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Evaluation and selection of highly antagonistic *Bacillus strains* for the development of probiotic formulations

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The relevance of this article is driven by the global challenge of identifying and developing alternative biological preparations to antibiotics – formulations that are affordable, effective, and natural – for maintaining a stable epizootic situation in poultry farming and reducing epidemic risks to humans within the framework of the "One Health" strategy. Accordingly, the materials presented in this study aim to identify, through in vitro screening, *Bacillus* strains isolated from quails that exhibited very high levels of antagonistic activity against both Gram-negative and Gram-positive indicator test cultures: *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 29630, and *Staphylococcus aureus* ATCC 6538. To address this objective, two complementary research methods were employed: the indirect perpendicular streak method and the quantitative delayed antagonism method, applied to experimental strains of *Bacillus subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* against the test cultures. Based on the results, 9 strains (7.3% of the 123 *Bacillus* isolates studied) demonstrated very high antagonistic activity simultaneously against all indicator test cultures. These included 4 strains (3.3%) of *B. subtilis*, 4 strains (3.3%) of *B. licheniformis*, and 1 strain (0.8%) of *B. amyloliquefaciens*. These 9 *Bacillus* strains were recognized as promising and selected for further research aimed at developing a synbiotic formulation for quails. The findings of this study have practical value, as the development and application of *Bacillus*-based synbiotics – administered as feed additives or via environmental spraying – represent a promising strategy for maintaining a stable epizootic status in poultry farms, improving the sanitary conditions of poultry housing, enhancing intestinal barrier function and immune responsiveness in birds, and enabling a complete transition away from antibiotic use in poultry production.

Keywords: isolates of non-pathogenic bacilli; *Bacillus subtilis*; *Bacillus licheniformis*; *Bacillus amyloliquefaciens*.

Introduction

Increasing the production of high-quality livestock products is one of the key objectives of the agricultural sector in the context of Ukraine's integration into the international community, as confirmed by data analysis in this field (Chemerovska, 2022; Boniuk, 2024). Poultry farming represents a vital component of the agricultural complex and currently plays an important role in ensuring the country's food security (Shkromada, 2022; Savchenko, 2023). According to industry development analysis, modern poultry farming is characterized by cost optimization through the realization of birds' biological potential and the implementation of resource-saving technologies for producing high-quality raw materials and products. However, the use of antibiotic preparations in poultry farming is increasingly restricted due to biological risks associated with the spread of multi-antibiotic-resistant bacterial pathogens. These microorganisms exhibit resistance to epidemiologically significant antibiotics of the latest generations and acquired resistance to beta-lactams and carbapenems, which can be horizontally transferred to other microbial species. The intestinal normobiota of animals, poultry, and humans is particularly susceptible to these processes (Chemerovska, 2024).

It is now well established that poultry health directly depends on the balance between normal, conditionally pathogenic, and potentially pathogenic intestinal microbiota. Disruption of this balance leads to functional disorders, reduced productivity, lower quality of raw materials and products, and increased susceptibility to infectious diseases due to weakened immunobiological responsiveness (Bodinga, 2020; Ruvalcaba-Gómez, 2022). To control pathogenic microorganisms and support poultry growth, alternative natural biological preparations are needed (Arif, 2021; Naeem, 2025). According to research findings (Iegorov, 2022; Hindle, 2025; Zhang, 2025), scientists emphasize that the gut microbiota and the immune system of the digestive organs form a powerful peripheral complex of immune defense in poultry. Based on the data, the gut microbiota plays a crucial role in regulating inflammatory, metabolic, immune, and neurobiological processes in the bodies of animals, poultry, and humans (Vinderola, 2022; Odriozola, 2024). Current trends in poultry farming indicate a growing interest in biologically active preparations based on *Bacillus* species. Researchers have shown that, unlike commonly used lactobacillus-based probiotics, non-pathogenic spore-forming *Bacillus* spp. possess high resistance to adverse environmental conditions and to the acidic internal environment of the gastrointestinal tract in poultry. They are also well-suited to technological production processes and retain viability when incorporated into feed as probiotic additives (Ramlucken, 2020; Ogbuewu, 2022; Naeem, 2025).

Therefore, a priority direction involves the development and application of synbiotic preparations based on natural substances with high antagonistic activity against infectious disease pathogens (Chechet, 2022; Guarner, 2024). Such preparations include innovative synbiotics derived from representatives of the genus *Bacillus*,

which possess both antagonistic and immunomodulatory properties. The development and implementation of biological products based on *Bacillus* bacteria offer advantages such as reduced production costs, alignment with modern technological processes, practical feasibility, and suitability for inclusion in poultry feed as additives (Costa, 2022).

Scientific data confirm that the use of multi-strain and dual-strain biological preparations based on *Bacillus* species – particularly *B. subtilis* and *B. licheniformis* – in poultry farming improves the morphological condition of the intestinal tract, positively influences growth, and significantly enhances the efficiency of raw material and product output in the industry (Anguara, 2022; Biswas, 2023).

Therefore, the aim of our study was to investigate cultures of *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* isolated from quails, and to select strains with a very high level of antagonism against Gram-positive and Gram-negative indicator test bacteria: *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 29630, and *Staphylococcus aureus* ATCC 6538.

To achieve this goal, microbiological studies were conducted on *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* strains isolated from quails to determine their level of antagonism following interaction with the indicator test bacteria (*P. aeruginosa* ATCC 15442, *E. coli* ATCC 25922, *S. typhimurium* ATCC 29630, and *S. aureus* ATCC 6538), and based on the obtained data, to select promising strains for the development of a probiotic preparation for quails.

Materials and methods

The study was conducted from November 1, 2023, to October 1, 2024, at the Bacteriological Research Department (BRD) of the State Research Institute for Laboratory Diagnostics and Veterinary-Sanitary Expertise (SRILDVSE), Kyiv; the Institute of Animal Biology of the National Academy of Agrarian Sciences of Ukraine (IAB NAAS), Lviv; and Bila Tserkva National Agrarian University (BNAU), Bila Tserkva.

