Research Article

The Distribution of *Bacillus* spp. in the Digestive Tract of Arab Chicken *(Gallus turcicus)* and the Evaluation as Indigenous Probiotic

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Abstract | Taliwang grilled chicken, a renowned dish in Lombok's culinary scene, utilizes Arab chickens *(Gallus turcicus*) as its primary ingredient. However, challenges in the growth of these chickens have been reported. The use of probiotics, particularly *Bacillus spp.,* has been proposed as a key solution due to their benefits in enhancing digestive organ weight and feed intake, which positively impact the chickens body weight. This study aims to investigate the distribution of *Bacillus* spp. within the chicken digestive tract and evaluate the potential as an indigenous probiotic for producing protease, amylase, lipase, and cellulase enzymes. The distribution was measured by isolating, counting, characterizing, and identifying *Bacillus* spp. bacteria in five segments of the digestive tract. The isolates were tested for their ability to inhibit the growth of pathogenic bacteria using the paper disk method, as well as for their capacity to hydrolyze protein, amylum, lipids, and cellulose. Moreover, the degree of pathogen inhibition and macromolecule hydrolysis was determined by the size of the clear zone formed. The results showed that a total of 27 isolates were found, with a distribution of 5 (18.51%), 7 (25.93%), 5 (18.51%), 8 (29.63%) and 2 (7.41%) isolates in the proventriculus, duodenum, jejunum, colon, and ileum respectively. Of the 20 *Bacillus* spp. isolates, 6 isolates (30%) demonstrated the ability to inhibit *Salmonella* sp., while 3 isolates (15%) exhibited the capacity to inhibit *E. coli*. The hydrolysis ability of the isolates included 10 isolates (50%) for protein, 4 isolates (20%) for amylum, 8 isolates (40%) for cellulose, and 1 isolate (5%) for lipids. Therefore, it can be concluded that *Bacillus* spp. isolates from *G. turcicus* effectively inhibit the growth of *Salmonella* sp. and *E. coli* while also hydrolyze protein, amylum, and cellulose compounds.

Keywords | Arab chicken (*Gallus turcicus*), *Bacillus* spp., Indigenous probiotic, Hydrolysing enzimes, Digestive tract, Pathogenic bacteria

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INTRODUCTION

Free-range chicken is a highly nutritious and adaptable source of animal protein, particularly for rural communities. The delicious meat and high protein content in eggs provide a valuable addition to any diet. Meanwhile, the strong body resistance and adaptability to diverse environmental conditions have been well-documented (Tantu

et al., 2022). The national population has consistently increased from 294.161.000 in 2016 to 311.912.000 in 2019, making a significant contribution of 4.58% to poultry production, as reported by the Directorate General of Livestock and Animal Health (2016).

The *Arab* chicken (*Gallus turcicus*) is a highly productive free-range chicken widely cultivated for eggs and meat. As stated by Tamzil and Indarsih (2022), it has a strong track record of producing high-quality eggs. Although the germplasm of chicken is not native to Indonesia, *G. turcicus* has proven to be a valuable addition to the poultry industry in the country. Chicken breed known as Brakel Silver and Brakel Gold was introduced to Indonesia in the 1990s through Saudi Arabia (Tamzil *et al*., 2015).

Recently, *G. turcicus* has gained popularity on Lombok island as a meat producer for the signature dish, *Ayam Taliwang* (Tamzil *et al*., 2015). The cultivation of free-range chicken has the advantages of simple maintenance, and requires little capital, making household-scale cultivation feasible. Moreover, it is easily marketed, and the commodities of eggs as well as meat have relatively stable prices (Tsafrakidou *et al*., 2023). In the cultivation of chicken, there are often obstacles in terms of growth, with the rate being relatively slower than that of laying breeds (Tamzil *et al*., 2013, 2015). The slow growth rate of the body can be attributed to the physiological function of the digestive tract, as well as genetic factors. Enzyme activity in the digestive tract, which is related to nutrient absorption, plays a crucial role. Therefore, probiotic supplementation is one of the solutions to address the growth rate problem.

Probiotic refers to non-pathogenic microorganisms that positively affect the health or physiology of the host when consumed in sufficient quantities (Pandey *et al*., 2015). These microorganisms effectively suppress microbial populations through competition, produce antimicrobial compounds, compete for nutrients, and increase enzyme activity in the gastrointestinal tract. Furthermore, the administration of probiotic improves the condition of the digestive tract by suppressing toxin formation reactions and stimulating enzyme reactions necessary for digesting feed and producing vitamins as well as other substances that may not be present in the feed (Jha *et al*., 2020; Gracia *et al*., 2023). Supplementation in chicken has been shown to provide several benefits, including increased digestive organ weight and enhanced feed intake, which positively affects overall weight (Sarwono *et al*., 2012). These results suggest that probiotic can be an effective tool to improve chicken health and productivity.

