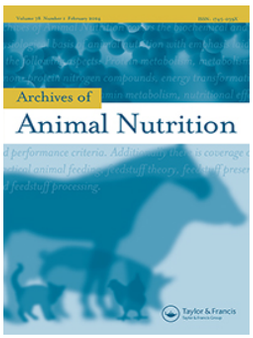

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Inclusion of a *Bacillus*-based probiotic in non-starch polysaccharides-rich broiler diets

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ABSTRACT

This study examined the effects of a 3-strain *Bacillus*-based probiotic (BP; *Bacillus amyloliquefaciens* and two *Bacillus subtilis*) in broiler diets with different rye levels on performance, mucus, viscosity, and nutrient digestibility. We distributed 720 one-d-old female broilers into 72 pens and designed nine diets using a 3×3 factorial approach, varying BP levels (0, 1.2×10^6, and 1.2×10^7 CFU/g) and rye concentrations (0, 200, 400 g/kg). On d 35, diets with 200 or 400 g/kg rye reduced broiler weight gain (BWG). Diets with 400 g/kg rye had the highest FCR, while rye-free diets had the lowest ($p \leq 0.05$). Adding BP increased feed intake and BWG in weeks two and three ($p \leq 0.05$). It should be noted that the overall performance fell below the goals of the breed. Including rye in diets reduced the coefficient of apparent ileal digestibility (CAID) for protein, ether extract (EE), calcium, phosphorus, and all amino acids ($p \leq 0.05$). Rye-free diets exhibited the highest CAID for all nutrients, except for methionine, EE, and calcium, while diets with 400 g/kg of rye demonstrated the lowest CAID ($p \leq 0.05$). BP in diets decreased phosphorus CAID ($p \leq 0.05$). Diets containing 1.2×10^7 CFU/g (10X) of BP exhibited higher CAID of methionine than the other two diets ($p \leq 0.05$). Diets containing 10X of BP showed higher CAID of cysteine than diets with no BP ($p \leq 0.05$). Ileal viscosity increased as the inclusion level of rye in the diets increased ($p \leq 0.05$). The ileal concentration of glucosamine in chickens fed diets with 400 g/kg of rye was higher than in those fed diets with no rye ($p \leq 0.05$). Furthermore, ileal galactosamine concentrations were elevated in diets with 200 and 400 g/kg of rye when compared to rye-free diets ($p \leq 0.05$). However, BP in diets had no impact on ileal viscosity, galactosamine, or glucosamine ($p > 0.05$). In conclusion, the applied *Bacillus* strains appeared to have a limited capacity to produce arabinoxylan-degrading enzymes and were only partially effective in mitigating the negative impacts of rye arabinoxylans on broilers.

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Arabinoxylans; enzyme; fermentation; fibre; non-starch polysaccharides

1. Introduction

Non-starch polysaccharides (NSP) are predominantly indigestible by broiler chickens, and soluble forms of NSP are primarily considered as anti-nutritional factors (ANF) for

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broilers (Hetland et al. 2004). The presence of soluble-viscous NSP in digesta reduces the rate of diffusion of digestive enzymes into the digesta and interferes with the diffusion and absorption of nutrients in the small intestine (Hetland et al. 2004). Indigested and unabsorbed nutrients, along with soluble NSP, serve as substrates for gut microbiota (Józefiak et al. 2004; Tejeda and Kim 2021).

Arabinoxylan is the major polymer in the cell walls of most cereals. Rye, in particular, contains a relatively high amount of arabinoxylans (Józefiak et al. 2007; Knudsen 2014; Bederska-Łojewska et al. 2017). A significant proportion of the arabinoxylans in rye is soluble, mainly due to their structural features, such as the arabinose/xylose ratio (Knudsen 2014). In a study investigating the variation in the chemical composition of cereal grains from different genotypes, the mean concentration of soluble arabinoxylans was found to be 9.7 g/kg DM in barley, 12.6 g/kg DM in triticale, 13.9 g/kg DM in wheat, and 30.9 g/kg DM in rye (Rodehutscord et al. 2016). A high concentration of a soluble-viscous NSP source like rye in broiler diets can disrupt the entire digestive process in the gut and may disturb the gut microecology, potentially leading to dysbiosis in the gut microbial community, digestive malfunction and impaired growth performance (Józefiak et al. 2007; Van Krimpen et al. 2015; Bederska-Łojewska et al. 2017). The addition of microbial NSP-degrading enzymes to poultry feed has been proven to be an effective alternative for reducing the negative digestive impacts of NSP (Choct 2006).

Spore-forming bacteria are considered one of the most promising probiotics in poultry nutrition due to their resistance to environmental stresses, such as heat, disinfectants, and low pH (Sella et al. 2014; Goodarzi Boroojeni et al. 2016, 2018; Zentek and Goodarzi Boroojeni 2020). Furthermore, certain *Bacillus* strains, including *Bacillus subtilis* and *Bacillus amyloliquefaciens*, have the capability to produce a variable set of enzymes, including xylanases, amylases, lichenase, β-galactosidase, cellulases, alkaline serine proteases, and phytase (Latorre et al. 2016; Su et al. 2020). These strains have been commonly employed by biotechnology companies for the production of NSP-degrading enzymes (Latorre et al. 2016). Thus, it can be hypothesised that the inclusion of a *Bacillus*-based probiotic (BP) containing strains such as *Bacillus subtilis* and *Bacillus amyloliquefaciens* in NSP-rich broiler diets, especially at higher dosages, may efficiently reduce the negative impacts of NSP through both NSP degradation and modification of gut bacteria.

The objective of the current study was to investigate the effects of different inclusion levels of a 3-strain BP in broiler diets with varying concentrations of rye on growth performance, ileal nutrient digestibility, ileal viscosity, and mucus loss.