The slaughter of quails for intestinal sampling was performed by qualified slaughterhouse operators in accordance with stunning and bleeding requirements as outlined in Council Regulation (EC) No 1099/2009 on the protection of animals at the time of killing. The procedure also referenced Directive 93/119/EC and Directive 98/58/EC concerning the protection of animals kept for farming purposes, the resolution of the First Scientific Congress on Bioethics (Reznikov, 2003), and adhered to bioethical standards consistent with the Law of Ukraine "On the Protection of Animals from Cruelty" (dated March 23, 2006, No. 3447-IV), including recent amendments (2023–2024). Sample collection was carried out by specialists trained in the procedures for obtaining pathological and biological material from poultry, including packaging, labeling, and delivery timelines to the laboratory.

The study employed culture media and diagnostic tests manufactured by HiMedia (India). All media and tests were pre-evaluated for performance, selectivity, and specificity, yielding satisfactory results. Growth properties were verified using test cultures from the ATCC collection.

A total of 123 *Bacillus* isolates were obtained from microbiological studies of 167 samples of ligated large intestinal sections from quails. Based on species identification, the isolates were classified into strains as follows: 83 (67.5% of the total) cultures of *B. subtilis*, 33 (26.8%) cultures of *B. licheniformis*, and 7 (5.7%) cultures of *B. amyloliquefaciens* (Table 1).

Test cultures of *P. aeruginosa* ATCC 15442, *E. coli* ATCC 25922, *S. typhimurium* ATCC 29630, and *S. aureus* ATCC 6538 were cultivated in test tubes containing meat-peptone broth (MPB) at 37 ± 1 °C for 24 hours. Bacterial suspensions were prepared by washing the agar surface with sterile physiological saline and thorough mixing. The resulting suspensions were adjusted to a concentration of 0.5 McFarland standard and used in the main experiment according to the methods described below.

Two *in vitro* laboratory methods were used to assess the antagonistic activity of the tested strains (*B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*): the perpendicular streak method and the delayed antagonism method, which confirmed the results based on quantitative indicators (Trufanov, 2008; Lutgendorff, 2009).

Table 1
Types of experimental cultures of bacteria of the genus *Bacillus* and abbreviations of their names

Species – full name / abbreviated name	Species abbreviation of strains	Examples of strain designations of different species	Origin of the material
Bacillus subtilis	Bsub	Bsub003, Bsub020, Bsub071, Bsub162	Quail intestines
Bacillus licheniformis	BLf	BLf022, BLf074, BLf112, BLf129	
Bacillus amyloliquefaciens	Bamyl	Bamyl064, Bamyl088, Bamyl143	

Note: all investigations were performed using standard methods (www.nicd.ac.za/wp-content/uploads/2019/10/Manual_v_7.0_EUCAST_Disk_Test_2019.pdf?utm_source=chatgpt.com).

Microbiological studies to assess the antagonistic activity of *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* were initially conducted using the perpendicular streak method to confirm the presence of antagonistic properties. In triplicate, a drop of each freshly prepared bacterial suspension of the respective *Bacillus* strains was inoculated at the center of meat-peptone agar (MPA) plates using a bacteriological loop (3 mm diameter). Subsequently, daily test cultures of *P. aeruginosa* ATCC 15442, *E. coli* ATCC 25922, *S. typhimurium* ATCC 29630, and *S. aureus* ATCC 6538 were streaked from the edge of the plate toward the center – perpendicularly to the inoculated *Bacillus* drop. Streaks were spaced at defined intervals. Control plates were prepared in parallel by inoculating the indicator test cultures without the central *Bacillus* strains. All experimental and control plates were incubated in a thermostat at 37 ± 1 °C for 24 hours.

Studies on the determination of antagonistic activity levels using the delayed antagonism method were conducted in five replicates on MPA (meat-peptone agar) plates. Prior to the main experiment, each of the test strains – *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* – was titrated, and the concentration was standardized at 1.0×10^2 CFU/cm³ for each strain. This concentration of bacterial suspensions ensured the growth of isolated colonies after inoculation. The determination of the required concentrations of bacterial suspensions was carried out using the standard serial dilution method.

Bacterial suspensions of *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* at a concentration of 1×10^2 CFU/cm³ were inoculated onto meat-peptone agar (MPA) plates and incubated at 37 ± 1 °C for 24 hours. After incubation, plates with isolated colonies of the respective *Bacillus* strains were treated with 2.0–3.0 cm³ of chloroform, held for 5 minutes, and the residual chloroform was decanted. The agar surface was then dried for 30 minutes under aseptic conditions. Fresh bacterial suspensions of test cultures (*P. aeruginosa* ATCC 15442, *E. coli* ATCC 25922, *S. typhimurium* ATCC 29630, *S. aureus* ATCC 6538) were prepared at a concentration of 0.5 McFarland standard. A volume of 0.1 cm³ of each suspension was added to 5.0 cm³ of molten semi-solid agar (SSA) cooled to 45 °C. After thorough mixing, the SSA was evenly poured over the surface of the plates containing the *Bacillus* colonies. Once solidified, the plates were re-incubated at 37 ± 1 °C for 24 hours. Each of the test strains – *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* – was examined in five replicates.

The determination of antagonistic properties using the perpendicular streak method was performed visually, based on the length of the growth streak of the test culture from the edge of the Petri dish toward its center, in the direction of the growth of the respective strains – *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens*. The assessment also considered the size of the growth-free zones along this section of the surface.