Based on several reports, one of the bacteria capable of being used as probiotic is *Bacillus* spp. Huting *et al*. (2023) reported that the use of multi-strain *Bacillus* probiotic (*B. amyloliquefaciens* and *B.subtilis*) improved the nutrient efficiency of piglets. In addition, supplementation of *Bacillus*-based probiotic enhanced protein digestibility, improved broiler production performance significantly, and altered cecal gut microbiota (Gracia *et al*., 2023; Horyanto *et al*., 2024). *B. subtilis* as probiotic supplementation also augmented broiler growth performance and quality against enteric pathogens (Khalifa *et al*., 2023).

G. turcicus has not been previously explored as an isolated bacteria host for probiotic candidates. The potential for increased use, despite having a tendency towards lower body weight compared to other types of chicken, makes this species a promising choice. *Bacillus spp.* isolates have been previously identified in the digestive tract. According to Evangelista *et al*. (2024), *B. subtilis* showed great potential as probiotic. This study aimed to examine the distribution of *Bacillus* spp. in the digestive tract of *G. turcicus* and evaluate the potential as probiotic candidates for producing protease, amylase, lipase, and cellulase enzymes.

MATERIALS AND METHODS

Sampling

Chicken samples were collected from farmers in Bunut Baok Village, Praya District, Central Lombok. The selected samples were alive and kept freely or naturally in the community or breeder's yard (Nugroho *et al*., 2020).

Total Gastrointestinal Bacteria Enumeration and Isolation

Samples were taken from various parts of the digestive tract, including the proventriculus, duodenum, jejunum, ileum, and colon. Each 1 gram sample was homogenized with 9 mL (Milli Liter) of sterile physiological NaCl (Sodium Chloride) using a vortex. The sample was heated in a water bath at 80°C (Celsius) for 15-20 minutes, followed by dilution in duplicates ranging from 10-1 to 10-3. The isolates were then grown on NA (Nutrient Agar) media using the spread method with great confidence in the accuracy of the results. Subsequently, 0.1 mL of the suspension was transferred aseptically to the solid media, leveled with a sterile spreader, and incubated at 37°C for 48 hours. The colonies of *Bacillus* spp. were counted using a colony counter and then purified (Li *et al*., 2019).

Morphological Characterization

To thoroughly analyze colony morphology, various characteristics were observed including shape, elevation, color, edge shape, inner structure, and growth on NA growing media. Similarly, cell morphology was studied by examining gram reaction, cell shape, cell arrangement, spore position, and motility under a microscope at 1000× magnification. By conducting these observations, a comprehensive

understanding of the sample was obtained with confidence and accuracy.

Antibacterial Activity Assay

Antibacterial activity assay was conducted to select or determine the presence of antibacterial activity in the isolated *Bacillus* spp. The isolates were tested against two test bacteria, namely *Salmonella* sp. and *Escherichia coli* using the disc method. Paper discs were soaked for 15 minutes into the antimicrobial sample solution (*Bacillus* spp.) that had been centrifuged at 3000 rpm (Revolutions Per Minute). Sterile distilled water served as a negative control, while the positive control was amoxicillin antibiotics. Using sterile tweezers, soaked paper discs along with controls were carefully placed on the surface of MHA (Mueller-Hinton Agar) plates inoculated with test bacteria and incubated at 37°C for 24 hours. Bacteria culture in MHA media was observed for the presence or absence of inhibition zone formed, then measured using a caliper or ruler to determine the activity and antibacterial properties of *Bacillus* spp (Munshi *et al*., 2022).

Qualitative Assay of Proteolytic, Aminolytic, Lipolytic and Cellulolytic Enzyme Activities

The purpose of this qualitative assay was to select *Bacillus* spp. isolates capable of producing a range of extracellular enzymes, such as protease, amylase, lipase, and cellulase. The test entailed inoculating *Bacillus* spp. into the test media, and a positive result was shown by the presence of a clear zone around the bacteria colony on the media surface (Hossain *et al*., 2020).

Quantitative Assay of Proteolytic, Aminolytic, Lipolytic and Cellulolytic Enzyme Activities

Isolates that showed at least three hydrolytic activities from the four tests were subjected to further quantitative testing. The quantitative tests were conducted using the cell culture and well method with cell-free supernatant. Both methods were analyzed to calculate the enzyme hydrolysis index with precision and accuracy (Malik and Javed, 2021).

Statistical Analysis

The statistical analysis in this study was conducted using a two-factor ANOVA with replication to evaluate the effects of different methods on enzymatic activity across multiple isolates. This method allows for the assessment of both main effects and interactions between the factors namely, the cell culture method and cell-free supernatant method while accounting for potential variability across replicates. The analysis was performed to determine statistical significance ($p < 0.05$) in differences between the tested methods, providing a robust evaluation of their influence on proteolytic, amylolytic, and cellulolytic activities.

