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Effects of *Bacillus spp.* as a supplemental probiotic in diets for weaned piglets

M. Dumitru[1], M. Hăbeanu[1], I. Sorescu[1,2#] & C. Tabuc[1]

[1]National Research Development Institute for Biology and Animal Nutrition (IBNA), Bucharest, No. 1, Balotesti, Ilfov, 077015, Romania

[2]The Institute of Diagnostic and Animal Health, Bacteriology Laboratory, Street Dr. Staicovici, No. 63, District 5, 050557, Bucharest, Romania

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Abstract

This article evaluated the effects of supplemental probiotic *Bacillus subtilis (Bs)* ATCC 6051a ($1.6x10^9$ cfu/mL) in diets for weaned piglets on their performance and on the occurrence of diarrhoea. Sixty piglets, 30 ±3 days old with initial bodyweight of 8.41±0.92 kg, were allotted randomly to six pens of ten piglets. There were two replicates of each treatment, namely a control diet (C), a diet supplemented with 1% *Bs* (E1), and a diet supplemented with 3% *Bs* (E2). Feed was provided ad libitum as flour in two meals per day. Feed materials were examined for total numbers of fungi, aerobic mesophilic bacteria (TNG), Coliforms, *Escherichia coli* and *Salmonella* spp. The addition of *Bs* did not influence (*P* >0.05) bodyweight (BW) or average daily weight gain (ADWG). However, across the experimental period ADWG was greater in E2 and E1 than in C (>1.12 and 1.08 times compared with C). Feed intake (ADFI) by pigs fed C was greater than pigs fed E1 and E2. Feed efficiency was higher in E1 and E2 than the C diet. Addition of 1% *Bs* decreased (*P*<0.05) diarrhoea occurrence around 8% compared with C, and 4% compared with 3% *Bs*. A total of 23.4% of the piglets produced soft faeces. Diarrhoea scores of 2 (mild diarrhoea) and 3 (severe diarrhoea) were observed in 43.75% and 32.81% of the pigs. No differences (*P* >0.05) were detected between the treatments. The results suggested that E1 could positively affect growth performance and mitigate the occurrence of diarrhoea.

Keywords: feed additive, microbiology, performance, weaned pig, weaning diarrhoea
[#]Corresponding author: mihaela.dumitru22@yahoo.com

Introduction

Weaning is a difficult period for piglets owing to incomplete development of their enzymatic systems and digestive disturbances (Dlamini *et al.*, 2017), with these factors generating stress (Lee *et al.*, 2014; Habeanu *et al.*, 2015). At weaning, piglets must adjust to a solid diet instead of the milk provided by the sows, and their endogenous enzymatic system requires several days to adjust (Guevarra *et al*., 2019). Early weaning removes piglets from an easily digested feed source and the gastrointestinal tract (GIT) is susceptible to adverse consequences as a result (Taylor & Roese, 2006). A managerial challenge in weaning piglets is to reduce the incidence of digestive disorders. The first sign of gastrointestinal disorder is diarrhoea (Mach *et al.*, 2015; Nowland *et al*., 2019). Antibiotics have long been used in feeding piglets to maintain their digestive health, but routine feeding of antibiotics has led to problematic levels of microbial resistance.

Probiotics are non-pathogenic live organisms, which, when administered in sufficient amounts, produce beneficial effects on the health of the host (FAO, 2001). Among other effects, probiotics may reduce the number of pathogenic bacteria in the GIT, and maintain a stable population of intestinal microbiota (Yirga, 2015; Dumitru *et al*., 2020a). Thus, probiotics may be beneficial to piglets around the time of weaning (Corcionivoschi *et al*., 2010). Probiotic products can contain a single or multiple strains of the bacterial species *Bacillus* (*B. cereus* var. *toyoi, B. licheniformis, B. subtilis*), *Enterococcus* (*E. faecium), Lactobacillus* (*L. acidophilus, L. casei, L. farciminis, L. plantarum, L. rhamnosus*), *Pediococcus* (*P. acidilactici*), *Streptococcus* (*S. infantarius*), and microscopic fungi such as yeasts *Saccharomyces cerevisiae* and *Kluyveromyces* (FAO, 2016; Dumitru *et al*., 2018; Sorescu *et al*., 2019). Known as a strict aerobe (Hu *et al*., 2015), *Bacillus* was noticed for its advantages because it is spore forming (Kim *et al*., 2019). In addition, *Bacillus* spp. tolerates the

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low pH in the stomach, is resistant to bile salts, and has thermo-stability during processing and long-term storage of feed (Ragul *et al*., 2017). Studies that used *Bacillus* spp. as a direct-fed microbial supplement (Leser *et al*., 2008) reported favourable results in swine diet with beneficial effects on growth and feed efficiency (Wang *et al*., 2011; Link *et al*., 2016). In addition, *Bacillus* spp. was reported to be a possible antimicrobial growth promoter and alternative to antibiotics for animals (Bedford *et al*., 2014).