The level of antagonistic activity of the experimental strains, assessed using the remote antagonism method, was determined by measuring the diameters of growth inhibition zones or confirming their absence. The antagonistic activity of the tested strains was conditionally classified as follows: low activity – inhibition zone diameter

ranging from 7 to 14 mm; moderate activity – from 14 to 26 mm; high activity – from 27 to 36 mm; very high activity – greater than 36 mm. This classification was applied under conditions of intensive growth of both the indicator test cultures and the corresponding experimental strains of *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* in their respective growth controls (Kenakin, 2008).

Following the evaluation of antagonistic activity levels, the obtained data were analyzed using Statistica 8.0 software (StatSoft Inc., USA). Results in the tables are presented as x ± SE (mean value ± standard error). Differences in the antagonistic activity levels of the experimental strains *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* against the indicator test cultures *P. aeruginosa* ATCC 15442, *E. coli* ATCC 25922, *S. typhimurium* ATCC 29630, and *S. aureus* ATCC 6538 were assessed using ANOVA, with differences considered statistically significant at $P < 0.05$.

Results

The results of testing *Bacillus* isolates using the perpendicular streak method confirmed the presence of antagonistic properties in certain strains against indicator Gram-negative and Gram-positive test cultures: *E. coli* ATCC 25922, *S. typhimurium* ATCC 29630, *S. aureus* ATCC 6538, and *P. aeruginosa* ATCC 15442.

Specifically, based on the size of growth inhibition zones for *E. coli* ATCC 25922, antagonistic properties were confirmed in 17 out of 83 *B. subtilis* isolates (20.5%), in 17 out of 33 *B. licheniformis* strains (50.5%), and in 4 out of 7 *B. amyloliquefaciens* strains (57.1%). When using *P. aeruginosa* ATCC 15442 as the test culture, antagonism was observed in 16 *B. subtilis* strains (19.2%), 17 *B. licheniformis* strains (51.5%), and 3 *B. amyloliquefaciens* strains (42.9%). In tests against *S. typhimurium* ATCC 29630, antagonistic properties were found in 16 *B. subtilis* strains (19.3%), 20 *B. licheniformis* strains (66.6%), and 4 *B. amyloliquefaciens* strains (57.1%). Regarding *S. aureus* ATCC 6538, such properties were observed in 15 *B. subtilis* strains (18.1%), 3 *B. licheniformis* strains (42.9%), and 4 *B. amyloliquefaciens* strains (57.1%).

Thus, preliminary indirect testing using the perpendicular streak method to determine the presence of antagonistic properties in *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* strains after interaction with indicator test cultures – *E. coli* ATCC 25922, *P. aeruginosa* ATCC 15442, *S. typhimurium* ATCC 29630, and *S. aureus* ATCC 6538 – confirmed the presence of antagonism in certain *Bacillus* strains. Given these findings, there was considerable interest in determining the level of antagonistic activity using the quantitative delayed antagonism method.

The results of testing the antagonistic activity levels of *B. subtilis* strains against indicator Gram-positive and Gram-negative test cultures using the delayed antagonism method are presented in Table 2.

Table 2

Results of the delayed antagonism assay (diameter of growth inhibition zone, mm) showing the level of antagonistic activity of *B. subtilis* strains against Gram-positive and Gram-negative indicator test cultures (%, x ± SD, n = 83)

