry farms leads to an imbalance of symbiotic microbial communities in the digestive tract, promotes the development of dysbacteriosis, increases the resistance of pathogenic microorganisms to antibiotics and leads to the accumulation of residual amounts of antibiotics in livestock products. Antibacterial spectrum drugs, widely

* Corresponding author: yuraduban45@mail.ru

used to control infectious diseases in birds, have recently been recognized as a source of the global problem of antibiotic resistance. Under pharmacological influence, bacteria inhabiting the gastrointestinal tract and circulating in poultry farms increase their virulence to antibiotics, which has led to a change in resistance and a decrease in the effectiveness of industrial herd health improvement. Also, the presence of residual antibiotics in poultry products leads to the development of secondary drug resistance and allergic reactions in consumers of products, which has social significance at the global level [1].

In poultry farming, the use of various biopreparations helps to increase the productivity of poultry enterprises and ensure the veterinary health of birds. However, complex additives are often characterized by a narrow spectrum of action and high cost, which significantly reduces the economic efficiency of poultry enterprises. Today, probiotic additives are used as alternatives to antibiotics. Restoring the balance of the intestinal microbiome, improving digestive processes, and increasing natural resistance against the background of the microbial load on the bird's body is an important stage in the context of the intensification of poultry farms. The use of probiotic additives contributes to increased productivity of animals and poultry, the effectiveness of treatment of gastrointestinal diseases, and a decrease in morbidity. The development of effective probiotic additives based on autochthonous microflora is one of the priority areas in agriculture [2-3].

Live microorganisms contained in probiotic biopreparations that inhabit the normal intestinal microflora of poultry (*Lactobacillus, Bifidobacterium, Enterococcus*, etc.) help to displace pathogenic and opportunistic microflora, significantly reducing their numbers in the biotope. The bacteriocins they produce, as well as organic acids, have an antagonistic effect, neutralizing enterotoxins of the pathogenic environment. Numerous studies emphasize the importance of such probiotic strains in modulating the body's immune response. They stimulate the phagocytic activity of neutrophils and macrophages, participate in the synthesis of immunoglobulins, the formation of interferons, and also activate cellular immunity. Probiotic bacteria regulate nonspecific immunity, increasing the protection of mucous membranes [4-6].

Thus, the search for promising probiotic strains, in particular representatives of the genus *Lactobacillus*, inhabiting the natural environment of the gastrointestinal tract of farm animals and poultry, capable of exhibiting high probiotic properties for their inclusion in the composition of microbial biopreparations is a promising and urgent task.

The aim of the work is to study the morphological, biological and probiotic properties of autochthonous lactobacilli isolated from the intestines of pheasants.

2 Materials and methods

Microbiological laboratory studies were carried out at the Federal State Budgetary Educational Institution of Higher Education “Russian State Agrarian University – Moscow Agricultural Academy named after K. A. Timiryazev” and the Federal State Budgetary Educational Institution of Higher Education “Kuban State Agrarian University named after I. T. Trubilin”.

The objects of the studies were samples of pure cultures of lactic acid bacteria (*Loigolactobacillus coryniformis, Lactobacillus johnsonii, Lactobacillus reuteri*), which were isolated from the contents of the caecum of domesticated pheasants using microbiological methods and identified to the species level using modern molecular genetic methods.

To study the morphological properties of lactobacilli, microorganism preparations on a smear were fixed in Carnoy’s solution, and stained according to Gram. Microscopic studies were also carried out using oil immersion, a 100× objective and a 10× eyepiece. Studies of the ability of lactobacilli to ferment carbohydrates were also carried out according to the works of [7].

The study of the manifestation of acid-forming activity by the objects of study was carried out *in vitro* according to the scientific work [8], as well as the resistance of lactobacilli to simulated aggressive environments and antibiotics according to the works [9].

The antagonistic activity of lactobacilli strains was studied *in vitro* using diffusion methods in various modifications [10].

The study of the anti-adhesive and adhesive activity of lactobacilli strains was carried out in relation to enterocytes of the intestine of a chicken embryo according to the requirements of [11].

The analysis and interpretation of the results were carried out using biometric methods. For this, Microsoft Excel 2019 software on the Windows 10 platform was used. During processing, the arithmetic mean (M), standard error of the mean (m) and standard deviation (σ) were calculated. Comparative analysis of quantitative characteristics was carried out by comparing the average values of two samples using Student's t-test and determining the significance level (p). Differences were considered statistically significant at $p \leq 0.05$.