2. Materials and methods

2.1. Animals and experimental diets

The study was conducted in full adherence to the recommendations of the National Ethics Commission in Warsaw, Poland. All procedures and experiments were conducted in accordance with established guidelines and received approval from the Local Ethics Commission at Poznań University of Life Sciences in Poznań, Poland, specifically concerning animal experimentation and the care of the animals involved. Every effort was made to minimise any potential suffering during the course of the study.

Nine experimental diets were formulated by incorporating varying levels of BP: 0 CFU/g (designated as 0X), 1.2×10^6 CFU/g (1X, following the manufacturer's recommended dosage), and 1.2×10^7 CFU/g (10X, representing a tenfold increase over the manufacturer's recommendation) into broiler diets containing 0 g/kg, 200 g/kg, and 400 g/kg of rye. The BP used in this study was GalliProFit (Chr. Hansen, Denmark), comprising one strain of *Bacillus amyloliquefaciens* (DSM25840) and two strains of *Bacillus subtilis* (DSM32324 and DSM32325).

The experimental diets (as shown in Table 1) were isocaloric and isonitrogenous, and they were formulated to meet or exceed the recommendations outlined by Smulikowska and Rutkowski (2018). These diets were tailored for specific broiler growth stages: starter diets for d 0–7, grower diets for d 8–21, and finisher diets (chemical composition is shown in Table 2) for d 22–35. The diets were provided in mash form.

Table 1. Feed ingredients and calculated proximate composition of the experimental diets (as fed, in fresh basis).

Ingredients [g/kg]	Starter (d 1–7)			Grower (d 8–21)			Finisher (d 22–35)		
Rye inclusion level [g/kg]	0.0	200.0	400.0	0.0	200.0	400.0	0.0	200.0	400.0
Wheat	580.3	354.9	129.5	650.2	424.8	199.5	667.5	442.3	216.9
Rye	0.0	200.0	400.0	0.0	200.0	400.0	0.0	200.0	400.0
Soybean meal 46.8%	320.3	339.4	358.7	236.6	255.9	275.2	214.0	233.2	252.5
Soybean oil	48.1	55.5	62.9	65.1	72.4	79.8	69.5	76.9	84.3
Vitamins and minerals premix[1]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Monocalcium Phosphate	17.0	16.9	16.8	14.6	14.5	14.4	14.1	13.9	13.8
Calcium carbonate	13.3	13.1	12.9	12.8	12.5	12.3	11.6	11.3	11.1
Sodium chloride	1.5	1.7	2.0	1.4	1.7	2.0	1.7	2.0	2.2
Sodium sulphate	2.8	2.5	2.2	2.9	2.6	2.3	2.6	2.3	2.0
L-Lysine HCl	4.1	3.6	3.1	4.3	3.8	3.3	3.5	3.0	2.5
L- Methionine	3.3	3.4	3.4	2.9	2.9	2.9	2.5	2.5	2.5
L-Threonine	1.7	1.6	1.5	1.7	1.6	1.5	1.4	1.3	1.2
Tryptophan	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.2
L-Valine	1.8	1.8	1.8	1.8	1.8	1.7	1.3	1.3	1.3
L-Arginine	2.6	2.3	2.0	2.6	2.3	1.9	2.2	1.8	1.5
Titanium dioxide[2]	0.0	0.0	0.0	0.0	0.0	0.0	5.0	5.0	5.0
	Calculated nutritional value [g/kg, unless noted]								
Dry matter	882.7	884.7	886.6	883.8	885.8	887.7	884.3	886.2	888.1
Ash	66.1	66.9	67.7	59.2	59.9	60.7	56.2	56.8	57.6
Crude protein	230.0	230.0	230.0	200.0	200.0	200.0	190.0	190.0	190.0
Ether extract	61.4	68.0	74.7	78.1	84.7	91.3	82.4	89.1	95.7
Crude fibre	25.3	24.9	24.4	23.5	23.1	22.7	23.0	22.6	22.2
Sodium	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
Calcium	9.5	9.5	9.5	8.7	8.7	8.7	8.1	8.1	8.1
Phosphorus	7.7	7.8	7.8	6.9	6.9	7.0	6.7	6.7	6.7
Digestible lysine	13.0	13.0	13.0	11.2	11.2	11.2	10.1	10.1	10.1
Digestible methionine	6.6	6.6	6.6	5.8	5.8	5.8	5.2	5.2	5.2
Digestible cysteine	3.2	3.2	3.1	3.0	2.9	2.8	2.9	2.8	2.7
Digestible threonine	8.4	8.4	8.4	7.3	7.3	7.3	6.7	6.7	6.7
Digestible tryptophane	2.7	2.7	2.7	2.4	2.4	2.4	2.2	2.2	2.2
AME_n[3] [MJ]	12.4	12.4	12.4	13.1	13.1	13.1	13.3	13.3	13.3

[1]Contents per kg diet: 11000 I.U. Vit. A (retinyl acetate); 2500 I.U. Vit. D3 (cholecalciferol); 50.0 mg Vit. E (α-tocopherole acetate); 2.49 mg Vit. K3 (MNB); 2.00 mg Vit. B1 (mononitrate thamines); 7.00 mg Vit. B2 (cryst. riboflavin); 4.00,00 mg Vit. B6 (pyridoxine hydrochloride); 21 µg Vit. B12 (cyanocobalamin); 40 mg niacin (nicotinic acid); 201 µg biotin (commercial, feed grade); 1.00 mg folic acid (cryst., commercial, feed grade); 300 mg choline (chloride); 45 mg iron (Sulfate(II) monohydrate); 95 mg zinc (zinc oxide); 70 mg manganese (manganese (II) oxide); 20 mg copper (copper oxide); 0.60 mg iodine (calcium iodate anhydrous granular); 0.351 mg selenium (coated granulated sodium selenium); 0.21 mg 25-hydroxy cholecalciferol; 0.09 mg propyl gallate; 0.083 mg butylated hydroxyanisole; 1.15 mg Butylated hydroxytoluene (BHT); 13.61 mg calcium D-pantothenate; carrier: calcium carbonate (calcium min 21,9%).[2] Indigestible marker (Sigma Aldrich, St. Louis, MO).[3] Nitrogen-corrected apparent metabolisable energy.