Strain name	*E coli* ATCC 25922	*Ps. aeruginosa* ATCC 15442	*S. typhimurium* ATCC 29630	*S. aureus* ATCC 6538
Bsub3	22.3 ± 0.3	17.6 ± 0.3	37.0 ± 0.7*	15.7 ± 0.7
Bsub6	19.3 ± 0.3	22.7 ± 0.7	22.3 ± 0.3	18.3 ± 0.3
Bsub9	21.3 ± 0.3	20.0 ± 0.7	19.3 ± 0.3	22.3 ± 0.3
Bsub12	32.7 ± 0.3	16.7 ± 0.3	27.3 ± 0.3	14.0 ± 0.7
Bsub20	37.3 ± 0.3*	28.0 ± 0.7	37.3 ± 0.3*	34.0 ± 0.3
Bsub23	23.0 ± 0.7	15.0 ± 0.7	31.0 ± 0.7	16.0 ± 0.7
Bsub26	21.3 ± 1.0	16.7 ± 0.3	22.3 ± 0.3	21.0 ± 0.3
Bsub27	19.0 ± 0.7	11.3 ± 0.7	19.7 ± 0.3	26.3 ± 0.3
Bsub37	20.3 ± 0.3	19.3 ± 0.3	20.3 ± 0.3	29.7 ± 0.3
Bsub42	11.3 ± 0,7	6.7 ± 0.3	25.7 ± 0.3	12.7 ± 0.3
Bsub44	26.7 ± 1.0	26.3 ± 1.0	17.3 ± 0.3	8.3 ± 0.7
Bsub49	36.6 ± 0.3*	38.7 ± 0.3*	39.0 ± 0.3*	39.7 ± 0.3*
Bsub51	39.0 ± 0.7*	39.7 ± 0.3*	40.3 ± 0.3*	40.0 ± 0.7*
Bsu54,	13.7 ± 0.3	17.7 ± 0.3	20.3 ± 0.3	24.7 ± 0.3
Bsub55	27.3 ± 0.3	32.3 ± 0.3	28.7 ± 0.3	13.3 ± 0.3
Bsub57	10.7 ± 1.0	15.3 ± 0.3	9.7 ± 0.3	20.3 ± 0.3
Bsub58	24.0 ± 0.7	20.3 ± 0.3	18.0 ± 0.3	20,3 ± 0.7
Bsub59	23.7 ± 0.3	25.3 ± 0.3	28.0 ± 0.7	11.7 ± 1.0
Bsub60	10.3 ± 0.3	10.3 ± 0.3	15.7 ± 0.3	19.3 ± 0.3
Bsub62	10.7 ± 0.3	10.7 ± 0.3	12.7 ± 0.3	13.7 ± 0.7
Bsub63	10.3 ± 0.3	10.0 ± 0.3	12.3 ± 0.3	21.3 ± 0.3
Bsub70	27.3 ± 0.3	30.3 ± 0.3	23.3 ± 0.3	29.7 ± 0.3
Bsub71	39.7 ± 0.3*	41.7 ± 0.3*	37.3 ± 0.3*	40.0 ± 0.3*
Bsub78	40.7 ± 0.3*	36.7 ± 0.3*	39.0 ± 0.3*	39.3 ± 0.3*
Bsub87	15.7 ± 0.3	27.3 ± 0.3	30.7 ± 0.3	24.7 ± 0.3
Bsub90	11.0 ± 0.7	12.3 ± 0.3	11.0 ± 0.7	15.7 ± 0.3
Bsub92	10.7 ± 1.0	14.7 ± 0.3	18.7 ± 0.3	10.3 ± 0.3
Bsub95	11.0 ± 0.7	11.3 ± 0.7	25.7 ± 0.3	12.7 ± 0.3
Bsub97	32.3 ± 0.3	29.7 ± 0.7	16.7 ± 0.3	20.3 ± 0.3
Bsub103	31.7 ± 0.3	23.3 ± 0.3	20.7 ± 0.3	19.7 ± 0.3
Bsub116	36.7 ± 0.3*	40.3 ± 0.3*	28.7 ± 0.3	40.7 ± 0.3*
Bsub123	22.3 ± 0.3	28.7 ± 0.3	30.7 ± 0.3	34.7 ± 0.3
Bsub126	11.0 ± 0.7	11.3 ± 0.3	27.3 ± 0.3	30.7 ± 0.3
Bsub127	11.0 ± 0.7	23.3 ± 0.3	10.7 ± 0.3	12.7 ± 0.3
Bsub128	10.7 ± 0.7	15.7 ± 0.3	18.7 ± 0.3	22.7 ± 0.3
Bsub131	10.0 ± 0.3	19.3 ± 0.3	8.7 ± 0.3	11.7 ± 0.3
Bsub138	36.7 ± 0.3*	40.3 ± 0.3*	35.7 ± 0.3	30.7 ± 0.3
Bsub139	14.3 ± 0.3	20.7 ± 0.3	24.3 ± 0.3	20.3 ± 0.3
Bsub142	35.3 ± 0.3	23.7 ± 0.3	27,7 ± 0.3	22.3 ± 0.3
Bsub147	10.7 ± 0.7	10.7 ± 0.3	22.7 ± 0.3	16.3 ± 0.3
Bsub149	11.0 ± 0.7	7.3 ± 0.3	20.3 ± 0.3	10.3 ± 0.3
Bsub156	41.3 ± 0.7*	36.3 ± 0.3*	35.7 ± 0.3	25.3 ± 0.3
Bsub157	20.7 ± 0.3	18.3 ± 0.3	23.3 ± 0.7	18.7 ± 0.3
Bsub160	10.3 ± 0.3	27.3 ± 0,3	29.3 ± 0.3	30.3 ± 0.3
Bsub162	40.3 ± 0.3*	38.7 ± 0.3*	39.0 ± 0.3*	25.7 ± 0.3
Bsub165	11.3 ± 0.3	9.7 ± 0.3	10.7 ± 0.3	12.7 ± 0.3
Bsub166	10.7 ± 0.7	13.3 ± 0.3	14.3 ± 0.3	22.3 ± 0.3
Bsub14	22.3 ± 0.3	21.7 ± 0.3	29.7 ± 0.3	15.7 ± 0.3
Bsub17	22.3 ± 0.3	21.7 ± 0.3	29.7 ± 0.3	15.7 ± 0.3
Bsub32	35.3 ± 0.3	15.7 ± 0.3	17.3 ± 0.3	28.7 ± 0.3
Bsub34	9.7 ± 0.3	12.7 ± 0.3	17.3 ± 0.3	27.3 ± 0.3
Bsub35	10.0 ± 0.7	16.3 ± 0.3	21.3 ± 0.3	10.7 ± 0.3
Bsub46	25.3 ± 0.3	31.7 ± 0.3	30.7 ± 0.3	24.3 ± 0.3
Bsub50	27.7 ± 0.3	17.7 ± 0.3	21.3 ± 0.3	34.7 ± 0.3
Bsub82	11.0 ± 0.7	9.3 ± 0.3	14.3 ± 0.3	8.3 ± 0.3
Bsub84	39.3 ± 0.3*	32.7 ± 0.3	29.3 ± 0.3	24.7 ± 0.3
Bsub86	30.7 ± 0.3	24.3 ± 0.3	21.3 ± 0.3	20.3 ± 0.3
Bsub104	10.3 ± 0.3	7.3 ± 0.3	14.7 ± 0.3	17.3 ± 0.3
Bsub106	11.7 ± 0.3	20.3 ± 0.3	23.7 ± 0.3	19.7 ± 0.3
Bsub111	10.7 ± 0.3	9.3 ± 0,3	10.3 ± 0.3	15.7 ± 0.3
Bsub112	11.7 ± 1.0	20.3 ± 0.3	7.7 ± 0.3	8.7 ± 0.3
Bsub117	11.3 ± 0.3	21.7 ± 0.3	18.7 ± 0.3	10.7 ± 0.3
Bsub119	27.3 ± 0.3	34.7 ± 0.3	30.7 ± 0.3	32.7 ± 0.3
Bsub124	20.3 ± 0.3	32.3 ± 0.3	28.7 ± 0.3	25.3 ± 0.3
Bsub134	37.7 ± 0.3*	32.3 ± 0.3	30.3 ± 0.3	32.7 ± 0.3
Bsub136	21.7 ± 0.3	33.3 ± 0,3	30.3 ± 0.3	20.3 ± 0.3
Bsub145	20.7 ± 0.3	31.7 ± 0.3	28.7 ± 0.3	19.7 ± 0.3
Bsub150	30.7 ± 0,3*	27.7 ± 0.3	22.3 ± 0.3	30.3 ± 0.3
Bsub153	10.0 ± 1.0	8.3 ± 0.3	10.7 ± 0.3	12.3 ± 0.3
Bsub158	41.7 ± 0.3*	27.7 ± 0.3	33.3 ± 0.3	30.7 ± 0.3
Bsub163	10.3 ± 0.3	12.3 ± 0.3	20.3 ± 0.3	17.7 ± 0.3
Bsub15	36.3 ± 0.3*	25.3 ± 0.3	30.7 ± 0.3	30.3 ± 0.3
Bsub38	25.3 ± 0.3	28.3 ± 0.3	32.3 ± 0.3	30.7 ± 0.3
Bsub39	30.7 ± 0.3	29.7 ± 0.6	24.7 ± 0.3	20.7 ± 0.3
Bsub64	11.0 ± 0.7	12.3 ± 0.3	15.7 ± 0.3	19.7 ± 0.3
Bsub65	10.7 ± 1.3	28.7 ± 0.3	12.7 ± 0.3	16.7 ± 0.3
Bsub99	28.7 ± 0.3	33.3 ± 0.3	21.3 ± 0.3	33.7 ± 0.3
Bsub101	37.3 ± 0.3*	34.7 ± 0.3	30.7 ± 0.3	33.3 ± 0.3
Bsub110	20.7 ± 0.3	19.7 ± 0.3	23.7 ± 0.3	24.7 ± 0.3
Bsub113	10.0 ± 0.7	20.3 ± 0.3	23.3 ± 0.3	16.3 ± 0.3
Bsub115	9.7 ± 0.7	9.7 ± 0.3	14.7 ± 0.3	19.3 ± 0.3
Bsub144	25.3 ± 0.3	32.7 ± 0.3	19.7 ± 0.3	24.7 ± 0.3
Bsub146	10.7 ± 0.3	12.7 ± 0.3	14.3 ± 0.3	17.7 ± 0.3