RESULTS AND DISCUSSION

This study successfully isolated 20 *Bacillus* spp. from five segments of the digestive tract, namely the proventriculus, duodenum, jejunum, ileum, and colon. The bacteria colony density varied among the different segments by the total plate count (TPC) as shown in Table 1. The total plate count of *Bacillus spp.* isolated from different regions of the digestive tract revealed significant variation, with the proventriculus showing the highest concentration at 1.15 × 10^4 cfu/g, while the jejunum exhibited the lowest count at 1.32 × 10^3 cfu/g. These findings indicate differential microbial colonization along the digestive tract.

Table 1: TPC data of *Bacillus* spp colonies.

No	Parameters	Total Plate Numbers Colony-Forming Units Per Gram (cfu/g)
1	Proventriculus	1.15 × 104
2	Duodenum	8.09 × 103
3	Jejunum	1.32 × 103
4	Ileum	4.9 × 103
5	Large Intestine	1.06 × 104

Bacillus spp. colonies were morphologically characterized by observing the shape, edge, color, texture, and elevation on agar media, while incubation was carried out for 2 × 24 hours at 37°C. The results obtained from the isolation and observation of colony morphological characteristics are presented in Table 2. The morphological characteristics of the *Bacillus spp.* colonies exhibited diverse phenotypes across isolates, ranging from rhizoid to circular shapes, with edges that varied from lobate to irregular. Most colonies displayed a flat elevation and rough texture, predominantly with milky white or cloudy white coloration.

The cell shape was demonstrated by the morphological characteristics of the isolated cells, as well as endospore and gram staining. Specifically, gram staining showed two types of cell morphology, namely gram-positive and negative bacteria. Further investigation into the colony morphological characteristics revealed that isolates DDASP.9, DDASP.9-1, DDASP.9-2, and DDASP.9-3, as well as UBASP.18, UBASP.18-1, UBASP.18-2, UBASP.18-3, and UBASP.18-4, are of the same type. Endospore staining presented round, oval, and cylindrical cell morphology, as illustrated in Table 3.

Table 3 showed that all isolates grew efficiently on media with a pH (Potential of Hydrogen) level of 5.7. Only 14 isolates showed positive growth at a pH level of 11.5, while six experienced no growth. The results provide clear evidence of the isolates' ability to thrive at a range of pH levels.

Table 2: Colony morphology characteristics of *Bacillus* spp.

No	Parameters	Colony Morphology					
		Isolate	Shape	Edges	Elevation	Texture	Color
1	Proventriculus (PVASP)	1	Rhizoid	Rhizoid	Flat	Rough	Milky white
2		2	Circular	Lobate	Flat	Rough	Milky white
3		3	Irregular	Irregular	Flat	Rough	Cloudy white
4		4	Circular	Lobate	Flat	Rough	Cloudy white
5		5	Irregular	Irregular	Flat	Rough	Cloudy white
6	Duodenum (DDASP)	6	Circular	Lobate	Flat	Rough	Milky white
7		7	Irregular	Irregular	Flat	Rough	Milky white
8		8	Circular	Lobate	Flat	Rough	Milky white
9		9	Irregular	Lobate	Flat	Rough	Milky white
10	Jejunum (JJASP)	10	Irregular	Irregular	Flat	Rough	Milky white
11		11	Irregular	Irregular	Flat	Rough	Milky white
12		12	Circular	Lobate	Flat	Rough	Cloudy white
13		13	Circular	Lobate	Flat	Rough	Cloudy white
14		14	Irregular	Lobate	Flat	Rough	Cloudy white
15	Large Intestine (UBASP)	15	Irregular	Rhizoid	Flat	Dry	Transparent white
16		16	Irregular	Irregular	Flat	Rough	Transparent white
17		17	Circular	Lobate	Flat	Rough	Milky white
18		18	Circular	Irregular	Flat	Rough	Milky white
19	Ileum (ILAPS)	19	Irregular	Irregular	Flat	Rough	Milky white
20		20	Irregular	Irregular	Flat	Rough	Milky white

The isolates were positive for catalase activity and exhibited motility in most cases. Additionally, their physiological tests indicated survival under various conditions, such as a temperature of 65°C, a saline concentration of up to 12.5%, and pH levels ranging from 5.7 to 11.5, suggesting robust environmental adaptability, as illustrated in Table 4.

Table 3: Cell characteristics of *Bacillus* spp.