Therefore, this study was designed to investigate the efficacy of *Bacillus subtilis* ATCC 6051a as a probiotic by studying its effects on growth performance and the incidence of diarrhoea when fed to weanling piglets.

Materials and Methods

The animals were provided by INCDBNA Balotesti Experimental Farm, Romania. The experiment was carried out according to the protocol approved by the Ethical Committee of the National Research-Development Institute for Animal Nutrition and Biology Balotesti (INCDBNA), Romania (Habeanu *et al*., 2015). The procedures were in agreement with Council Directive 2010/63/EU legislation for the protection of animals used for scientific purposes. No antibiotic was supplied to the animals during the experiment.

Piglets used in the experiment were a hybrid of Topigs germplasm TN Talent x (Large White × (Large White × Pietrain). At the end of the study, the piglets were maintained at the Experimental Farm IBNA Balotesti, Romania, until they reached a bodyweight of 25 ± 3 kg, when they were sold.

The crude protein (CP) of the diet was determined using a semi-automatic classical Kjeldahl method with a Tecator Kjeltek auto analyser 1030 (FOSS – Tecator AB, Hoganas, Sweden). The fat was established as ether extract (EE) by continuous extraction in solvent with a Soxhlet apparatus. The crude fibre (CF) was determined with the classical semi-automatic Fibertec-Tecator method (FOSS – Tecator AB, Hoganas, Sweden) and the ash content was determined by incineration at 550 °C until the sample reached a constant mass. The nitrogen-free extract (NFE) was calculated with the formula:

$$NFE = \text{Dry matter} - (CP + EE + CF + Ash).$$

Metabolizable energy (ME) was calculated with the regression equation developed by the Oskar Kellner Institute of Animal Nutrition:

$$ME = 5.01 \times dCP + 8.93\ dEE + 3.44\ dCF + 4.08\ dNFE$$

where: dCP, dEE, dCF and dNFE are digestible CP, EE, CF and NFE.

Microbiological examination of the samples followed the protocols described in the Romanian standards STAS 6953-81, namely SR 13178-1: total number of fungi (TNF) SR 13178-2: total number of germs (TNG), SR 13178-2: total number of Coliforms) (*E. coli*), and SR 12824: *Salmonella* spp. Plates were incubated aerobically. Results were expressed as the average of three dilutions as logarithm (base 10) colony-forming units per gram of sample (cfu/g).

The microbial feed additive in the current study was based on *Bacillus subtilis* ATCC 6051a *(Bs).*The *Bs* strain was grown in nutritive medium (Merck KGaA, Darmstadt, Germany), at 37 °C for 24 hours under shaking agitation (150 rpm) and aerobic conditions. The culture strain was prepared in liquid form every two days and stored at 4 °C in a sterile bottle. The optical density of the culture was measured and re-suspended in sterile physiological saline (0.85%, w/v) to a concentration of 1.6 x 10^9 cfu/mL/g^{-1} feed viable spore. Each day the supplement was mixed manually with the basal diet for the piglets.

Growth performance was evaluated with 60 Topigs piglets weaned at 30 ± 3 days old with an average initial bodyweight of 8.41 ± 0.92 kg. The piglets were divided randomly into three groups distributed in six pens of ten piglets, two replicates for each group, namely the control group (C) and two experimental groups each receiving a supplement of *Bs* strain at 1% (E1) and 3% (E2) level. The minimum bacterial concentration of *Bs* was 1.6 x 10^9 cfu/mL/g^{-1} feed. The pens measured 4.3 m^2, each with slatted plastic flooring. Each pen had one self-feeder and a nipple-type drinking fountain. Ventilation was delivered by a mechanical system with automatic adjustments. The room temperature was approximately 25 °C. The experiment lasted 16 days, although generally after weaning, the mortality rate because of digestive disorders is highest in the first seven days (Habeanu *et al.*, 2015). The feed (Table 1) was provided in flour form twice daily according to appetite and water was provided ad libitum. The intake and refusals of feed were recorded daily. Animals from C group were fed the control diet without *Bs* supplementation. In E1 and E2 the concentrate portion of the ration was unchanged, but different levels of supplement were used.