Note: * – $P < 0.05$, compared to the established growth inhibition range values in the respective test cultures.

According to the results of *in vitro* testing using the delayed antagonism method, following interaction with the indicator test culture *E. coli* ATCC 25922, a very high level of antagonism was identified in 15 out of 83 *B. subtilis* strains (18.1%): Bsub20, Bsub49, Bsub51, Bsub71, Bsub78, Bsub116, Bsub138, Bsub156, Bsub162, Bsub84, Bsub134, Bsub150, Bsub158, Bsub15, Bsub101. This was confirmed

by growth inhibition zone diameters ranging from 36.6 ± 0.3 to 41.7 ± 0.3 mm.

Data obtained from the assessment of antagonistic properties following interaction with the test culture *P. aeruginosa* ATCC 15442 revealed a very high level of antagonism in 8 *B. subtilis* strains (9.6%): Bsub49, Bsub51, Bsub71, Bsub78, Bsub116, Bsub138, Bsub156, Bsub162, with inhibition zone diameters ranging from 38.7 ± 0.3 to 41.4 ± 0.3 mm.

The results of studies on the antagonistic effect against the test culture *S. typhimurium* ATCC 29630 revealed 7 *B. subtilis* strains (5.7%): Bsub3, Bsub20, Bsub49, Bsub51, Bsub71, Bsub78, Bsub162 with a very high level of antagonism. These conclusions were based on growth inhibition zone diameters ranging from 37.0 ± 0.7 to 40.3 ± 0.3 mm following interaction with *B. subtilis* strains.

According to the results of delayed antagonism testing, very high antagonistic properties against the Gram-positive test culture *S. aureus* ATCC 6538 were identified in 5 *B. subtilis* strains (71.4%): Bsub49, Bsub51, Bsub71, Bsub78, Bsub116, based on growth inhibition zone diameters ranging from 25.7 ± 0.3 to 40.0 ± 0.7 mm.

Based on the results of antagonism assays, among 33 experimental *B. licheniformis* cultures, 7 strains (21.2%) – BLf22, BLf75, BLf112, BLf131, BLf66, BLf74, and BLf129 – demonstrated a very high level of antagonism against the test culture *E. coli* ATCC 25922. This was confirmed by the diameters of growth inhibition zones ranging from 37.0 ± 0.3 to 43.3 ± 0.3 mm. Following interaction with *P. aeruginosa* ATCC 15442, 9 *B. licheniformis* strains – BLf22, BLf44, BLf75, BLf105, BLf106, BLf112, BLf125, BLf74, and BLf129 – also exhibited a very high level of antagonism against the test culture, with inhibition zone diameters ranging from 36.7 ± 0.3 to 42.3 ± 0.3 mm (Table 3).

Table 3

Results of remote antagonism assay (diameter of growth inhibition zone, mm) for the level of antagonistic activity of experimental *B. licheniformis* strains against gram-positive and gram-negative indicator test cultures (%, x ± SD, n = 33)