Table 2. Analysed chemical composition of the finisher diets (d 22–35, as fed basis) used for digestibility analysis.

Diet	T1	T2	T3	T4	T5	T6	T7	T8	T9
Rye [g/kg]	0	0	0	200	200	200	400	400	400
Probiotic[1]	0	1X	10X	0	1X	10X	0	1X	10X
Dry matter	902	902	901	903	904	903	908	908	908
Ash	50.7	51.8	54.3	54.2	49.6	57.8	55.0	52.3	57.5
Crude protein	192.3	195.9	194.1	195.9	192.0	197.7	192.6	191.1	192.6
Ether extract	77.7	76.4	75.7	86.0	84.9	84.9	94.1	93.6	95.9
Alanine	7.5	7.4	7.1	7.9	7.6	7.8	8.0	7.7	7.6
Arginine	13.0	12.7	12.0	13.4	12.6	13.2	13.1	12.3	12.3
Asparagine	4.7	4.8	4.5	5.3	4.9	5.0	5.4	5.1	5.0
Cysteine	5.2	5.6	5.2	4.9	5.1	5.2	4.8	4.8	5.3
Glutamic acid	44.1	43.3	41.8	42.0	40.6	41.3	39.1	37.6	37.2
Glycine	7.9	7.7	7.4	8.1	7.8	8.0	8.1	7.7	7.7
Histidine	4.7	4.3	4.4	4.8	4.6	4.7	4.7	4.4	4.5
Isoleucine	7.2	7.4	7.0	7.6	7.2	7.7	7.8	7.0	7.4
Leucine	13.4	13.2	12.7	13.6	13.0	13.5	13.4	12.7	12.7
Lysine	11.1	11.1	10.3	11.7	11.2	11.7	11.7	11.0	11.2
Methionine	8.4	7.5	8.4	7.3	7.5	8.0	7.4	7.1	9.6
Phenylalanine	9.5	9.3	8.9	9.7	9.2	9.5	9.6	9.0	9.1
Proline	12.5	12.3	11.9	12.1	11.8	11.9	11.5	11.0	10.9
Serine	9.5	9.0	8.7	9.5	9.0	9.1	9.0	9.0	8.6
Threonine	8.1	7.4	7.1	7.9	7.6	7.9	7.8	7.8	7.7
Tyrosine	5.5	5.4	5.1	5.7	6.0	5.6	5.5	5.2	5.1
Valine	18.6	19.0	18.1	19.3	19.0	19.8	20.1	18.4	19.1

[1]1X = GalliProFit $1.2 \times 1\ 0^6$ CFU/g feed, 10X = GalliProFit 1.2×10^7 CFU/g feed – GalliProFit (Chr. Hansen, Denmark) consists of two *Bacillus subtilis* strains, DSM32324 and DSM32325 and a *Bacillus amyloliquefaciens* strain DSM25840.

A total of 720 one-d-old female broiler chicks (Ross 308®) were randomly distributed into 72 pens equipped with softwood shaving flooring. Employing a completely randomised design with a 3×3 factorial arrangement of treatments, nine distinct experimental diets were randomly assigned to the birds within these pens (with eight replicate pens for each diet, housing 10 birds per pen). The study lasted 35 d. The birds received vaccinations in the hatchery against Infectious Bronchitis, using Nobilis IB Ma5 and Nobilis IB 4/91 vaccines. Subsequently, at d 12 and d 20 after hatching, they were vaccinated against Gumboro disease, with the administration of HIPRAGUMBORO-GM97 and AviPro PRECISE vaccines.

All birds had unrestricted access to both the experimental diets and water. In the finisher diets, 5 grams of titanium dioxide (Sigma Aldrich, St. Louis, MO) per kg of feed were included as an indigestible marker. This marker was utilised to facilitate the determination of the coefficient of apparent ileal digestibility (CAID) of nutrients and to enable the comparison of ileal concentrations of galactosamine and glucosamine between the different treatment groups.

2.2. Performance measurements

The body weights of the chicks were recorded on the first d of the experiment and subsequently on a weekly basis throughout the study. Additionally, the feed intake (FI) was recorded on a weekly basis, and from these data, the feed conversion ratio (FCR) was calculated.

The health of the birds was maintained throughout the entire duration of the experiment, with no reported health issues or concerns. Moreover, the overall mortality rate among the birds remained low, with total mortality remaining below 2%.

2.3. Litter quality

At the end of the experiment, the assessment of litter quality for all birds was conducted by trained personnel following the procedures outlined by the Welfare Quality Consortium (2009). This method for evaluating litter quality was based on a scoring system ranging from 0 to 4, where 0 indicated completely dry and flaky litter (i.e. easily moved with the foot), and 4 indicated litter that adhered to boots once the cap or compacted crust was disrupted.

2.4. Apparent ileal digestibility of nutrient

At the end of the experiment, a total of five birds per pen were randomly selected, stunned, and euthanised by exsanguination. The excreta from these birds were collected for analysis. Additionally, the ileum, spanning from Meckel's diverticulum to the ileo – ceco–colic junction, was carefully dissected, and the digesta from the distal 2/3 of the ileum were collected for the determination of the CAID of nutrients.