3 Results and Discussion

3.1 Study of morphological properties of lactobacilli

The results of laboratory study of morphological properties of lactobacilli (*Loigolactobacillus coryniformis, Lactobacillus johnsonii, Lactobacillus reuteri*) isolated from the cecum of domesticated pheasants showed that the studied lactobacilli are gram-positive; do not show signs of movement in a native smear; when fixed in a smear under immersion, separately located small rod-shaped cells of lactic acid bacteria measuring 1.5-2.0 × 0.3-0.5 μm are most often found; they carry out their energy life cycles both in the absence of oxygen and are also capable of growing in its presence (facultative anaerobes). When growing on a dense nutrient medium, colonies with the following characteristics appear: round shape, 1.0-1.5 mm in size, fuzzy edge contour, flat relief, smooth and matte surface, light gray color, granular structure and soft consistency. When analyzing the characteristics of microorganisms, in all cases they were similar and characteristic of direct representatives of the genus *Lactobacillus*.

3.2 Study of biological properties of lactobacilli

The following were attributed to the biological characteristics of lactobacilli:

- The ability to ferment various carbohydrates.
- An indicator of acid-forming activity.
- Resistance to aggressive environments and antibiotics.

Glucose, galactose, fructose, maltose, mannitol, sucrose, xylose, arabinose and lactose were used as carbohydrates in the studies. As a result of the studies, it was established that Loigolactobacillus coryniformis and Lactobacillus johnsonii are capable of utilizing all carbohydrates, however, Lactobacillus reuteri did not use sucrose, xylose and lactose as a food factor during the growth process. Thus, Loigolactobacillus coryniformis and Lactobacillus johnsonii exhibit the best ability to adapt to various substrates. An important indicator of the specific activity of lactic acid bacteria is their ability to exhibit acid-forming activity. The results of titratable acidity during the cultivation of probiotic strains are presented in Table 1.

Table 1. Results of acid-forming activity of *Lactobacillus coryniformis*, *Lactobacillus johnsonii* and *Lactobacillus reuteri*, M±m.

Object of study	**Titratable acidity, °T**		
	6 hours	**12 hours**	**24 hours**
Loigolactobacillus coryniformis	35.6 ± 0.8	142.3 ± 1.9	231.2 ± 3.1
Lactobacillus johnsonii	41.3 ± 0.6	163.6 ± 2.0	271.1 ± 3.3
Lactobacillus reuteri	24.7 ± 0.5	89.4 ± 1.7	179.8 ± 2.7

The results of the studies demonstrated that by the end of the cultivation period in a liquid nutrient medium (for 24 hours), the maximum value of the titratable acidity index was established when growing Lactobacillus johnsonii, which was 271.1 ± 3.3 °T and *Loigolactobacillus coryniformis* – 231.2 ± 3.1 °T, while the lowest value was found in *Lactobacillus reuteri* – 179.8 ± 2.7 °T. As is known, the value of the acid formation index is directly related to the ability of lactic acid bacteria to synthesize a complex of organic acids (especially lactic acid) in the external environment, which also affects their sensitivity to hydrochloric acid in the gastrointestinal tract. It has been established that more pronounced acid formation activity increases the resistance of potential probiont strains to the antimicrobial action of gastric juice [9].

In this regard, the tolerance of the research bioobjects to the action of biological fluids of the gastrointestinal tract in simulated aggressive environments was studied. The analyzed parameter was assessed by visually determining the presence or absence of growth of *Loigolactobacillus coryniformis, Lactobacillus johnsonii* and *Lactobacillus reuteri* in a liquid nutrient substrate, which additionally included various concentrations of the following components: natural gastric juice (the drug "Equin", in a concentration of 10%), medical bile (in concentrations of 20; 30 and 40%), phenol (in a concentration of 0.4%) and sodium chloride (in concentrations of 2.0 and 4.0%) (Table 2).

Table 2. Resistance of research objects to simulated aggressive nutrient media.