Name of Isolate	Cell Morphology					
	Cell Shape	Bacterial Formation	Gram reaction	Endospore Reaction	Endospore Shape	Endospore Formation
PVASP.1	Bacilli	Single	+	+	Cylinder	Central
PVASP.2	Bacilli	Single	+	+	Oval	Subterminal
PVASP.3	Bacilli	Single	+	+	Round	Subterminal
PVASP.4	Bacilli	Single	+	+	Oval	Central
PVASP.5	Bacilli	Single	+	+	Cylinder	Central
DDASP.6	Bacilli	Single	+	+	Cylinder	Central
DDASP.7	Bacilli	Single	+	+	Cylinder	Central
DDASP.8	Bacilli	Single	+	+	Cylinder	Central
DDASP.9	Bacilli	Single	+	+	Cylinder	Central
JJASP.10	Bacilli	Single	+	+	Round	Subterminal
JJASP.11	Streptobacilli	Chain	+	+	Oval	Central
JJASP.12	Bacilli	Single	+	+	Cylinder	Central
JJASP.13	Bacilli	Single	+	+	Cylinder	Subterminal
JJASP.14	Bacilli	Single	+	+	Oval	Central
UBASP.15	Diplobacilli	Chain	+	+	Cylinder	Central
UBASP.16	Diplobacilli	Chain	+	+	Cylinder	Central
UBASP.17	Bacilli	Single	+	+	Oval	Subterminal
UBASP.18	Diplobacilli	Chain	+	+	Oval	Subterminal
ILASP.19	Bacilli	Single	+	+	Oval	Central
ILASP.20	Bacilli	Single	+	+	Cylinder	Central

The antagonistic activity of *Bacillus spp.* isolates against *Salmonella sp.* and *Escherichia coli* revealed weak inhibitory effects, with inhibition zone diameters averaging between 9 and 10 mm, except for the control, which displayed a much stronger inhibition at 20 mm, as illustrated in Table 5.

Among the *Bacillus spp.* isolates, protease and cellulase activities were more prevalent, with only a few isolates demonstrating lipase activity. Notably, isolate PVASP.5 exhibited multiple hydrolytic activities, including protease, amylase, and cellulase, as illustrated in Table 6 and Figure 1.

The proteolytic assay of the five *Bacillus* spp. isolates were performed using cell culture as well as cell-free supernatant containing protease enzyme crude extract. This test showed the presence of clear zones with different sizes. Differences in the ability of isolates to hydrolyze proteins are present-

ed in Table 7. The protein hydrolysis index of *Bacillus spp.* isolates revealed significant enzyme activity, with isolate PVASP.5 showing the highest index using both the cell culture method (2.4) and the cell-free supernatant method (1.55), indicating its strong proteolytic capacity.

Table 4: Biochemical and physiological tests of *Bacillus* spp.

Name of Isolate	Biochemical Test			Physiological Test					
	Catalase	Motility	Indole	Temperature 10°C	Temperature 65°C	2% Salt	12.5 % salt	pH 5.7	pH 11.5
PVASP.1	+	-	+	+	-	+	-	+	+
PVASP.2	+	+	+	+	+	+	+	+	+
PVASP.3	+	-	+	+	+	+	+	+	+
PVASP.4	+	+	+	+	-	+	-	+	-
PVASP.5	+	+	+	+	+	+	+	+	+
DDASP.6	+	+	-	+	-	+	-	+	-
DDASP.7	+	+	+	+	-	-	-	+	+
DDASP.8	+	+	+	+	-	+	-	+	+
DDASP.9	+	+	+	+	-	+	-	+	+
JJASP.10	+	+	-	+	-	+	-	+	-
JJASP.11	-	+	+	+	-	+	-	+	+
JJASP.12	+	+	+	+	-	+	-	+	+
JJASP.13	+	+	+	+	+	+	+	+	+
JJASP.14	+	+	+	+	-	+	-	+	-
UBASP.15	+	+	+	+	-	+	-	+	+
UBASP.16	-	+	+	+	-	+	-	+	-
UBASP.17	+	+	+	+	-	+	-	+	-
UBASP.18	+	+	+	+	-	+	-	+	+
ILASP.19	+	+	+	+	-	+	-	+	+
ILASP.20	+	+	+	+	-	+	-	+	+

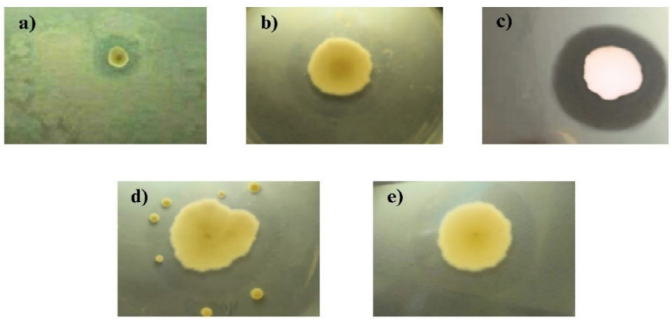

Figure 1: Hydrolytic activity test results of *Bacillus* spp. Isolates; **a:** Isolate PVASP5; **b:** Isolate JJASP.10; **c:** JJASP.12; **d:** UBASP.15; **e:** UBASP.18.