The digesta and excreta from all the birds within each pen were pooled and promptly frozen at −80°C for subsequent analysis. A portion of the pooled digesta samples was utilised for viscosity analysis, while the remainder was subjected to freeze-drying before chemical analysis.

The CAID calculation was performed using the following formula:

$$\begin{aligned}\text{The } CAID \text{ of nutrient} = 1 - [&(\text{concentration of marker in feed}/\\ &\text{concentration of marker in ileum})\\ &\cdot (\text{concentration of nutrient in ileum}/\\ &\text{concentration of nutrient in feed})]\end{aligned}$$

2.5. Viscosity

To assess viscosity, 1.5 grams of previously frozen pooled ileal samples and 1.2 grams of previously frozen excreta samples were thawed, then diluted with a factor of 1.33 using distilled water, followed by thorough homogenisation. Afterward, these samples were subjected to centrifugation at 13,000 revolutions per min (rpm) for 10 min. Subsequently, 532 µl of the supernatants were retrieved and subjected to viscosity analysis using a rotational viscometer (40°C) known as the DV-II Viscometer (Brookfield Engineering Laboratories Inc., MA, USA) with a cone spindle (CPA-40Z) at a rotational speed of 100 rpm (Ellner et al. 2021).

2.6. Chemical and physical analysis

The content of titanium dioxide was determined following the method outlined by Short et al. (1996). Basic composition analyses were conducted using established standard procedures as described by Naumann and Bassler (2004). Phosphorus content was measured utilising the ammonium vanadate/molybdate method (Gericke and Kurmies 1952). Calcium was analysed using an atomic absorption spectrophotometer (AAS

vario®, Analytik Jena, Jena, Germany). Amino acid (AA) analyses were carried out according to the standard method specified by VDLUFA (2003) with a Biochem 30 Plus AA analyser (Amersham Pharmacia Biotech, Piscataway, USA). Prior to analysis, the samples were hydrolysed in a 6 M aqueous HCl solution at 110°C for 24 h (Naumann and Bassler 2004). The content of methionine and cysteine was determined after the oxidation of samples using a mixture of H2O2 and formic acid. For the evaluation of mucin content, galactosamine and glucosamine were measured as mucin markers (as described by Tsirtsikos et al. (2012)) using the method employed for AA analysis.

2.7. Statistical analysis

The data were analysed using ANOVA with the GLM procedure in SPSS 19.0 (SPSS Inc., Chicago, IL). The analysis was structured as a 3 × 3 factorial arrangement of treatments, which incorporated three levels of BP (0, 1X, and 10X) and three inclusion levels of rye (0, 200, or 400 g/kg) as the main factors, along with their interactions.

To discern significant differences between treatment groups, treatment means were separated utilising the Least Significant Difference as a post hoc test, with a significance level set at $p \leq 0.05$. For all measured variables, the replicate-pen was considered the experimental unit.

3. Results

During the starter and grower periods, broilers fed diets containing 400 g/kg of rye exhibited lower ($p \leq 0.05$) body weight gain (BWG) compared to those receiving diets with 0 and 200 g/kg of rye (Table 3). However, the inclusion of either 200 or 400 g/kg of rye in broiler diets resulted in reduced BWG during the finisher and the entire

Table 3. The effect of experimental diets (mean values) on the growth performance variables[1] of female broiler chickens.

	Rye (RY [g/kg])			Probiotic (BP)[2]				*p*-value		
	0	200	400	0	1X	10X	SEM*	BP	RY	BP × RY
BWG 1–7 d[3]	123[a]	122[a]	116[b]	121	119	121	1.1	0.660	0.034	0.576
FI 1–7 d[3]	136	136	138	136	136	138	0.6	0.248	0.371	0.140
FCR 1–7 d[3]	1.11[b]	1.12[b]	1.19[a]	1.13	1.14	1.14	0.008	0.690	<0.001	0.913
BWG 8–21 d	697[a]	684[a]	663[b]	665[b]	692[a]	688[a]	4.6	0.019	0.005	0.239
FI 8–21 d	903	905	886	880[b]	915[a]	899[ab]	5.1	0.017	0.217	0.231
FCR 8–21 d	1.30[b]	1.32[a]	1.34[a]	1.33	1.32	1.31	0.004	0.120	<0.001	0.830
BWG 22–35 d	992[a]	945[b]	933[b]	941	946	982	9.8	0.167	0.036	0.931
FI 22–35 d	1568	1558	1590	1545	1571	1601	12.4	0.211	0.586	0.877
FCR 22–35 d	1.58[b]	1.65[b]	1.71[a]	1.64	1.66	1.63	0.008	0.118	<0.001	0.557
BWG 1–35 d	1811[a]	1751[b]	1712[b]	1727	1757	1791	12.1	0.069	0.002	0.783
FI 1–35 d	2607	2599	2614	2562	2621	2637	15.4	0.123	0.932	0.721
FCR 1–35 d	1.44[c]	1.49[b]	1.53[a]	1.48	1.49	1.47	0.005	0.058	<0.001	0.671

[1]Data are means of 8 replicate-pens with 10 birds per pen.
[2]1X = GalliProFit 1.2 × 106 CFU/g feed, 10X = GalliProFit 1.2 × 107 CFU/g feed – GalliProFit (Chr. Hansen, Denmark) consists of two Bacillus subtilis strains, DSM32324 and DSM32325 and a Bacillus amyloliquefaciens strain, DSM25840.
[3]BWG = body weight gain (g); FI = feed intake (g); FCR = feed conversion ratio (g of feed intake / g of body weight gain).
[a,b,c]Means of each main factor with different superscripts (a, b, c) in a row differ significantly ($p \leq 0.05$).
*Pooled standard error of mean.

experimental periods ($p \leq 0.05$). FI was not influenced by the inclusion of rye in broiler diets during various experimental periods ($p > 0.05$).