The animals were monitored daily and the severity of diarrhoea was recorded. The faeces of every animal were examined visually in the morning at 08h00 after the piglets had been fed. A subjective scoring system was used to determine the severity of diarrhoea, ranging from 1 to 3, namely 1: soft faeces; 2: mild

diarrhoea, 3: severe diarrhoea, which was monitored by the same evaluator. The incidence of diarrhoea was expressed as the average number of days with diarrhoea related to the total monitoring days (Habeanu *et al.*, 2015). Throughout the experiment, final bodyweight (FBW), average daily feed intake (ADFI) (g feed/piglet/day), ADWG (g/piglet/day), and feed conversion ratio (FCR) (g feed/g gain) were also recorded.

The data were fit to a general linear model using SPSS version 20.0 (IBM Corp., Armonk, New York, USA). The results were expressed as mean and standard error of mean. The diets were considered fixed factors. The effects were considered significant at P-value ≤ 0.05, and were regarded as a trend when $0.05 < P < 0.10$. If a fixed effect was significant, the means were compared with the Tukey post hoc test. Pearson correlation coefficients were used to assess relationships among the traits.

Table 1 Compound feed formula and chemical composition for piglets after weaning

Ingredients, %	Control	E1 1% *Bacillus subtilis*	E2 3% *Bacillus subtilis*
Maize	33.48	33.15	32.50
Sorghum	25.00	24.75	24.27
Peas	17.00	16.83	16.50
Soybean meal	13.00	12.87	12.62
Maize gluten	3.00	2.97	2.91
Milk replacer	5.00	4.95	4.85
DL-methionine	0.10	0.10	0.10
L-lysine	0.21	0.21	0.20
Calcium carbonate	1.60	1.58	1.55
Phytase	0.01	0.01	0.01
Mono-calcium phosphate	0.40	0.40	0.39
Salt	0.10	0.10	0.10
Premix choline	0.10	0.10	0.10
Vitamin-mineral premix[1]	1.00	0.99	0.97
Bacillus subtilis ATCC 6051a	-	1.00	3.00
Calculated chemical composition, g/kg feed			
Metabolizable energy, ME kcal/kg	3,237	3,237	3,237
Crude protein	18.23	18.23	18.23
Lysine	1.20	1.20	1.20
Digestible lysine	0.95	0.95	0.95
Methionine + cysteine	0.69	0.69	0.69
Digestible methionine + cysteine	0.61	0.61	0.61
Ether extract	2.20	2.20	2.20
Calcium	0.90	0.90	0.90
Total phosphorus	0.70	0.70	0.70
Available phosphorus	0.32	0.32	0.32
Cellulose	4.11	4.11	4.11

[1]Vitamin A: 10 000 IU, vitamin D_3: 2 000 IU, vitamin E: 30 IU vitamin K_3: 3 mg vitamin B_1: 2 mg vitamin B_2: 6 mg. vitamin B_3: 20 mg, vitamin B_5: 13.5 mg, vitamin B_6: 3 mg, vitamin B_7: 0.06 mg, vitamin B_9: 0.8 mg, vitamin B_{12}: 0.05 mg, vitamin C: 10 mg, manganese 30 mg, iron: 110 mg, copper: 25 mg, zinc: 100 mg, iodine: 0.38 mg, selenium: 0.36 mg, cobalt: 0.3 mg, antioxidant: 60 mg per kg of complete diet

Results and Discussion

At the beginning of testing, the animals had not exhibited any signs of illness and were kept in similar environmental conditions. The results of the bacteriological examination of the raw materials in the diets are described in Table 2.