Amylolytic activity was prominent in isolate UBASP.15, which exhibited the highest hydrolysis index using the cell-free supernatant method (1.55), while isolate JJASP.12 demonstrated the lowest index (0.3), indicating variation in starch degradation abilities among the isolates, as illustrated in Table 8.

The fat hydrolysis test showed no significant lipolytic activity across most isolates, with no clear zone formation in either the cell culture or cell-free supernatant methods, suggesting limited fat-degrading capabilities of the *Bacillus spp.* Isolates, as illustrated in Table 9.

Cellulolytic assay on five isolates of *Bacillus* spp. both using cell culture and cell-free supernatant showed variations in the size of the clear zone formed. Differences in the ability to hydrolyze cellulose are presented in Table 10. Isolate JJASP.10 exhibited the highest cellulolytic activity with a hydrolysis index of 2.0 using the cell culture method, while the cell-free supernatant method revealed a reduced hydrolytic efficiency, highlighting the influence of culture conditions on enzyme activity.

Table 5: Mean diameter of inhibition zone for *Bacillus* spp. isolates against the growth of *Salmonella* sp. and *Escherichia coli* using the disk method.

Test Bacteria	Isolate Code	Pitting diameter (mm)	Diameter of clear zone(mm)		Average (mm)	Category
			Horizontal	Vertical		
	DDASP.7	7	10	10	3	Weak
	ILASP.20	7	10	10	3	Weak
	JJASP.10	7	9	9	2	Weak
Salmonella sp.	JJASP.11	7	10	10	3	Weak
	PVASP.4	7	9	9	2	Weak
	UBASP.18	7	11	9	3	Weak
	Control +	7	20	20	13	Strong
	Cock -	7	0	0	0	None
	PVASP.5	7	9	9	2	Weak
	PVASP.4	7	11	11	4	Weak
E. coli	UBASP.17	7	9	9	2	Weak
	Control +	7	10	10	3	Weak
	Control -	7	0	0	0	None

Based on Table 11 the ANOVA analysis of enzymatic activity assays revealed no significant differences in proteolytic and amylolytic activities between the cell culture and cell-free supernatant methods, with p-values of 0.348616 and 0.364539 (proteolytic) and 0.10338 and 0.920293 (amylolytic) for sample and column factors, respectively, and F values below the critical threshold. Conversely, a significant difference was observed in cellulolytic activity (p-value = 0.002835 for the column factor), suggesting that the method employed significantly influences cellulolytic activity.

The different functions of bacteria in each digestive tract may explain the variation in colonies. In the gastrointestinal tract of *G. turcicus*, *Bacillus* spp. bacteria have varying

functions and roles depending on the location. Specifically, in the proventriculus, the bacteria aid in the production of protease enzymes, which facilitate digestive processes (Ogbuewu *et al*., 2022), while in the intestine, the function is to suppress the growth of pathogenic microorganisms, such as *E-coli* and *Salmonella* sp. According to Bahaddad *et al*. (2023), the bacteria in the intestine block or suppress pathogenic microorganisms and improve feed efficiency by releasing enzymes such as amylase, protease, lipase, and cellulose. These enzymes aid in breaking down complex molecules, including carbohydrates, proteins, and fats, into simpler molecules to facilitate digestion and nutrient absorption. Additionally, Šimunović *et al*. (2022) showed the effectiveness of *B. Subtilis* in reducing pathogenic microbes in chicken feces.

Table 6: Hydrolytic activity test of *Bacillus* spp. isolates from the digestive tract of *G. Tarcicus*.

No	Isolate Code	Protease	Amylase	Lipase	Cellulose
1.	PVASP.1	+	-	-	+
2.	PVASP.2	+	-	-	-
3.	PVASP.3	+	-	+	-
4.	PVASP.4	+	-	-	+
5.	PVASP.5	+	+	-	+
6.	DDASP.6	+	-	-	+
7.	DDASP.7	-	-	-	-
8.	DDASP.8	-	-	-	+
9.	DDASP.9	+	+	-	-
10.	JJASP.10	+	+	-	+
11.	JJASP.11	+	-	-	+
12.	JJASP.12	+	+	-	+
13.	JJASP.13	+	-	-	-
14.	JJASP.14	+	+	-	-
15.	UBASP.15	+	+	-	+
16.	UBASP.16	-	-	-	-
17.	UBASP.17	-	+	-	-
18.	UBASP.18	+	+	-	+
19.	ILASP.19	-	-	-	+
20.	ILASP.20	+	-	-	-

According to Mukamto *et al*. (2015), *Bacillus* spp. colonies had a variety of shapes and elevations, including raised, flat, and convex as well as punctiform, irregular, and circular forms. The margins of the colonies were observed to be smooth (entire), undulate (notched), or lobed (lobed), while the height varied between raised, flat, and convex. The isolates obtained showed a range of colors, including white, cream, and yellow. The morphological characteristics of the colonies were similar to those found in the study conducted by Mukamto *et al*. (2015), despite being isolated from different samples.