The FCR of broilers consuming diets with 400 g/kg of rye was higher than the other two groups during the starter and finisher periods ($p \leq 0.05$). Conversely, the FCR of broilers receiving diets with no rye was better than those on diets containing rye during the grower period ($p \leq 0.05$). At the end of the trial, broiler chickens consuming diets with 200 g/kg of rye (FCR of 1.49) exhibited better FCR ($p \leq 0.05$) compared to those receiving diets with 400 g/kg of rye (FCR of 1.53). Conversely, broilers fed 200 g/kg of rye diets displayed a worse FCR ($p \leq 0.05$) than those consuming diets with no rye (FCR of 1.44).

The inclusion of BP in diets did not have a significant impact on growth performance variables ($p > 0.05$), except for BWG and FI during the grower period. During the grower period, broilers on diets containing 1X and 10X of BP achieved higher BWG (692 and 688 g, respectively) than those on diets without BP (665 g). Additionally, FI of birds fed diets without BP (880 g) was lower ($p \leq 0.05$) than those fed diets containing 1X of BP (915 g). There were no observed interactions between rye and BP inclusion levels for the performance variables measured ($p > 0.05$).

As presented in Table 4, the inclusion of rye in diets led to a decrease in the CAID of crude protein, ether extract (EE), Ca, P, and all AA ($p \leq 0.05$). For most of these nutrients, except methionine, EE, and Ca, diets without rye exhibited the highest CAID, while diets with 400 g/kg of rye showed the lowest CAID ($p \leq 0.05$). In the case

Table 4. The effect of experimental diets on apparent ileal nutrient digestibility coefficients (mean[1] values) in broilers (d 35).

	Rye (RY [g/kg])			Probiotic (BP)[2]				*p*-value		
	0	200	400	0	1X	10X	SEM*	BP	RY	BP × RY
Crude protein	0.820[a]	0.771[b]	0.732[c]	0.768	0.771	0.783	0.0055	0.209	<0.001	0.979
Ether extract	0.879[a]	0.858[a]	0.740[b]	0.826	0.799	0.847	0.0106	0.072	<0.001	0.997
Phosphorus	0.534[a]	0.482[b]	0.449[c]	0.523[a]	0.480[b]	0.457[b]	0.0086	<0.001	<0.001	0.085
Calcium	0.411[a]	0.374[a]	0.325[b]	0.389	0.354	0.366	0.0100	0.217	0.001	0.126
Alanine	0.783[a]	0.738[b]	0.696[c]	0.735	0.736	0.746	0.0061	0.540	<0.001	0.592
Arginine	0.873[a]	0.850[b]	0.824[c]	0.851	0.846	0.852	0.0037	0.660	<0.001	0.757
Asparagine	0.787[a]	0.745 [b]	0.715[c]	0.750	0.750	0.746	0.0053	0.883	<0.001	0.406
Cysteine	0.834[a]	0.776[b]	0.745[c]	0.775[b]	0.783[ab]	0.798[a]	0.0060	0.041	<0.001	0.057
Glutamic acid	0.893[a]	0.847[b]	0.808[c]	0.848	0.847	0.853	0.0053	0.719	<0.001	0.736
Glycine	0.768[a]	0.718[b]	0.671[c]	0.717	0.716	0.725	0.0065	0.666	<0.001	0.830
Histidine	0.829[a]	0.796[b]	0.755[c]	0.793	0.788	0.799	0.0051	0.513	<0.001	0.658
Isoleucine	0.822[a]	0.772[b]	0.730[c]	0.774	0.767	0.782	0.0060	0.310	<0.001	0.812
Leucine	0.833[a]	0.787[b]	0.745[c]	0.787	0.784	0.795	0.0057	0.526	<0.001	0.767
Lysine	0.865[a]	0.839[b]	0.804[c]	0.834	0.834	0.840	0.0041	0.601	<0.001	0.455
Methionine	0.952[a]	0.930[b]	0.924[b]	0.931[b]	0.932[b]	0.943[a]	0.0020	0.000	<0.001	0.005
Phenylalanine	0.859[a]	0.817[b]	0.783[c]	0.818	0.815	0.826	0.0051	0.497	<0.001	0.704
Proline	0.857[a]	0.804[b]	0.757[c]	0.803	0.804	0.811	0.0064	0.694	<0.001	0.522
Serine	0.802[a]	0.751[b]	0.699[c]	0.747	0.748	0.757	0.0067	0.642	<0.001	0.429
Threonine	0.781[a]	0.729[b]	0.683[c]	0.727	0.727	0.738	0.0066	0.535	<0.001	0.172
Tyrosine	0.808[a]	0.770[b]	0.707[c]	0.759	0.764	0.761	0.0065	0.902	<0.001	0.708
Valine	0.922[a]	0.904[b]	0.888[c]	0.904	0.903	0.907	0.0023	0.564	<0.001	0.888
Total amino acids	0.846[a]	0.811[b]	0.786[c]	0.810	0.814	0.819	0.0044	0.597	<0.001	0.157

[1]Data are means of 8 replicate-pens with 5 birds per pen.
[2]1X = GalliProFit 1.2×10^6 CFU/g feed, 10X = GalliProFit 1.2×10^7 CFU/g feed – GalliProFit (Chr. Hansen, Denmark) consists of two *Bacillus subtilis* strains, DSM32324 and DSM32325 and a *Bacillus amyloliquefaciens* strain, DSM25840.
[a,b,c]Means of each main factor with different superscripts (a, b, c) in a row differ significantly ($p \leq 0.05$).
*Pooled standard error of mean.

