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Addition of probiotic *Bacillus subtilis* QST 713 to low protein diets for fattening pigs

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ABSTRACT

Objective: To evaluate growth performance, serum metabolites, and bacterial populations in fattening pigs fed standard and low protein (CP) diets supplemented with probiotics (GROBIGTM *Bacillus subtilis*) instead of antibiotics.

Design/Methodology: Twenty-eight pigs-gilts and barrows —were grouped according to a completely randomized design. The treatments involved two levels of protein in their diet, standard protein (SP) and low protein (LP), and two supplements, antibiotic (ANT) and probiotic (PROB) *B. subtilis*.

Results: The probiotic supplemented to standard diets affected the growth performance of nursery pigs. In the following stages (growing, finishing I, and finishing II), neither the CP level, the antibiotic, nor the probiotic affected growth performance in any way. In finishing phases I and II the concentration of triglycerides was lower with the standard CP diet and antibiotic. Urea in plasma was not affected by the CP level.

Study limitations/Implications: The large number of bacteria in pigs' intestines limits the ability to specifically identify the type of populations modified when providing an antibiotic, reducing the percentage of CP, or adding the probiotic to the feed.

Findings/Conclusions: Including *B. subtilis* in a standard protein diet affects the growth performance variables of nursery pigs and serum metabolites in the finishing stage. The probiotic can replace the antibiotic in the diet of fattening pigs.

Key words: Bacteria, Pathogens, Probiotics, Safety, Synergy.

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INTRODUCTION

To reduce excessive nitrogen (N) excretion and the use of antibiotics in pig farming (Upadhaya *et al.*, 2022), some researchers have proposed reducing protein in diets by using synthetic amino acids (Hartog and Sijtsma, 2007) while maintaining the same productive

performance (Rocha *et al.*, 2022) of traditional diets. Antibiotics have given rise to multi-resistant pathogens (Adekunle *et al.*, 2020), so probiotics constitute an alternative, even more so since their use improves health (Pereira *et al.*, 2022) and growth in pigs (Magnoli *et al.*, 2022) by helping to fight pathogenic bacteria (Kwak *et al.*, 2021). Strains of the *Bacillus* genus have been studied for zootechnical purposes (Milián *et al.*, 2017) because their thermostable spores survive transit through the gastrointestinal tract (GIT) (Cutting, 2011). *Bacillus subtilis* protects lactic acid bacteria in unfavorable environmental conditions and works strongly against pathogenic bacteria (Kimelman and Shemesh, 2019). Tested in production pigs, it improved the immune response (Ayala *et al.*, 2012), and in piglets it decreased cholesterol and triglycerides, and increased glucose levels (Patiño *et al.*, 2019). Our study aims to evaluate productive variables, serum metabolites, and populations of Coliforms, *E. coli*, and lactic acid bacteria in feces of fattening pigs fed diets with standard and low protein and supplemented with an antibiotic (PISAMIX® PLUS) or probiotic (GROBIGTM BS) based on *Bacillus subtilis* QST 713.

MATERIALS AND METHODS

This study was conducted at the Swine Unit of the Experimental Farm of the Colegio de Postgraduados, Montecillo, Campus, in Texcoco, State of Mexico. The handling of the animals followed the recommendations of the "Regulations for the use and care of animals intended for research" created by the Animal Welfare Committee of the Colegio de Postgraduados. A total of 28 pigs were used in the experiment, gilts and barrows (Large White×Hampshire×Duroc), housed in individual pens (1.2 m×1.5 m) with a nipple drinker and a hopper feeder to provide feed and water *ad libitum*.

The treatments consisted of a dietary combination of two protein levels, standard protein (SP) and low protein (LP), and two supplements, antibiotic (ANT)-PISAMIX® PLUS —and probiotic (PROB)-GROBIGTM *Bacillus subtilis* —administered under manufacturer instructions. There were four treatments: T1: SP+ANT, T2: SP+PROB, T3: LP+ANT, T4: LP+PROB. The evaluated stages were: nursing (21 days), growing (28 days), finishing I (21 days), and finishing II (21 days). The diets were based on corn, soybean meal, and synthetic amino acids (lysine, methionine, threonine, and tryptophan), which cover the requirements of each stage as recommended by the National Research Council (NRC, 2012), whose protein/stage percentages were used as the standard level and decreased by two percentage units to establish the low protein level. Diets were formulated using the Excel Solver command (Microsoft Excel, 2019). The SP and LP levels were as follows: nursing - 19% and 17%; growth - 16.5% and 14.5%; finishing I - 14% and 12%; and finishing II - 12% and 10%.