Table 2 Microbiological analysis of compounded feeds and ingredients in diets for piglets

Raw materials		TNG SR 13178-1	Total Coliforms SR 13178-2	*Escherichia coli* SR 13178-2	*Salmonella* spp. SR EN 12824
Maize		6.9×10^3	2.5	2.5	N
Sorghum		3.4×10^3	0.0	0.0	N
Peas		3.6×10^3	0.0	0.0	N
Soybean meal		3.6×10^3	0.0	0.0	N
Gluten		8.5×10^4	0.0	0.0	N
Milk replacer		6.6×10^3	0.0	0.0	N
Feed 01 B	Batch 1	9.0×10^3	0.5	0.0	N
	Batch 2	4.2×10^4	16.5	9.5	N

Allowed maximal limits: (MO 362 bis/2003) TNG: total number of aerobic mesophilic bacteria, maximum 1.5×10^7 cfu/g; Total coliforms: maximum 3×10^3 cfu/g, *E. coli:* maximum 1×10^2 cfu/g; *Salmonella* spp.: 0 cfu/g (N = negative); SR: Romanian standard, SR EN: European standards

The number of aerobic bacteria did not exceed 10^7 cfu/g for TNG, 10^3 cfu/g for Coliforms, and 10^2 cfu/g for *E. coli*. According to EN regulation *Salmonella* spp. must be excluded in 25 g feed materials. The total number of germs defines how many aerobic, mesophilic microorganism colonies such as bacteria, yeast and mould fungi will grow for 24--48 hours on agar nutritive at 37 °C. The mycological status of the raw materials indicated that contamination was within the normal range, that is, not exceeding 10^3 cfu/g (Table 3). Based on these results, the raw materials used in this study had low levels of contamination and could be used in the diets for piglets.

Table 3 Mycological analysis of compounded feeds and ingredients in diets for piglets

Raw materials		TNF (cfu/g) STAS 6953-81	Colonies identified
Maize		3.5×10^3	*Aspergillus flavus* (5×10^2 cfu/g); *Fusarium graminearum* (1×10^3 UFC/g); *Fusarium culmorum* (7.5×10^2 cfu/g); *Penicillium* sp.; yeasts
Sorghum		Sterile	-
Peas		Sterile	-
Soybean meal		2.5 x 10	*Aspergillus flavus* (2.5 x 10 cfu/g)
Gluten		Sterile	-
Milk replacer		Sterile	-
Feed 01 B	Batch 1	1.1×10^4	*Aspergillus flavus* (2.75×10^2 cfu/g); *Aspergillus niger*; *Fusarium graminearum* (2.5×10^2 cfu/g); *Rhizopus nigricans*; yeasts
	Batch 2	6.5×10^3	*Fusarium graminearum* (2.5×10^2 cfu/g); *Rhizopus nigricans; Absidia spp.*; Yeasts

Allowed maximal limits for TNF: total numbers of fungi in raw materials = 5×10^3 cfu/g, in feed = 5×10^4 cfu/g; and for fungal species known to produce mycotoxins = 5×10^2 cfu/g, STAS: state standards

No significant effects were noticed between groups (P >0.10) in bio-productive performances from the addition of probiotic bacteria, although numerically greater values were registered for E1 and E2 throughout the experiment (Table 4). The values of FBW, ADWG and feed efficiency were similar to those of the literature on the addition of *Bacillus*-based probiotic when a concentration of 2×10^9 cfu/g^{-1} feed was used (Upadhaya *et al*., 2015).

Table 4 Effects of *Bacillus subtilis* ATCC 6051a supplementation on growth performance of piglets weaned at 30 ± 3 days old

Performance measure	Control	E1	E2	SE	*P*-value
BW at weaning, kg	8.44	8.42	8.38	0.14	0.47
BW at weaning + 7d, kg	9.80	9.76	9.70	0.15	0.92
BW at weaning + 16d, kg	12.72	13.02	12.63	0.23	0.75
ADG 1st week, kg/d	0.20	0.18	0.22	0.01	0.22
ADG 16 d, kg/d	0.26	0.29	0.28	0.23	0.52
ADFI, kg/day	0.52	0.48	0.45		
Feed efficiency	0.50	0.61	0.58		

BW: bodyweight, ADG: average daily gain, ADFI: average daily feed intake, *Bs*= *Bacillus subtilis* ATCC 6051a, 1.6 x 10^9 UFC/mL/g^{-1} feed; E1: basal diet + 1% *Bs,* E2: basal diet + 3% *Bs*.

In this study, no serious digestive disorders were observed and differences among the treatments were not significant (Figure 1). Some piglets in each group were affected by diarrhoea, with the mild score being predominant. E1 decreased diarrhoea incidence around 8% compared with C group and 4% compared with E2. A total of 23.4% piglets had soft faeces, 43.75% had score 2 (mild diarrhoea), and 32.81% had score 3 (severe diarrhoea). In the present study, GP may be linked to diarrhoea occurrence, which was noted daily. Some numerical differences between groups might be associated with the probiotic treatment. However, some studies have found that the use of probiotics could significantly improve intestinal health and promote growth of weanling piglets (Liu *et al*., 2018).