Strain name	*E coli* ATCC 25922	*Ps. aeruginosa* ATCC 15442	*S. typhimurium* ATCC 29630	*S. aureus* ATCC 6538
BLf-8	19.6 ± 0.3	25.3 ± 0.3	23.3 ± 0.3	17.0 ± 1.0
BLf-22	37.3 ± 0.3*	40.0 ± 0.3*	39.3 ± 0.3*	41.3 ± 0.7*
BLf-44	28.3 ± 0.3	39.0 ± 0.3*	42.3 ± 0.3*	36.3 ± 0.3*
BLf-47	25.3 ± 0.3	30.0 ± 0.7	31.0 ± 0.3	25.3 ± 0.3
BLf-56	7.0 ± 0.3	14.7 ± 0.3	8.0 ± 0.3	10.3 ± 0.3
BLf-57	15.7 ± 0.3	20.3 ± 0.3	23.3 ± 0.3	7.7 ± 0.3
BLf-63	7.7 ± 0.3	11.3 ± 0.3	19.7 ± 0.3	9.3 ± 0.3
BLf-73	10.7 ± 0.3	12.3 ± 0.3	21.3 ± 0.3	31.3 ± 0.3
BLf-75	40.7 ± 0.3*	39.7 ± 0.3*	25.3 ± 0.3	34.7 ± 0.3
BLf-93	15.7 ± 0.7	20.0 ± 0.3	23.7 ± 0.3	19.3 ± 0.3
BLf-105	35.3 ± 0.3	41.0 ± 0.7*	39.7 ± 0.3*	23.7 ± 0.3
BLf-106	23.7 ± 0.3	42.3 ± 0.3*	35.3 ± 0.3	17.3 ± 0.3
BLf-109	14.7 ± 0.3	20.7 ± 0.3	31.7 ± 0.3	19.7 ± 0.3
BLf-112	40.3 ± 0.3*	40.0 ± 0.7*	38.7 ± 0.3*	41.3 ± 0.7*
BLf-117	7.7 ± 0.3	19.3 ± 0.3	16.7 ± 0.3	11.3 ± 0.3
BLf-123	25.7 ± 0.3	19.0 ± 0.3	17.7 ± 0.3	21.0 ± 0.3
BLf-125	34.3 ± 0.7	41.7 ± 0.3*	38.3 ± 0.3*	32.7 ± 0.3
BLf-131	39.3 ± 0.3*	32.7 ± 0.3	28.7 ± 0.3	30.7 ± 0.3
BLf-151	7.3 ± 0.3	30.0 ± 0.7	32.0 ± 0.3	23.7 ± 0.3
BLf-152	20.7 ± 0.3	17.7 ± 0.3	33.0 ± 0.3	21.3 ± 0.3
BLf-153	17.3 ± 0.3	30.3 ± 0.3	21.3 ± 0.3	23.3 ± 0.3
BLf-165	23.7 ± 0.3	20.0 ± 0.7	19.0 ± 0.7	34.7 ± 0.3
BLf-21	19.7 ± 0.3	30.3 ± 0.3	33.0 ± 0.3	27.7 ± 0.3
BLf-23	23.0 ± 0.3	20.7 ± 0.3	23.3 ± 0.3	16.7 ± 0.3
BLf-36	25.7 ± 0.7	20.3 ± 0.3	37.3 ± 0.3*	17.7 ± 03
BLf-43	30.3 ± 0.3	31.3 ± 0.3	29.7 ± 0.3	33.7 ± 0.3
BLf-66	39.7 ± 0.3*	17.7 ± 0.3	27.7 ± 0.3	29.3 ± 0.3
BLf-74	37.0 ± 0.3*	40.7 ± 0.3*	39.7 ± 0.3*	41.7 ± 0.3*
BLf-118	31.0 ± 0.3	32.7 ± 0.3	28.0 ± 0.7	31.7 ± 0.3
BLf-121	39.7 ± 0.3*	27.7 ± 0.3	38.7 ± 0.3*	24.0 ± 0.7
BLf-129	43.3 ± 0.3*	36.7 ± 0.3*	39.3 ± 0.3*	43.3 ± 0.3*
BLf-133	28.7 ± 0.3	15.7 ± 0.3	29.3 ± 0.3	21.7 ± 0.3
BLf-164	18.7 ± 0.3	34.3 ± 0.3	17.3 ± 0.3	20.7 ± 0.3

Note: see Table 2.

In the study of *B. licheniformis* strains for their antagonistic activity against the indicator test culture *S. typhimurium* ATCC 29630, a very high level of antagonistic activity was observed in 9 strains (27.3%) – BLf22, BLf44, BLf105, BLf112, BLf125, BLf36, BLf74, BLf121, and BLf129. This was confirmed by growth inhibition zone diameters ranging from 37.3 ± 0.3 to 39.7 ± 0.3 mm.

Among *B. licheniformis* strains, 5 strains (15.2%) – BLf22, BLf44, BLf112, BLf74, and BLf129 – demonstrated a very high level of antagonism against the indicator test culture *S. aureus* ATCC 6538. This was supported by inhibition zone diameters ranging from 36.3 ± 0.3 to 43.3 ± 0.3 mm.

According to the results of the *in vitro* remote antagonism assay involving the interaction between the indicator test culture *E. coli* ATCC 25922 and experimental *B. amyloliquefaciens* strains, a very high level of antagonistic activity was observed in 2 cases (28.6%) – strains Bamyl-88 and Bamyl-143. This was confirmed by inhibition zone diameters ranging from 37.3 ± 0.3 to 39.3 ± 0.3 mm (Table 4).

Table 4

Results of delayed antagonism testing (diameter of growth inhibition zone, mm) of *B. amyloliquefaciens* strains for antagonistic activity against Gram-positive and Gram-negative indicator test cultures (%, x ± SD, n = 7)

Strain name	*E. coli* ATCC 25922	*Ps. aeruginosa* ATCC 15442	*S. typhimurium* ATCC 29630	*S. aureus* ATCC 6538
Bamyl-64	11.0 ± 0.7	15.3 ± 0.3	21.7 ± 0.3	19.3 ± 0.3
Bamyl-75	17.3 ± 0.3	10.3 ± 0.3	18.3 ± 0.3	25.3 ± 0.3
Bamyl-88	37.3 ± 0.3*	40.7 ± 0.3*	42.7 ± 0.3*	41.0 ± 1,0*
Bamyl-117	21.7 ± 0.3	16.0 ± 0.7	23.3 ± 0.3	21.7 ± 0.3
Bamyl-143	39.3 ± 0.3*	27.3 ± 0.3	43.3 ± 0.3*	37.0 ± 0.7*
Bamyl-156	34.7 ± 0.3	18.7 ± 0.7	28.0 ± 0.7	27.7 ± 0.3
Bamyl-167	36.7 ± 0.3	27.7 ± 0.3	28.0 ± 0.7	30.7 ± 0.3

Note: see Table 2.