Table 7: Protein hydrolysis index of *Bacillus* spp. isolated from the digestive tract of *G. Tarcicus*.

Isolate Code	Proteolytic Test					
	Cell culture method			Cell-free supernatant method		
	clear zone diameter (mm)	colony diameter (mm)	hydrolysis index	clear zone diameter (mm)	well diameter (mm)	hydrolysis index
PVASP.5	12	5	2.4	14	9	1.55
JJASP.10	8	7	1.14	17	9	1.88
JJASP.12	9	5	1.8	11	9	1.22
UBASP.15	9	7	1.28	18	9	1
UBASP.18	9	6	1.5	12	9	1.33

Table 8: Amylum hydrolysis index of *Bacillus* spp. isolates from the digestive tract of *G. Tarcicus*.

Isolate Code	Amylolytic assay					
	Cell culture method			The cell-free supernatant method		
	diameter of the clear zone (mm)	colony diameter (mm)	hydrolysis index	diameter of the clear zone (mm)	colony diameter (mm)	hydrolysis index
PVASP.5	8	6	1.33	12	9	1.33
JJASP.10	15	13	1.15	13	9	1.44
JJASP.12	8	16	0.5	3	9	0.3
UBASP.15	16	12	1.33	14	9	1.55
UBASP.18	17	13	1.30	10	9	1.11

Table 9: Fat hydrolysis index of *Bacillus* spp. isolates from the digestive tract of *G. Tarcicus*.

Isolate Code	Lipolytic test					
	Cell culture method			The cell-free supernatant method		
	diameter of the clear zone (mm)	colony diameter (mm)	hydrolysis index	diameter of the clear zone (mm)	colony diameter (mm)	hydrolysis index
PVASP.5	-	7	-	-	9	-
JASP.10	-	6	-	-	9	-
JJASP.12	-	13	-	-	9	-
UBASP.15	-	8	-	-	9	-
UBASP.18	-	10	-	-	9	-

To assess bacteria spore production, this study also used endospore cell staining in conjunction with gram staining. Endospores were visualized in green under a microscope, while vegetative cells were visualized in red. The malachite green reagent was selected for endospore staining due to the solubility in high-pH water. This property is necessary

for effective staining of endospores that are typically resistant to ordinary dyes. The test was performed on isolates three days old after the stationary phase, following Shrestha *et al*. (2023).

Table 10: Cellulose hydrolysis index for *Bacillus spp*. isolates from the digestive tract of *G. Tarcicus*.

Isolate Code	Cellulolytic Test					
	Cell culture method			The cell-free supernatant method		
	diameter of the clear zone (mm)	colony diameter (mm)	hydrolysis index	diameter of the clear zone (mm)	colony diameter (mm)	hydrolysis index
PVASP.5	16	7	2.28	16	9	1.77
JJASP.10	22	11	2	12	9	1.33
JJASP.12	19	16	1.8	6	9	0.6
UBASP.15	8	5	1.6	7	9	0.77
UBASP.18	18	11	1.63	9	9	1

Table 11: Statistical analysis using ANOVA: two-factor with replication for hydrolisis index.

Proteolytic Test						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	1.4486	4	0.36215	1.257290654	0.348616	3.47805
Columns	0.25992	1	0.25992	0.90237467	0.364539	4.964603
Interaction	1.45388	4	0.36347	1.261873351	0.347023	3.47805
Within	2.8804	10	0.28804			
Total	6.0428	19				
Amylolytic Assay						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	2.80708	4	0.70177	2.566074302	0.10338	3.47805
Columns	0.00288	1	0.00288	0.010530935	0.920293	4.964603
Interaction	0.20572	4	0.05143	0.188057628	0.939241	3.47805
Within	2.7348	10	0.27348			
Total	5.75048	19				
Cellulolytic Test						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	2.09552	4	0.52388	2.739098609	0.089416	3.47805
Columns	2.94912	1	2.94912	15.41942905	0.002835	4.964603
Interaction	0.28568	4	0.07142	0.373418383	0.822492	3.47805
Within	1.9126	10	0.19126			
Total	7.24292	19				

Bacillus spp. bacteria isolates showed either motile or non-motile growth around the ose puncture. The indole assay results varied among isolates, with some producing indole and others not, while the catalase assay showed positive results or catalase activity. Bacteria isolates grew optimally at 10°C, with only four showing growth at 65°C and 16 indicating no growth (negative). Growth tests were conducted at different salt levels and based on the results, most isolates were able to grow on media with a salt content of 2% (positive). However, when the salt content was increased to 12.5%, only four isolates were able to grow (positive), and the remaining 16 did not (negative). These results show a clear correlation between salt concentration and the growth of the isolates.