Response variables and sampling. Growth performance: average daily gain, ADG; average daily feed intake, ADFI; feed conversion, FC; final weight, FW. Serum metabolites: cholesterol, triglycerides, glucose, and urea. Populations of Coliforms, *E. coli*, and lactic acid bacteria in finishing stage II. At the beginning and end of each stage, we measured the back fat thickness (BFT) and the area of the *Longissimus dorsi* muscle (LMA) at the last floating rib, using ultrasonography equipment (Sonovet 600) with a convex transducer of 3.5 Mhz (Medison, Inc., Cypress, CA. USA) to calculate Fat

Free Lean gain (FFLG) and lean meat percentage (LMP) (Burson and Berg, 2001). At the end of each stage, blood samples were taken by puncture of the anterior vena cava from each animal, using Vacutainer® tubes with heparin (BD® Vacutainer Systems, NJ, USA). The blood samples were then centrifuged (Sigma 2-16k centrifuge, Germany) for 20 min at 2683 *g*. The plasma was placed in polypropylene tubes and stored at −20 °C pending analysis in the laboratory. Also feces samples were taken in the second-to-last and last week of finishing stage II and one week after removing the antibiotic or probiotic from the diets. Samples were from four animals per treatment at 8:00 a.m. using rectal stimulation. The samples were kept in sterile polypropylene containers and stored in a cooler for immediate analysis.

Laboratory analyses. Total levels of cholesterol (TC), triglycerides (TRIG), glucose (GLU), and urea in plasma (UP) were quantified in duplicate using commercial SPINREACT® kits. Readings were made with a visible light spectrophotometer (Beckham DU65) at a wavelength of 505 nm for TC, TRIG, and GLU, and 510 nm for UP.

Feces samples were analyzed under aseptic conditions, by diluting a 10 g sample in 90 mL of sterile peptone saline (0.1% peptone and 0.85% sodium chloride) and homogenizing it. Serial dilutions were made from the supernatant up to 10^{-6}. Then 1 mL of each dilution was seeded in duplicate on Petrifilm™ plates (3M™ 6404) to quantify Coliforms and *E. coli*; and 500 μL per plate with MRS agar (Difco™ Lactobacilli MRS Agar) to quantify lactic acid bacteria. All plates were incubated at 37 °C. The Petrifilm™ plates were read after 24 h to quantify Coliforms and 48 h to quantify *E. coli*; MRS plates were monitored until colony growth stopped. The data were expressed as colony-forming units per gram of dry matter (CFU g^{-1} DM) and as $\log^{10}$.

Statistical analysis. The experiment had a completely randomized design. To analyze the growth performance variables (ADG, ADFI, FC, BFT, final LMP, and LMA), the initial weight of each stage was used as a covariate. Bacterial population count was analyzed as a generalized block design. The LSMEANS procedure was used to analyze all variables with the statistical package SAS, 2010. For means comparison, the Tukey test was used with a significance level of $P \leq 0.05$. Shapiro-Wilk normality tests ($\alpha = 0.05$) were performed on all variables per treatment.

RESULTS AND DISCUSSION

During the nursery stage (Table 1), the pigs consuming the SP+PROB diet showed a lower ADG ($P \leq 0.05$), which could have negatively affected final weight ($P \leq 0.05$) and increased FC ($P \leq 0.05$). Results show that supplementing a SP diet with *B. subtilis* QST 713 might have affected protein digestion, even though the level of urea in plasma was similar ($P > 0.05$) between treatments. Likewise, we observed a higher LMA ($P \leq 0.05$) in pigs consuming the SP+ANT diet, compared to those with the SP+PROB diet. Thus, considering the same CP level, antibiotics increased dietary protein usage, although the final LMP was not affected ($P > 0.05$). Moreover, it was observed that by decreasing CP in diets by 2 percentage units, the *B. subtilis* probiotic promoted a higher final LMP ($P \leq 0.05$) and increased TC ($P \leq 0.05$). Higher glucose levels in plasma ($P \leq 0.05$) in pigs consuming nursery diets with LP (T3 and T4), compared to those fed with the SP+PROB diet, concur

with the observations of Spring *et al.* (2020): reducing CP affects nitrogen, starch, and sucrose metabolism, provoking high levels of glucose and serum cholesterol.