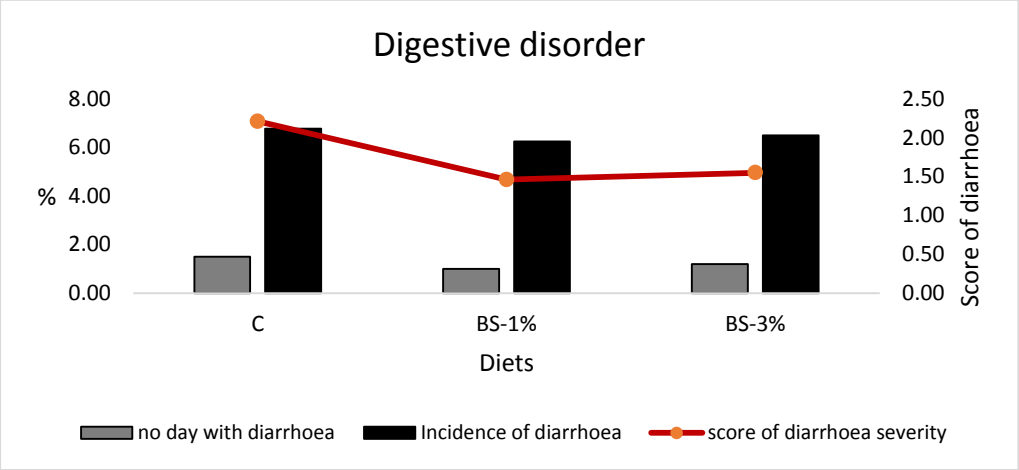

Figure 1 Incidence and severity score of diarrhoea in weaned piglets

Weaning is a worrying period in the life cycle of pigs because it is associated with changes in diet, environment and gut morphology, and may result in low growth percentage, high diarrhoea incidence and imbalanced intestinal ecology (Giang *et al*., 2012). Hu *et al*. (2014) supposed that *B. subtilis* strain could balance piglet microbiota by stimulating beneficial bacteria, improving health of the GIT, diminishing diarrhoea incidence and enhancing GP. In addition to separation from their dam at weaning, several other factors can influence the piglets' lives including mixing with other litters in an unfamiliar environment and changing their diet from easily digestible milk to less digestible solid ingredients (Lalles, 2007; Giang *et al*., 2012; Pluske *et al*., 2018).

Animal feed can be exposed to numerous factors (biological, chemical, physical and other agents), which can affect their health status, and, indirectly, that of human beings (Giang *et al*., 2012). The microbiological quality of feed is important in the health status of animals (Kwiatek, 2011). Feed is considered one of the main carriers of bacteria pathogens in animal production (Fink-Gremmels, 2012). Additionally, the

use of raw materials in feed must be safe for and appropriate to animal nutrition. Generally, raw materials are recognized as safe or considered acceptable for use in feed (Pluske *et al*., 2018). Feed composition and feeding handling are critical issues that influence the health status of piglets after weaning (Nuntapaitoon *et al*., 2018). In this study, analyses of raw materials did not find microbial contamination exceeding the maximum allowable cfu/g (Table 2). For example, total number of microbes per gram provides an indication of feed quality that results from production processes and sanitary conditions during plant growth, harvest, storage and transport (Kwiatek, 2011). Contamination of the feed with *E. coli* and *Salmonella* spp. bacteria are considered indicators during the feed production process (Kelley & Walker, 1999). Generally, plants are the principal source of fungi and contamination may take place during storage (Kwiatek, 2011). Like bacteria, the presence of moulds in feeds implies a risk to animal health arising from ingredients of animal origin.

In the current study, 13% (w/w) soybean meal was included as the main source of protein for animal production (Lallès, 2008). The addition of *Bs* as a direct-fed microbial product could ferment the soybean meal through hydrolysis of protein to amino acids and peptides (Dong *et al*., 2014; Kiers *et al.*, 2003). The vegetative cells of *Bacillus* could secrete extracellular products (Bernardeau *et al*., 2017) as enzymes (carbohydrase, lipase, protease, etc.) that are involved in the degradation of anti-nutritive fractions from feed (Asmare, 2014). For example, the *Bacillus* group can produce extracellular enzymes as protease (Degering *et al*., 2010), which are involved in the process of digestion with an improvement in the GP of animal (Kaewtapee *et al*., 2017).