Bacillus spp. bacteria isolates showed great ability to produce catalase enzymes, as well as indole, motile, and non-motile properties. Based on the catalase test using Hydrogen Peroxide (H_2O_2), all isolates produced enzymes, characterized by the emergence of bubbles when the reagent was dripped. Liu *et al*. (2023) reported that the catalase activity of *Bacillus baekryungensis* as probiotic was significantly higher compared to the control group, resulting in better growth, changes in nutrients of the body wall, and improved digestibility. The results also showed that some isolates produced indole, while others did not. In the motility test, the isolates were found to have flagellum as a means of movement. These results were in line with Feng *et al*. (2023) stating that endophytic *Bacillus* spp. had motile and non-motile properties with flagellum as a means of movement.

The influence of environmental factors such as temperature, pH, and salt content on bacteria growth was also examined. Based on the results, *Bacillus* spp. isolates optimally grew at 10°C and pH 5.7. Ramiah *et al*. (2020) found that the optimal body temperature of broiler chicken was between 20-25°C, suggesting the reason for bacteria growth at 10°C was due to the adaptation to the host. Bacteria growth decreased when the temperature was higher than the optimal range for chicken.

The antibacterial activity assay found that six out of 20 *Bacillus* spp. isolates, namely DDASP.7, ILASP.20, JJASP.10, JJASP.11 PVASP.4 and UBASP.18 effectively inhibited the growth of *Salmonella* sp. bacteria (Table 5). This result was in line with a previous study stating that *Bacillus* spp. significantly reduced *Salmonella* sp. population (Evangelista *et al*., 2024). Three isolates, namely PVASP.4, PVASP.5 and UBASP.17 also inhibited the growth of *E. coli* bacteria although the average inhibition was categorized as weak. The weak antibacterial activity against *E. coli* is presumed to be due to the use of bacterial strains in this study that are not commonly employed in standard testing procedures, which may have led to stronger sensitivity or resistance. Additionally, it is hypothesized that the metabolites produced by *Bacillus* spp. are specifically more effective in inhibiting the growth of *Salmonella* sp. compared

to *E. coli*. According to a study by Khalifa *et al.* (2023), *B. subtilis* significantly binds to peritoneal macrophage cells and inhibits the surface adhesion of *Salmonella enterica*. This underscores the potential of *B. subtilis* to aggregate in macrophage cells, suggesting that the use as probiotic supplement can improve the growth performance and quality of broilers against enteric pathogens.

Complex macromolecules such as carbohydrates, proteins, and fats are hydrolyzed into smaller molecules that can be absorbed and used by body cells (Lucas, 2015). Artha *et al.* (2019) stated that *Bacillus* spp. has the ability to produce protease, amylase, and cellulase enzymes. Therefore, hydrolytic activity test was conducted to measure the potential of isolates as probiotic candidates. The results for the 20 isolates from the digestive tract of *G. turcicus* are shown in Table 6. The more types of macromolecules hydrolyzed by an isolate, the better the utility as probiotic candidate.

The ability to hydrolyze macromolecular compounds into simpler compounds facilitates the digestive system in absorbing nutrients into the body. The hydrolytic ability of an isolate to break down or degrade macromolecular compounds determines the potential as probiotic candidate. Probiotic provides functional benefits by aiding the digestive system in absorbing nutrients for growth. The hydrolytic activity test results showed that five isolates, namely PVASP.5, JJASP.10, JJASP.12, UBASP.15, and UBASP.18, possessed complex hydrolytic activity.

Based on the hydrolytic activity assay results (Table 6), certain isolates were selected for further quantitative tests. The isolates selected were those with the most significant potential as probiotic candidates, fulfilling the requirement of showing at least three hydrolytic activities from the four tests conducted. These isolates were PVASP.5, JJASP.10, JJASP.12, UBASP.15 and UBASP.18.