In the growing stage (Table 1), no differences were observed ($P>0.05$) between treatments in the analyzed growth performance variables, concurring with Kerr *et al.* (2003), according to whom CP levels can be reduced in the growing-finishing stages by 2 or 4 percentage units without affecting the response. Another study posits that growth is not affected, but rendering is (Gonzalo *et al.*, 2022). These variations depend on the quantity of protein subtracted. As stated by Martínez-Aispuro *et al.* (2009), reducing the CP concentration in a diet by up to 11.5% negatively affects ADG and FC, and decreases UP. Nevertheless, UP and other serum metabolites in our study were similar ($P>0.05$) between treatments. In Table 1 can be observed that SP and LP levels in finishing I stage diets did not affect the growth performance ($P>0.05$) or UP levels. Hence, the reduction must be higher to increase the efficacy of N utilization (Goodarzi *et al.*, 2023).

The data obtained in our study using *Bacillus subtilis* QST 713 in finishing stages I and II differ from those observed in other studies (Hao *et al.*, 2020) that used *Bacillus subtilis* and *Pediococcus pentosaceus* in finishing stage pigs, where levels of glucose, triglycerides, and urea in serum were not affected.

In finishing stage II (Table 1), pigs fed with the SP+PROB diet had higher levels of TRIG, GLU, and UP ($P\leq0.05$), compared to animals fed with the SP+ANT diet. This indicates that, in diets with 12% CP, *B. subtilis* impacted pig metabolism to a higher degree than antibiotics. Increased GLU levels ($P\leq0.05$) may be due to the probiotic, as proved by the administration of *B. subtilis* in piglets (Patiño *et al.*, 2019) and chickens (Ortega *et al.*, 2020). However, TC and TRIG did not decrease, as in these two studies. Additionally, higher TRIG levels ($P\leq0.05$) in the SP+PROB diet, compared to the LP+PROB diet, indicate that reducing protein levels may promote a better performance of *B. subtilis* on the diet components and a better nutrients utilization.

It was also observed that UP levels showed no changes ($P>0.05$) in diets with SP and LP, which contrasts with the results obtained by Figueroa *et al.* (2019), who found that low protein diets improved or maintained growth performance variables (as in our study) but lowered the UP level —an effect we did not accomplish.

Low CP diets reduce substrate availability for bacteria such as Coliforms and *E. coli* (Rodríguez *et al.*, 2012; García *et al.*, 2013). In turn, the lactate produced by acid lactic bacteria helps reduce the GIT's pH level, thus blocking the growth of potentially pathogenic bacteria such as *E. coli* (Vasquez *et al.*, 2022). When comparing the effects of diets with the same level of protein, the counting of microbe population in feces during finishing stage II (Figure 1) showed that both antibiotics and probiotics had the same efficacy (in the first sampling—M1) against Coliforms since Coliform population was similar ($P>0.05$) both among pigs fed with SP diets (T1 and T2) and among those fed with LP diets (T3 and T4). However, the latter favored a lower count of Coliforms compared to the SP + PROB diet, and feces of animals fed with the SP + ANT diet had a higher count ($P\leq0.05$) of Coliform than those of pigs fed with the LP + PROB diet.

Table 1. Effects of pig diets considering two protein levels supplemented with antibiotics or probiotics.