Object of study	**Nutrient medium + NaCl**		**Nutrient medium + medical bile**			**Nutrient medium + phenol (0.4%)**	**Nutrient medium + gastric juice (10%)**
	2 %	**4 %**	**20 %**	**30 %**	**40 %**		
Loigolactobacillus coryniformis	+	+	+	+	+	+	+
Lactobacillus johnsonii	+	+	+	+	+	+	+
Lactobacillus reuteri	+	+	±	–	–	±	±

* "+" – moderate growth; "±" – average growth; "–" – no growth in liquid medium

It was found that when growing *Loigolactobacillus coryniformis* and *Lactobacillus johnsonii* in liquid nutrient media with the addition of aggressive components, in all cases, according to the McFarland turbidity standard, good (moderate) growth of lactobacilli cultures was detected, however, it was found that *Lactobacillus reuteri* showed weak growth when adding gastric juice, phenol and medical bile to the nutrient substrate at a concentration of 20%. A complete absence of *Lactobacillus reuteri* growth was noted when adding medical bile to the liquid culture medium at concentrations of 30 and 40%, respectively.

A study was conducted on the antibiotic resistance of the analyzed lactobacilli strains to therapeutic doses of some antimicrobial drugs. The research results are presented in Table 3.

Table 3. Sensitivity of lactobacilli to antimicrobial substances, M±m.

Antibiotic	Dose, mcg	Size of growth inhibition of lactobacilli around antibiotic, mm		
		Loigolactobacillus coryniformis	*Lactobacillus johnsonii*	*Lactobacillus reuteri*
Cephalothin	30	15.0 ± 0.4	13.0 ± 0.5	18.0 ± 0.4
Ampicillin	10	4.0 ± 0.1	6.0 ± 0.2	9.0 ± 0.3
Lincomycin	15	9.0 ± 0.4	9.0 ± 0.3	8.0 ± 0.3
Erythromycin	15	7.0 ± 0.2	6.0 ± 0.2	7.0 ± 0.3
Tetracycline	30	11.0 ± 0.4	7.0 ± 0.3	10.0 ± 0.4
Streptomycin	10	6.0 ± 0.2	3.0 ± 0.1	7.0 ± 0.3
Penicillin	10	12.0 ± 0.3	9.0 ± 0.2	10.0 ± 0.4

The results of the studies showed that the growth inhibition zone for *Loigolactobacillus coryniformis* from the antimicrobial compounds used was within 4.0-15.0 mm, for *Lactobacillus johnsonii* – 3.0-13.0 mm, and for *Lactobacillus reuteri* – 7.0-18.0 mm. According to the lactic acid bacteria sensitivity assessment indicator, they can be classified as “intermediately resistant” microorganisms.

3.3 Study of probiotic properties of lactobacilli

The following parameters were subjected to assessment of probiotic properties of the objects of research:

- Antagonistic and antiadhesive action of the studied lactobacilli strains in relation to field opportunistic microflora.
- Antagonistic action of the studied lactobacilli strains in relation to beneficial intestinal microflora and to each other.
- Adhesiveness of the studied lactobacilli strains in relation to intestinal enterocytes of chicken embryos.

The opportunistic field test culture was Staphylococcus aureus isolated from birds at the Primorskaya Poultry Farm and Escherichia coli A-20/O137 isolated from birds at the Novomyshastovskaya Poultry Farm [11]. The results of microbiological studies are reflected in Table 4.

Table 4. Zone of growth inhibition of field opportunistic microorganisms from the action of the studied lactobacilli, mm.

Field opportunistic pathogenic test culture	Object of study		
	Loigolactobacillus coryniformis	*Lactobacillus johnsonii*	*Lactobacillus reuteri*
Growth inhibition zone during the agar layer method, mm			
Escherichia coli A-20/O$_{137}$	6.0 ± 0.2	7.0± 0.3	–
Staphylococcus aureus	5.0 ± 0.2	5.0± 0.3	–
Growth inhibition zone when performing the well method, mm			
Escherichia coli A-20/O$_{137}$	5.0 ± 0.2	6.0± 0.2	1.0± 0.1
Staphylococcus aureus	6.0 ± 0.2	7.0± 0.3	1.0± 0.1
Growth inhibition zone when performing the perpendicular stroke method, mm			
Escherichia coli A-20/O$_{137}$	5.0 ± 0.2	6.0± 0.2	1.0± 0.1
Staphylococcus aureus	7.0 ± 0.2	8.0± 0.3	1.0± 0.1
Growth inhibition zone during the drop method, mm			
Escherichia coli A-20/O$_{137}$	5.0 ± 0.2	7.0± 0.3	1.0± 0.1
Staphylococcus aureus	3.0 ± 0.1	6.0± 0.2	–

The results of the microbiological studies demonstrated that the best antagonistic activity was shown by *Lactobacillus johnsonii* strains, the growth inhibition zone of field test cultures depending on the study method was observed in the range of 5.0-8.0 mm and *Loigolactobacillus coryniformis* with an inhibition zone of opportunistic test strains within 3.0-7.0 mm. *Lactobacillus reuteri* in some cases did not show antagonism, and in others with a minimum value of up to 1.0 mm.