Based on the hydrolysis index analysis in the proteolytic activity assay, *Bacillus* spp., bacteria isolates from G. *turcicus* showed the ability to hydrolyze proteins at low to medium levels, both with the cell culture and the cell-free supernatant methods. Classification of the hydrolysis index value is as follows: < 2.1 (low), 2.1 - 3.1 (moderate), and > 3.1 (high) (Ahmad *et al.*, 2013). The isolates showed a significant ability to hydrolyze proteins at low to moderate levels. This result was in line with Ciurko *et al.* (2021) stated that *B. cereus* and *B. lentus* also have a fairly high proteolytic ability. Soeka and Sulistiani (2020) reported that B. Subtilis from Samarinda Shrimp Paste showed a remarkable ability to hydrolyze proteins at low to moderate levels, with a hydrolysis index value between 1.50 to 2.90.

Amylolytic assay performed on five isolates using cell culture and cell-free supernatant containing crude extract of amylase enzyme showed differences in the ability to hydrolyze amylum. This was indicated by the presence of clear zones with different sizes, as presented in Table 8.

Based on the result, *Bacillus* spp. effectively hydrolyzed amylum and the highest index was found in UBASP.15 isolate. *B. velezensis* strains are capable of breaking down polysaccharides through oxidation, which aids in the absorption of nutrients (Srivastava *et al.*, 2023). According to Ahmad *et al.* (2013), *Bacillus* spp. bacteria isolates from the digestive tract of chicken have low amylum hydrolysis ability, shown by the index value of < 2.1. Some reports showed that high amylolytic properties were obtained in *Bacillus* spp. isolated from soil, higher than that reported from the chicken digestive tract (Klinfoong *et al.*, 2022).

The lipolytic assay results obtained using cell culture and cell-free supernatant containing crude extract of lipase enzyme are presented in Table 9. Based on the results, the five *Bacillus* spp. isolates grown on NA media with the addition of 1% fat from olive oil did not form a clear zone. Negative test results may be caused by the possibility that the lipase they possess is an intracellular lipase, so its products are not detected in the test medium (Papackova and Cahova, 2015). However, almost all bacterial lipases are extracellular, the results will be visible on the test media during testing. Mazhar *et al.* (2018) showed that *Bacillus cereus* was lipolytic and formed a clear zone around bacteria colonies on Tween-80 agar media. Demirkan *et al.* (2021) also reported that *B. cereus* ATA179 isolated from soil in Turkey has very potent lipolytic activity. Cellulolytic activity assays on *Bacillus* spp. bacteria isolates showed the ability to hydrolyze cellulose at low to moderate levels, both by cell culture and cell-free supernatant methods. A study by Oladiti *et al.* (2020) on the isolation of cellulolytic bacteria from cassava peel waste reported that *B. Subtilis* showed very high activity in cellulose hydrolysis and improved its nutritional content.

This showed that the bacteria were able to effectively hydrolyze cellulose. According to Oktiarni *et al.* (2021), *Bacillus* spp. isolates from termite gut can produce cellulase enzyme, which acts as a biocatalyst in the enzymatic hydrolysis process, facilitating the degradation of lignocellulosic biomass into glucose.

Cellulolytic activity assay on *Bacillus* spp. bacteria isolates showed the ability to hydrolyze cellulose at low to moderate levels, both by cell culture and cell-free supernatant methods. A study by Oladipo *et al.* (2020) on the isolation of cellulolytic bacteria from cassava peel waste reported that Bacillus group bacteria, specifically *B. subtilis*, had a high cellulose hydrolysis. According to Oktiarni *et al.* (2021), *Bacillus* spp. isolates from termite gut can produce cellulase enzyme, which acts as a biocatalyst in the enzy-

matic hydrolysis process, facilitating the degradation of lignocellulosic biomass into glucose.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, this study successfully obtained 20 *Bacillus* spp. isolates from the digestive tract of *G. turcicus*, with the majority being found in the jejunum and ventriculus. Among the isolates, DDASP.7, ILASP.20, JJASP.10, JJASP.11, PVASP.4, and UBASP.18, effectively inhibited the growth of *Salmonella sp*. Three isolates, namely PVASP.5, PVASP.4, and UBASP.17, inhibited the growth of *E. coli* bacteria, but with a weak inhibition zone. The selected isolates, including JJASP.10, JJASP.12, PVASP.8, UBASP.15, and UBASP.18, were able to hydrolyze protein, amylum, and cellulose compounds, but did not show lipolytic activity. The hydrolysis index values of protein, amylum, and cellulose varied based on the cell culture and cell-free supernatant methods.

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NOVELTY STATEMENTS

This research is looking for indigenous probiotic multispecies multiaction candidates that will be expected to be able to attach to the digestive tract segments according to their role, can improve the digestibility and resistance of Arab chickens against pathogenic bacteria so that it has an impact on improving the growth performance, immunity, and fitness of Arab chickens.

AUTHOR'S CONTRIBUTIONS

All authors contributed equally to the manuscript.

Conflict of Interest

The authors have declared no conflict of interest.

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