Table 5. The effect of experimental diets (mean values) on the ileal and excreta viscosity[1] and litter quality score[2](d 35).

	Rye (RY [g/kg])			Probiotic (BP)				*p*-value		
	0	200	400	0	1X	10X	SEM*	BP	RY	BP × RY
Ileal viscosity [mP.s][3]	3.10^{c}	8.85^{b}	28.43^{a}	15.75	13.61	11.02	1.608	0.123	<0.001	0.069
Excreta viscosity [mP.s]	2.32^{b}	3.70^{b}	5.64^{a}	3.26	3.92	4.48	0.344	0.284	<0.001	0.687
Litter quality score	1.17^{c}	1.71^{b}	2.83^{a}	1.88	1.79	2.04	0.116	0.468	<0.001	0.750

[1]Data are means of 8 replicate-pens with 5 birds per pen.
[2]1X = GalliProFit 1.2×10^{6} CFU/g feed, 10X = GalliProFit 1.2×10^{7} CFU/g feed – GalliProFit (Chr. Hansen, Denmark) consists of two *Bacillus subtilis* strains, DSM32324 and DSM32325 and a *Bacillus amyloliquefaciens* strain DSM25840.
[3]mP.s = millipascal-seconds.
[a,b,c]Means of each main factor with different superscripts (a, b, c) in a row differ significantly ($p \leq 0.05$). *Pooled standard error of mean.

of methionine, diets with rye had lower CAID compared to rye-free diets ($p \leq 0.05$), and there was no significant difference in CAID of methionine between diets with 200 g/kg and 400 g/kg of rye ($p > 0.05$). Diets containing 400 g/kg of rye exhibited lower CAID of EE and calcium compared to the other two diets ($p \leq 0.05$), while there were no significant differences in these variables between diets with 0 g/kg and 200 g/kg of rye ($p > 0.05$).

The addition of BP to broiler diets did not significantly impact the CAID of most nutrients ($p > 0.05$), except for P, methionine, and cysteine. The inclusion of BP reduced the CAID of phosphorus ($p \leq 0.05$), with no significant difference between diets containing 1X and 10X of BP (0.523 *vs.* 0.480 and 0.457, respectively). Diets with 10X of BP showed higher CAID of methionine (0.943) compared to diets containing 0 (0.931) and 1X (0.932) of BP ($p \leq 0.05$). Furthermore, diets containing 10X of BP (0.798) displayed higher CAID of cysteine than diets without BP (0.775), with no significant difference between diets with 1X of BP (0.783) and the other two diets ($p > 0.05$).

For most of the measured variables, there was no interaction between rye and BP inclusion levels ($p > 0.05$), except for CAID of methionine. However, the interaction effect between rye and BP on CAID of methionine did not exhibit a clear trend ($p \leq 0.05$).

At the end of the trial, it was observed that the litter quality score (Table 5) increased as the inclusion level of rye in the diets increased ($p \leq 0.05$). Notably, the litter quality score for the group receiving 200 g/kg of rye (1.71) was superior ($p \leq 0.05$) to that of the 400 g/kg rye group (2.83) but inferior ($p \leq 0.05$) to the group with no rye (1.17). Similarly, ileal viscosity exhibited the same pattern, increasing with higher levels of rye in broiler diets (3.10, 8.85, and 28.43 mP.s, respectively) ($p \leq 0.05$). Excreta viscosity in broilers consuming 400 g/kg of rye (5.64 mP.s) was higher ($p \leq 0.05$) than in those on diets with 0 or 200 g/kg of rye (2.32 and 3.70 mP.s, respectively).

In Table 6, it is evident that the ileal concentration of glucosamine in broiler chickens receiving diets with 400 g/kg of rye (1165 mg/kg DM) was higher ($p \leq 0.05$) than in those on diets with no rye (852 mg/kg DM). Additionally, the ileal concentration of galactosamine in broiler chickens fed diets with 200 g/kg (519 mg/kg DM) and 400 g/kg (522 mg/kg DM) of rye was higher ($p \leq 0.05$) than in those fed diets with no rye (410 mg/kg DM).

Notably, the inclusion of BP in broiler diets did not have any significant impact on litter quality, ileal and excreta viscosity, as well as ileal concentration of galactosamine

Table 6. The effect of experimental diets (mean[1] values) on ileal concentration of glucosamine and galactosamine (mg/kg dry matter of ileal digesta – d 35).

	Rye (RY [g/kg])			Probiotic (BP)[2]				*p*-value		
	0	200	400	0	1X	10X	SEM*	BP	RY	BP × RY
Glucosamine	852[b]	1010[ab]	1165[a]	1091	1013	919	33.1	0.057	<0.001	0.956
Galactosamine	410[b]	519[a]	522[a]	502	507	442	15.1	0.110	0.002	0.797

[1]Data are means of 8 replicate-pens with 5 birds per pen.
[2]1X = GalliProFit 1.2×10^6 CFU/g feed, 10X = GalliProFit 1.2×10^7 CFU/g feed – GalliProFit (Chr. Hansen, Denmark) consists of two *Bacillus subtilis* strains, DSM32324 and DSM32325 and a *Bacillus amyloliquefaciens* strain, DSM25840.
[a,b]Means of each main factor with different superscripts (a, b) in a row differ significantly ($p \leq 0.05$).
*Pooled standard error of mean.

and glucosamine ($p > 0.05$). Moreover, there were no statistically significant interactions observed between rye and BP inclusion levels for these variables ($p > 0.05$).