	PVI (kg)	PVF (kg)	CDA (kgd^{-1})	CA	GDP ($kg\ d^{-1}$)	PCMF (%)	GCM ($kg\ d^{-1}$)	GD (mm)	AML (cm^2)	CT ($mg\ dL^{-1}$)	TRIG ($mg\ dL^{-1}$)	GLU ($mg\ dL^{-1}$)	UP ($mg\ dL^{-1}$)
Nursery													
T1	24.00	38.52^{a}	1.53	2.10^{b}	0.74^{a}	40.11^{ab}	0.25^{ab}	7.16	16.03	86.46^{b}	43.49	139.59^{ab}	47.29
T2	22.64	33.66^{b}	1.46	2.89^{a}	0.51^{b}	42.46^{a}	0.19^{c}	7.33	16.84	102.12^{bc}	51.69	126.47^{b}	47.21
T3	22.71	37.52^{a}	1.52	2.21^{b}	0.69^{a}	40.05^{b}	0.23^{bc}	7.46	15.87	100.08^{bc}	54.20	160.10^{a}	50.86
T4	22.64	36.63^{a}	1.54	2.40^{b}	0.65^{a}	41.05^{ab}	0.22^{bc}	7.33	16.46	107.28^{ac}	44.81	159.45^{a}	52.67
EE	0.93	1.11	0.03	0.08	0.02	0.38	0.01	0.15	0.65	2.83	1.94	5.12	2.16
P	-	<0.01	0.73	<0.01	<0.01	0.03	0.04	0.73	0.64	0.05	0.14	0.05	0.76
Growing													
T1	-	61.65	2.38	2.67	0.89	39.13	0.34	11.03	23.96	117.67	57.69	124.01	36.62
T2	-	63.01	2.19	2.37	0.94	39.46	0.33	11.30	25.30	125.72	88.57	141.06	47.95
T3	-	60.55	2.22	2.62	0.86	39.16	0.33	11.20	23.91	122.08	93.94	138.10	42.09
T4	-	61.40	2.44	2.76	0.89	39.47	0.33	11.33	24.71	119.15	87.78	149.58	48.28
EE	-	1.60	0.06	0.05	0.03	0.27	0.01	0.23	0.75	1.98	6.11	4.78	1.97
P	-	0.74	0.27	0.06	0.75	0.96	0.96	0.96	0.71	0.50	0.14	0.30	0.11
Finishing I													
T1	-	80.09	2.46	2.75	0.88	38.96	0.33	13.73	30.49	138.50	43.66^{c}	111.97	51.18
T2	-	77.50	2.38	2.97	0.76	39.88	0.29	13.46	31.54	171.98	77.29^{b}	123.21	56.23
T3	-	79.78	2.45	2.87	0.86	38.62	0.31	14.17	30.13	159.06	82.57^{ab}	133.65	47.80
T4	-	80.30	2.59	2.95	0.89	38.95	0.33	13.93	30.83	164.65	60.09^{bc}	119.06	57.73
EE	-	1.91	0.04	0.08	0.04	0.25	0.02	0.27	0.83	4.62	3.68	3.20	2.09
P	-	0.59	0.21	0.79	0.60	0.35	0.76	0.69	0.81	0.06	<0.01	0.11	0.31
Finishing II													
T1	-	99.67	2.84	3.43	0.96	37.99	0.33	16.01	34.84	116.94	35.02^{b}	93.98^{b}	35.08^{b}
T2	-	100.22	2.81	3.42	0.99	37.84	0.30	16.15	34.61	109.72	61.79^{a}	119.58^{a}	44.51^{a}
T3	-	99.09	2.95	3.17	0.94	37.45	0.31	16.85	33.91	114.16	51.46^{ab}	104.15^{ab}	39.31^{ab}
T4	-	99.38	3.03	3.23	0.95	37.62	0.31	16.98	34.60	118.49	87.78	106.85^{ab}	41.69^{ab}
EE	-	2.28	0.05	0.25	0.05	0.15	0.02	0.36	0.83	2.93	2.68	2.72	1.08
P	-	0.98	0.20	0.97	0.98	0.64	0.94	0.58	0.93	0.74	<0.01	<0.01	0.01

T1: Standard protein + antibiotic; T2: Standard protein + probiotic; T3: Low protein + antibiotic; T4: Low protein + probiotic; IW (PVI): Initial weight; FW (PVF): Final weight; ADFI (CDA): Average Daily feed Intake; FC (CA): Feed conversion; ADG (GDP): Average Daily Gain; LMC (PCM): Lean meat percentage; FFLG (GCM): Fat Free Lean gain; BFT (GD): Back Fat Thickness; LMA (AML): *Longissimus dorsi* muscle area; TC (CT): Total cholesterol; TRIG: Triglycerides; GLU: Glucose; UP: Urea in plasma; $^{a, b, c}$ Means with different letters are significantly different ($P \leq 0.05$).

The abovementioned results indicate that reducing CP in the diet by 2 percentage units can also reduce the substrate needed for Coliform proliferation. Still, this measurement did not affect (P>0.05) *E. coli* and acid lactic bacteria populations between treatments in M1.

As for the second sampling (M2), Coliform populations dropped (P≤0.05) in pigs consuming SP diets (T1 and T2), compared to M1, while populations remained stable in LP diets (T3 and T4). This behavior can also be observed for *E. coli*. In both cases, no differences between treatments (P>0.05) were observed. During this same period, the acid lactic bacteria count remained stable (P>0.05) in both treatments and samplings. These results show that microbe populations fluctuate from one week to another, even in the same animals, under the same conditions, and following the same diets.