The concept of "anti-adhesion" of beneficial microflora implies the ability of microflora to prevent or reduce the attachment effect (adhesion) of a pathogenic test strain to a cellular model, in our case to enterocytes of the intestine of a chicken embryo. The results of the anti-adhesiveness of the studied lactobacilli strains in relation to *Staphylococcus aureus* and *Escherichia coli* A-20/O_{137} are reflected in Table 5.

Table 5. Results of anti-adhesive properties of research objects in relation to test cultures *E. coli* A-20/O_{137} and *S. aureus*.

Object of study	**Anti-adhesive properties to control, %**	
	***Escherichia coli* A-20/O_{137}**	***Staphylococcus aureus***
Control (enterocytes + test culture)	100.0	100.0
Enterocytes + test culture + *Loigolactobacillus coryniformis*	87.4	83.9
Enterocytes + test culture + *Lactobacillus johnsonii*	90.6	86.3
Enterocytes + test culture + *Lactobacillus reuteri*	23.1	19.7

It was established that the probiont strains *Loigolactobacillus coryniformis* and *Lactobacillus johnsonii* exhibit high anti-adhesive properties in relation to test cultures of field opportunistic strains, and this indicator is within 83.9-90.6%. *Lactobacillus reuteri* showed a low anti-adhesive effect in relation to test cultures, which was 19.7% in relation to *Staphylococcus aureus* and 23.1% in relation to *Escherichia coli* A-20/O_{137}.

To study the effect of the research objects on the inhibition of the growth of beneficial microflora and in relation to each other, a microbiological research method was used - "perpendicular streaks". The probiont strain was sown in the form of a streak along the diameter on a Petri dish, and streaks of other test-analyzed bioobjects were made perpendicular to it. The cultures were grown at 37±1.0 °C for 1-2 days. The results of the studies were assessed by the growth inhibition zone of the test-analyzed biological objects in relation to the probiotic strain isolated from the pheasant cecum.

It was established that the zones of inhibition of growth of test objects of beneficial microflora (*Lactobacillus acidophilus* (drug "Linex"), *Lactobacillus acidophilus* strain n.v. Ep 317/402 (drug "Narine"), *Lactobacillus galinarum* VKPM-10134), as well as the objects of research themselves in relation to each other (*Loigolactobacillus coryniformis*, *Lactobacillus johnsonii*, *Lactobacillus reuteri*) – were not visualized.

The results of the study of the adhesive ability of the studied lactobacilli to enterocytes of the intestine of the chicken embryo are shown in Table 6.

Table 6. Results of adhesive activity of research objects to enterocytes.

Object of study	**Average adhesion index (AAI) to enterocytes, units**
Loigolactobacillus coryniformis	19.1 ± 0.52
Lactobacillus johnsonii	18.3 ± 0.46
Lactobacillus reuteri	8.8 ± 0.37

It was established that the studied lactobacilli strains are capable of exhibiting a high adhesion index in relation to the intestinal cells of birds, since the AAI in the context of the objects of study was 8.8-19.1 units. The highest adhesive activity was demonstrated by the strain – *Loigolactobacillus coryniformis* (the AAI was 19.1 units), then *Lactobacillus johnsonii* (the AAI was 18.3 units) and the lowest value was found in *Lactobacillus reuteri* (the AAI was 8.8 units).

4 Conclusion

Thus, the results of the conducted studies demonstrated that lactic acid cultures isolated from the caecum of pheasants, in particular *Lactobacillus johnsonii* and *Limosilactobacillus coryniformis*, have all the characteristics characteristic of lactobacilli, are resistant to aggressive environmental conditions and exhibit moderate tolerance to antimicrobial drugs, exhibit high probiotic potential, since they do not have an inhibitory effect on the normal intestinal flora, exhibit antagonistic activity against field opportunistic microflora, have high anti-adhesive and adhesive properties, in connection with which, these lactobacilli can serve as a basis for the development of microbial biopreparations with probiotic action.

Acknowledgement

The study was supported by the Russian Science Foundation grant No. 25-26-00061, https://rscf.ru/project/25-26-00061/.

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