4. Discussion

It has been well-documented that a high concentration of soluble-viscous NSP interferes with the digestion process and the diffusion and absorption of nutrients in the small intestine (Tejeda and Kim 2021). Incorporating *Bacillus*-based probiotics into NSP-rich broiler diets offers a potential solution to mitigate NSP-induced disturbances in nutrient digestibility and absorption. This mitigation can be attributed to the functional properties of probiotics and their capacity to produce a diverse array of enzymes, including NSP-degrading enzymes.

In this study, the incorporation of rye (200 and 400 g/kg) into wheat-soybean broiler diets (mash) from d 1 to 35 resulted in several noteworthy outcomes. It increased ileal digesta and excreta viscosity, reduced nutrient digestibility, and had a detrimental negative impact on litter quality and overall growth performance of the broilers. It is apparent that the elevated digesta viscosity induced by rye inclusion in the diets played a pivotal role in diminishing nutrient digestibility, subsequently leading to impaired BWG and FCR in broilers. This heightened digesta viscosity also rendered broiler excreta more adhesive, which, in turn, lowered litter quality. The present findings align with previous researches, where the inclusion of more than 100 g/kg of rye in broiler diets has consistently demonstrated a reduction in nutrient digestibility and overall growth performance (Bederska-Łojewska et al. 2017). The primary explanation for these adverse effects in prior studies has been the capacity of rye's arabinoxylans to retain water and increase digesta viscosity (Rahmatnejad and Saki 2016; Bederska-Łojewska et al. 2017). These effects have also been linked to the production of sticky-viscous faeces, contributing to decreased litter quality (Abd El-Wahab et al. 2020). It's important to note that the presence of wet litter and sticky-viscous faeces can elevate the prevalence and severity of footpad dermatitis (Abd El-Wahab et al. 2013). However, it is crucial to acknowledge that the magnitude of these adverse impacts can vary across studies due to differences in the chemical composition of rye varieties, the inclusion level of rye in experimental diets, the physical form and composition of diets, as well as variations in the experimental period and the age of broilers (Anderson and Sunderland 2002; Józefiak et al. 2007; Bederska-Łojewska et al. 2017).

Ileal viscosity increased significantly (3.10, 8.85, and 28.43 mP.s, respectively) as the inclusion level of rye in the diets rose ($p \leq 0.05$). Broilers consuming 400 g/kg of rye exhibited higher excreta viscosity ($p \leq 0.05$) compared to those on diets with 0 or 200 g/kg of rye (5.64 mP.s, 2.32 mP.s, and 3.70 mP.s, respectively). Notably, there was a substantial difference between ileal and excreta viscosity in the present study, likely attributed to the dense microbial population and intense bacterial fermentation occurring in the hindgut of broilers (Rinttilä and Apajalahti 2013). The pronounced disparity in ileal viscosity between diets containing 200 g/kg and 400 g/kg of rye can be attributed to the nonlinear relationship between the concentration of soluble viscous arabinoxylans in the diets and the resulting viscosity in the digestive tract. The initial 200 g/kg increment likely brought the rye concentration to a threshold level, introducing a significant amount of additional soluble NSP into the digestive system. This increase in soluble NSP led to a considerable rise in viscosity compared to the control diet with 0 g/kg rye, but it might not have saturated the system completely. Subsequently, the second 200 g/kg increase pushed the rye concentration beyond that initial threshold, introducing a substantial additional load of soluble NSP into the digestive tract. Consequently, this resulted in a more pronounced increase in viscosity compared to the diet with 200 g/kg of rye, as the cumulative effect of NSP from both increments contributed to a more substantial rise in viscosity.

It has been discussed in various publications that elevated digesta viscosity may extend the retention time of digesta in the gut, potentially reducing the FI of broilers (Lázaro et al. 2003, b; Goodarzi Boroojeni et al. 2016; Zentek and Goodarzi Boroojeni 2020). Interestingly, in the current study, while digesta viscosity increased with rye inclusion in the diets, FI of broilers remained unaffected.

In this study, glucosamine and galactosamine in the ileal digesta were also assessed as mucin markers, providing insights into how the applied treatments influenced mucin secretion and loss in the small intestine of broilers. The current data demonstrated that the inclusion of rye in the experimental diets resulted in increased mucus loss. This phenomenon has been discussed by Duangnumsawang et al. (2021), who proposed that the presence of bulky and viscous digesta, formed by NSP, can elevate luminal pressure and flow resistance. Consequently, this may lead to more abrasion of the mucus layer and, as a compensatory response, a higher differentiation of goblet cells to augment mucin secretion. Goblet cells are specialised columnar epithelial cells, playing a crucial role in mucin secretion (Duangnumsawang et al. 2021). In line with the present findings, previous studies have shown that the addition of either insoluble (2–4% cellulose) or soluble fibre (2–4% carboxymethyl cellulose) to broiler diets can stimulate goblet cell differentiation in the ileum (Rahmatnejad and Saki 2016; Murai et al. 2018). For instance, feeding diets containing insoluble dietary fibre compounds, such as rice hull (at 100 g/kg diet), to chickens led to increased MUC2 expression, an elevated number of goblet cells per villus, and increased mucin secretion in both the jejunum and ileum compared to control diets with cornstarch (Murai et al. 2018). Furthermore, the inclusion of various types of dietary fibre in pig diets has been shown to increase the excretion of mucins in the terminal part of the ileum (Montagne et al. 2003). It's important to note that the mucins released in the gut are resistant to the digestion process, signifying that the sloughed mucins can be regarded as endogenous losses of protein and energy for birds (Duangnumsawang et al. 2021).