One week after removing antibiotics or probiotics from diets (M3), the Coliform, *E. coli*, and acid lactic bacteria counts did not vary (P>0.05) when compared to M2. This may be due to a residual effect of the products, which must have continued to act on the Coliform populations, whose count was similar (P>0.05) between treatments. Still, the *E. coli* count was higher in feces of pigs fed with the SP + PROB diet, compared to those of pigs fed with

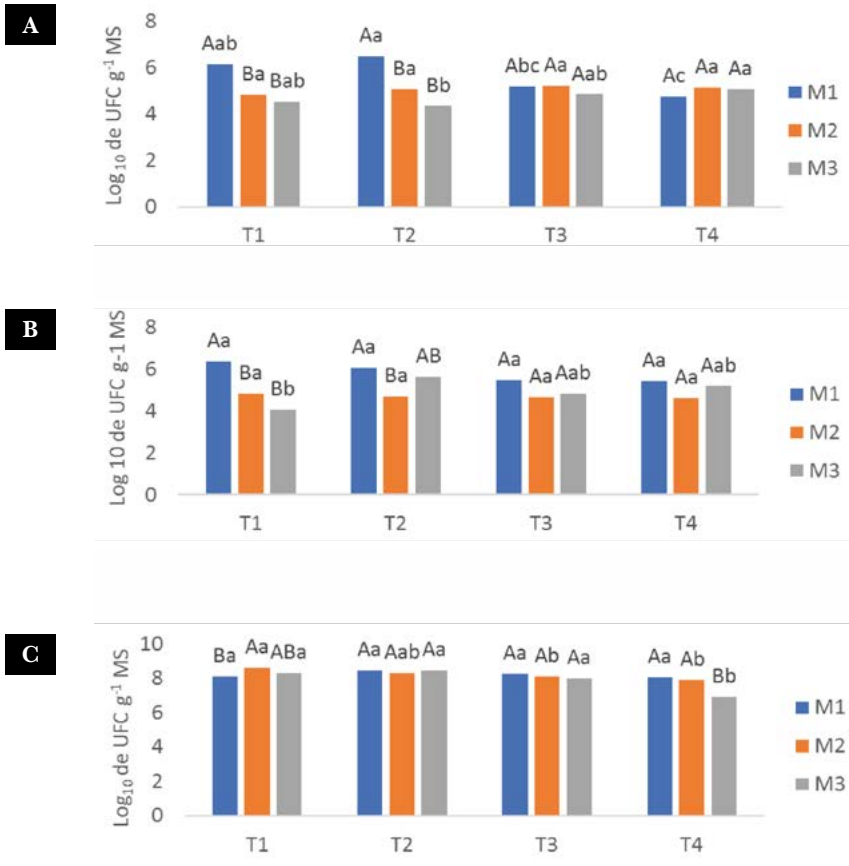

Figure 1. Coliform (A), *E. coli* (B), and acid lactic bacteria (C) counts in feces of pigs in finishing stage II fed with two levels of protein supplemented with antibiotic or probiotic during the two last weeks of this stage (M1 and M2) and a week after removing the products (M3). A, B, C Different uppercase letters are statistically different means in each treatment of the different samplings. a, b, c Different lowercase letters are statistically different means between treatments in the same sampling.

the SP + ANT diet, which shows that in diets with 12% CP the residual effect of probiotics was lower than that of antibiotics.

The acid lactic bacteria count in feces of animals fed with the LP+PROB diet was lower ($P \leq 0.05$) compared to the other treatments, which can be due to the protective effect of *B. subtilis* over these bacteria with this kind of diet. As some authors point out (Kimelman and Shemesh, 2019), this effect decreased when the products were removed from the diet, something that was not observed in animals consuming probiotics in the SP diet.

CONCLUSIONS

Reducing protein in pigs' diets by two percentage units does not lead to a decrease in the levels of urea in plasma. Using *B. subtilis* in standard protein diets for pigs affects growth performance variables in the nursery stage and serum metabolites during the finishing stages. Antibiotics and probiotics have the same efficacy in diets with standard and low protein during the growing stage. Protein levels affect the protective activity of *B. subtilis* and its residual effect. Probiotics can replace antibiotics in the feed of fattening pigs.

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