Among *B. amyloliquefaciens* cultures, a very high level of antagonism against the test culture *P. aeruginosa* ATCC 15442 was identified in 1 strain (14.3%): Bamyl-88. The diameter of the growth inhibition zone for this test culture was 40.7 ± 0.3 mm, falling within the range defined for a very high level of antagonism among *Bacillus* species.

In studies involving the test culture *S. typhimurium* ATCC 29630 following interaction with *B. amyloliquefaciens* strains, a very high level of antagonism was observed in 2 strains (28.6%): Bamyl-88 and Bamyl-143, confirmed by growth inhibition zone diameters ranging from 42.7 ± 0.3 to 43.3 ± 0.3 mm.

Based on the data obtained from antagonism testing against the Gram-positive test culture *S. aureus* ATCC 6538, a very high level of antagonism was found in 2 *B. amyloliquefaciens* strains (28.6%): Bamyl-88 and Bamyl-143, with growth inhibition zone diameters ranging from 37.0 ± 0.7 to 41.0 ± 1.0 mm. These values indicated a very high level of antagonism against staphylococci.

Thus, a general analysis of the *in vitro* results allows us to conclude that among the tested *Bacillus strains*, *B. licheniformis* strains were the most effective against the Gram-negative test culture *E. coli* ATCC 25922. This was confirmed by growth inhibition zone diameters exceeding 40.0 mm in most cases. The *in vitro* results of *Bacillus* species following interaction with the Gram-negative test culture *P. aeruginosa* ATCC 15442 demonstrate effective antagonistic activity of *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens*, confirmed by growth inhibition zone diameters with only minor variation among them. It is evident that among all tested *Bacillus* strains, those of *B. amyloliquefaciens* were the most effective in terms of antagonistic properties against the test culture *S. typhimurium* ATCC 29630, as indicated by the highest growth inhibition zone diameters. Based on the obtained growth inhibition zone diameters, the greatest antagonistic activity against the Gram-positive test culture *S. aureus* ATCC 6538 was observed in *B. licheniformis* and *B. amyloliquefaciens* strains, as confirmed by the inhibition zone diameters for the respective test bacteria.

Therefore, for the development of an effective synbiotic formulation, the most promising candidates are *Bacillus* strains that simultaneously exhibit very high levels of antagonistic activity against all applied Gram-negative and Gram-positive indicator test cultures:

E. coli ATCC 25922, *P. aeruginosa* ATCC 15442, *S. typhimurium* ATCC 29630, and *S. aureus* ATCC 6538. Based on the results of the conducted studies, a total of 9 such strains were identified (7.3% of all tested). Specifically: 4 *B. subtilis* strains (3.3%) – Bsub49, Bsub51, Bsub71, Bsub78; 4 *B. licheniformis* strains (3.3%) – BLf22, BLf112, BLf74, BLf129; and 1 *B. amyloliquefaciens* strain (0.8%) – Bamyl-88, which were selected as promising candidates for further research.

Discussion

Members of the genus *Bacillus* are prolific producers of biologically active compounds, ranging from extracellular enzymes that enhance nutrient digestibility and absorption, to antagonistic substances that suppress pathogenic bacteria in the gastrointestinal environment. *Bacillus* bacteria are currently being considered as alternatives to antibiotic preparations. According to published data, experiments conducted on two strains of *B. subtilis* demonstrated the superior effectiveness of multi-strain applications of *Bacillus* species. The combined use of two *B. subtilis* strains showed significantly higher efficacy in terms of immune response, gut health, and microbiota balance compared to control groups and single-strain applications. Therefore, the authors emphasize the potential of combined *B. subtilis* strains as promising alternatives to antibiotic growth promoters in poultry production (Qiu, 2021).

In poultry, the benefits of synbiotic formulations include accelerated growth, improved feed efficiency, enhanced nutrient absorption, better intestinal morphology, and protection against oxidative stress. Numerous studies investigating the properties of *Bacillus* species – particularly their antagonism toward pathogenic bacteria and their systemic effects on the host – have opened new avenues for their use as therapeutic and prophylactic biological agents in poultry and other livestock sectors (Safronova, 2024).

The high level of antagonistic activity observed in our *Bacillus* strains – particularly *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* – is supported by findings from other researchers. Studies have demonstrated the effectiveness of *B. amyloliquefaciens* and *B. subtilis* when used as feed additives, confirming their antagonistic potential. Notably, *B. licheniformis* has shown superior antagonistic efficacy, attributed to its ability to reduce the abundance of pathogenic *Clostridium* spp., *Salmonella* spp., and *E. coli*. These microbial shifts were accompanied by an increased population of *B. licheniformis* in the intestinal tract of broiler chickens, enhanced body weight gain, and reduced feed conditioning compared to groups supplemented with *B. subtilis* or unsupplemented controls (Arif, 2021; Wang, 2021).

Similar findings were reported in studies where broiler chickens, pre-infected with *Salmonella*, received feed supplementation with *B. subtilis* and *B. licheniformis*. Both species exhibited strong antagonism against *Salmonella*, contributing to improved intestinal health – evidenced by increased villus height – and elevated serum levels of cholesterol, glucose, globulins, and total protein. Feed intake, body weight gain, and feed conversion ratio also improved compared to control groups. However, among the experimental groups, chickens supplemented with *B. licheniformis* showed significantly better performance indicators (Abudabos, 2020).