In a previous study, it has been demonstrated that feeding *Bacillus*-based probiotics to broiler chickens can enhance gut functionality and nutrient digestibility, primarily attributed to the reduction in nutritional stress-induced dysbiosis (Goodarzi Boroojeni et al. 2018). Moreover, certain *Bacillus* strains, including *Bacillus subtilis* and *Bacillus amyloliquefaciens*, have exhibited the capability to produce a diverse array of enzymes, such as xylanases, amylases, lichenase, β-galactosidase, cellulases, alkaline serine proteases, and phytase (Latorre et al. 2015, 2016; Su et al. 2020). *In vitro*, the inclusion of two *Bacillus amyloliquefaciens* strains and one *Bacillus subtilis* strain in five distinct sterile soybean-based poultry diets containing corn (viscosity of 0.96 cP), wheat (viscosity of 1.55 cP), barley (viscosity of 1.75 cP), rye (viscosity of 8.40 cP), and oats (viscosity of 36.9 cP) led to a substantial reduction in viscosity (Latorre et al. 2015). Therefore, in the present study, it was hypothesised that the addition of 3-strain *Bacillus*-based probiotics (especially at a high concentration; 1.2×10^7 CFU/g) to broiler diets containing high levels of soluble-viscous arabinoxylans might mitigate the adverse impact of NSP on nutrient digestion and mucus loss. This mitigation could occur through the degradation of NSP and the reduction of digesta viscosity in the gut, while simultaneously ameliorating potential bacterial dysbiosis. In the current study, the addition of BP to broiler diets resulted in increased FI and BWG during the second and third weeks of age. These findings align with previous research where the inclusion of 500 ppm of an enzyme complex (containing 858 IU of β -glucanase and 864 IU of xylanase/g) in broiler diets with 50% rye led to a reduction in digesta viscosity in young broilers at d 11 (1cP = 1 mP. s; from 609 cP to 157 cP) and 25 (from 321 cP to 86 cP). This reduction was attributed to the enzymatic degradation of NSP and resulted in shorter digesta retention time (17.1 *vs.* 18.8 h) and higher FI (66.7 *vs.* 63.6 g/d) (Lázaro et al. 2003). However, the stimulating effect of NSP-degrading enzymes on feed intake can be less pronounced in adult birds (Almirall and Esteve-Garcia 1994; Lázaro et al. 2003). In this study also, the positive effect of BP on FI and BWG diminished as the birds matured.

Turkey poults fed rye-based diets supplemented with a *Bacillus*-based probiotic experienced a reduction in the total number of coliforms in the liver (2.13 *vs.* 0.35 CFU Log10/g tissue) and a decrease in digesta viscosity (2.8 *vs.* 1.62 cP). Furthermore, they exhibited an increase in tibia diameter (4.45 *vs.* 5.82 mm), ash content (35.61 *vs.* 50.87%), breaking strength (0.26 *vs.* 0.44 kg/mm2), calcium content (27.35 *vs.* 40.31% of ash), and phosphorus content (22.67 *vs.* 16.35% of ash) compared to those fed rye-based diets without *Bacillus*-based probiotic supplementation (Latorre et al. 2014). In this study, the inclusion of BP at a concentration 10 times higher than standard resulted in a significant increase ($p \leq 0.05$) in the CAID of methionine (from 0.931 to 0.943) and cysteine (from 0.775 to 0.798). Additionally, there was a tendency ($p = 0.057$) for a reduction in ileal concentrations of glucosamine. However, no significant impact was observed on other measured variables, including digesta viscosity, litter quality, and the digestibility of other nutrients. These findings suggest that the capacity of the applied *Bacillus* strains in this study may have been limited in terms of producing arabinoxylan-degrading enzymes. Consequently, it raises questions about the suitability of *Bacillus subtilis* (DSM32324 and DSM32325) and *Bacillus amyloliquefaciens* (DSM25840) strains for inclusion in broiler diets containing high levels of arabinoxylans. Meanwhile, it is important to consider that the effectiveness of enzymes derived from these *Bacillus* strains depends on the presence and concentration of metabolically active cells in the

broiler gut, particularly their proportion within the intestinal microbiota. Previous research has shown rapid germination and metabolic activity of *Bacillus cereus* var. *toyoi* in the gut of broilers and piglets (Jadamus et al. 2001). Similar effects could be expected for other *Bacillus* strains, including the ones used in this study. However, regarding the proportion of metabolically active probiotic cells within the intestinal microbiota, studies have reported that probiotic strains, such as *Enterococcus faecium* NCIMB 10,415, reached concentrations of 10^4 to 10^5 CFU/g digesta/faeces in piglets, indicating a relatively low proportion within the intestinal microbiota (Macha et al. 2004). Thus, low proportion metabolically active *Bacillus* cells within the intestinal microbiota may also contribute to the absence of expected effects in our study, even for chickens receiving diets with a 10X concentration of BP.

In conclusion, the incorporation of 200 and 400 g/kg rye into broiler diets resulted in elevated digesta viscosity, leading to reduced litter quality, increased mucin loss, decreased nutrient digestibility, and ultimately, poorer growth performance. However, the supplementation of BP to broiler diets exhibited a positive effect by enhancing feed intake and body weight gain in young broilers and improving the apparent ileal digestibility of methionine and cysteine. Notably, BP had no discernible impact on other measured variables. Although the overall growth performance fell below the breed's performance benchmarks, it appears that the combination of *Bacillus subtilis* (DSM32324 and DSM32325) and *Bacillus amyloliquefaciens* strain (DSM25840) strains had limited efficacy in producing arabinoxylans-degrading enzymes. Consequently, they only offered partial mitigation of the adverse effects of rye arabinoxylans on broiler chickens.

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