The effectiveness of feed supplementation with *B. subtilis* and *B. licheniformis* (administered separately) was also confirmed in comparison with broiler chickens from the control group that received a standard compound feed. Experimental results showed that chickens supplemented with *B. subtilis* and *B. licheniformis* exhibited higher body weight, average daily gain, and serum concentrations of immunoglobulins IgA, IgY, and IgM. Additionally, elevated levels of anti-inflammatory markers (IL-10, IL-1β, and IL-6) were observed, along with enhanced antioxidant capacity due to increased activity of glutathione peroxidase, superoxide dismutase, and catalase. There was also a notable rise in the concentration of short-chain fatty acids (SCFAs). These factors contributed to the modulation of cecal microbiota in the experimental groups. Among them, chickens receiving *B. licheniformis* supplementation demonstrated superior performance indicators (Xu, 2021).

It is worth noting that our findings on the antagonistic properties of various *Bacillus* species corroborate those reported by other authors. *B. licheniformis* demonstrated greater antagonistic efficacy following interaction with indicator test bacteria *E. coli* ATCC 25922, *P. aeruginosa* ATCC 15442, *S. typhimurium* ATCC 29630, and *S. aureus* ATCC 6538. This was confirmed by growth inhibition zone diameters corresponding to very high levels of antagonism against both Gram-positive and Gram-negative test cultures.

The analysis of data from other researchers supports the validity of our research direction focused on evaluating the level of antagonistic activity of various *Bacillus* species for their potential combined use in the formulation of future probiotic preparations. Moreover, the relevance of our experimental approach is further confirmed by studies demonstrating the antagonistic efficacy of *B. subtilis* when used as a feed additive for broiler chickens experimentally infected with *C. perfringens* (Aljumaah, 2020; Bodinga, 2020). Compared to control groups, the antagonistic properties of *Bacillus* strains contributed to a reduction in *Clostridium* counts, less intestinal damage, and increased concentrations of propionic, acetic, and butyric acids in the cecum. Furthermore, several studies have shown that multi-strain *Bacillus*-based formulations exhibit greater antagonistic efficacy than single-strain applications of *B. licheniformis* (Konieczka, 2022).

Based on the obtained data, other researchers have demonstrated that *B. amyloliquefaciens* exhibits greater antagonistic activity in broiler chickens compared to *B. subtilis*. The antagonistic substances produced by *B. amyloliquefaciens* positively influenced growth performance, carcass traits, and both biochemical and immunological blood parameters (Ahmat, 2021).

In trials involving experimental infection of chicks with *S. typhimurium* and direct administration of *Bacillus*-based preparations, body weight significantly increased after 21 days of supplementation compared to control groups. Moreover, direct administration of *Bacillus*-based preparations contributed to the modulation of intestinal microbiota and alleviated the complex negative effects caused by pathogenic agents, as compared to the control group (Hernandez-Patlan, 2019).

In turn, Abdel-Moneim (2020) reported a high level of antagonistic activity following the use of *B. subtilis* in spore form as a feed additive for quails, demonstrating greater efficacy compared to its vegetative form and to control groups. The spore form of *B. subtilis* resulted in significantly higher levels of total protein, albumin, glucose, creatinine, aspartate aminotransferase, alanine aminotransferase, and enhanced enzymatic activity in the duodenum – including proteolytic, lipolytic, and amylolytic functions – as well as increased thyroid hormone activity.

Several researchers have reported that supplementation with *B. licheniformis*, combined with oral administration of *C. perfringens* to chicks over a 75-day period, significantly improved body weight gain, daily glucose balance, thymus index, immunoglobulin and antioxidant levels in serum, and reduced feed conversion ratio and pro-inflammatory cytokine levels in broiler chicks compared to control groups (Xiao, 2024).

The results of studies on the antagonistic activity of *Bacillus* species have practical relevance for poultry farming. Based on scientifically substantiated data, it is recommended to include *Bacillus* strains with very high antagonistic activity against Gram-negative and Gram-positive test cultures in synbiotic formulations for quails. Such application may enhance poultry productivity and production efficiency, supporting more sustainable and economically viable directions in poultry development. In addition, the positive results obtained from trials involving *Bacillus* species encourage further research and the development of biological preparations based on these bacteria for use in other animal species.

A comparative analysis of our findings and those of other researchers reveals some variability in data regarding the antagonistic activity of *Bacillus* species. Nevertheless, in all cases, the use of non-pathogenic spore-forming bacilli as feed additives or biological preparations has demonstrated beneficial effects as an alternative to antibiotics and warrants further investigation.

Conclusion

The results of our *in vitro* studies on antagonistic activity, conducted using the indirect diffusion method of perpendicular streaks and visual observation, demonstrated that among 123 tested strains of *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens*, antagonistic properties were confirmed against the Gram-positive test culture *S. aureus* ATCC 6538 in 22 strains (17.9%), and against the Gram-negative test cultures *E. coli* ATCC 25922 in 38 strains (30.9%), *P. aeruginosa* ATCC 15442 in 33 strains (25.6%), and *S. typhimurium* ATCC 29630 in 40 strains (32.5%).

The results of *in vitro* testing using the quantitative diffusion method of delayed antagonism confirmed the effectiveness and precision of this approach. Among the 123 *Bacillus* strains studied (*B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*), 9 strains (7.3%) were identified with a very high level of antagonism simultaneously against all indicator test cultures: the Gram-positive test culture *S. aureus* ATCC 6538 and the Gram-negative test cultures *E. coli* ATCC 25922, *P. aeruginosa* ATCC 15442, and *S. typhimurium* ATCC 29630.

Specifically, 4 strains (3.3%) of *B. subtilis*, 4 strains (3.3%) of *B. licheniformis*, and 1 strain (0.8%) of *B. amyloliquefaciens* were selected as promising candidates due to their very high antagonistic activity. These findings were confirmed by growth inhibition zone diameters exceeding 36 mm for all tested indicator bacteria.

There remains a need for further testing of the selected 9 promising *Bacillus* strains (7.3%) for their sensitivity to antibacterial agents, in order to prevent the inclusion of antibiotic-resistant *Bacillus* cultures in the development of a probiotic preparation.

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