In the current study, the addition of *Bs* to piglet feed was in accordance with European Food Safety Authority Guide (2021) which affirms that the minimum inclusion level in feedstuffs for piglets is 1 x 10^9 cfu/g^{-1} feed (Yuan *et al*., 2017). The presence of spores as bacterium protection enable *Bacillus* spp. to withstand environmental conditions such as pH, bile salts, temperature, radiation, pressure, and chemical agents, and factors that can destroy the vegetative form (Bernardeau *et al.*, 2017). Dumitru *et al*. (2019; 2018a) reported that *B. subtilis* ATCC 6051a tolerated well GIT conditions such as low pH and bile salts, making it a commensal bacterium for animals that ingest it (Vasques, 2016). The *Bacillus subtilis* ATCC 6051a strain also tolerated 80 °C temperature for two hours which allows its inclusion in animal diets even if it is ground and pelleted (Dumitru *et al*., 2019). However, the results of supplementation of feed with *Bacillus* spp. are variable. Chen *et al*. (2013) reported that the supplementation of *B.subtilis* var. *natto* improved the BW and ADFI of geese in a concentration of 10^8 cfu/kg feed and AWDG of broilers (Zhang *et al*., 2012). Other researchers reported that the supplementation of *B. subtilis* to diet improved the growth performance of pigs (Alexopoulos *et al*., 2004; Wang *et al*., 2011; Lee *et al*., 2014). *Bacillus*-based probiotic had significant effects on ADWG and ADFI of weaned piglets by reducing the feed conversion ratio (Nuntapaitoon *et al*., 2018; Liao & Nyachoti, 2017). *Bacillus* spp. supplementation had a significant effect on performance parameters, nutrient digestibility, and faecal microbiota in pig diets (Cheng & Kim, 2019; Balasubramanian *et al*., 2016). For example, the addition of two levels of *Bacillus licheniformis* (1.6 x 10^9 cfu and 4.8 x 10^9 cfu) in piglet feed improved BW and AWDG significantly when a high concentration was added (Dumitru *et al*., 2020b). The variations in the results of these studies could be attributed to factors such as diet composition, feed form and their interaction with other bacterial strains used as dietary additives (Chesson, 1994).

The effect of a probiotic could be affected by strain composition and inclusion levels, but positive effects of probiotics on growth performance were always observed in the early period after weaning (Kiers *et al.*, 2003). Ahmed *et al*. (2014) reported that the inclusion of *Bacillus* in piglet feed increased ADWG and ADFI through 0 to 28 days after weaning (3.2 x 10^6 cfu/g).

The faecal and intestinal microbial population of piglets with and without *Bacillus subtilis* ATCC 6051a was evaluated by Dumitru *et al.* (2020b). The results showed that *Lactobacillus* counts were higher in the ileum and faeces of piglets on the diet with *Bs*1% with a decrease in pathogenic bacteria such as *Escherichia coli* biotype *β*-hemolytic, which is a major source of serious illnesses.

Fluctuations in the GIT microbiota after weaning and the time taken to adapt to the new feed may cause a gastrointestinal infection, mostly *Colibacillosis* diarrhoea, which produces extensive morbidity and mortality (around 17% of piglets born in Europe) in the most serious cases (Nam *et al*., 2012). The results of this study indicated that feeding E1 to piglets could balance diarrhoea incidence during weaning (Figure 1), with an effects that was more pronounced than for E2 probably because of variation in the response of individual animals Furthermore, a negative Pearson correlation was found between level of *Bacillus* and AWDG over 16 days (r = -0.41).

In the literature (Cai *et al*., 2015), it was stipulated that *Bacillus* spp. is not a principal member of normal animal microbiota and could not colonize the intestine for long periods. It consumes oxygen rapidly and reduces the intestinal pH which favours Lactobacilli and inhibits pathogens such as *E. coli* and *Salmonella* spp. As a gram-positive bacterium with the ability to form endospores, *Bs* can endure high temperatures of animal feed pelleting and remain stable for long-time storage (Rychen *et al*., 2018).

Post-weaning diarrhoea in piglets is characterized by the frequent ejection of soggy faeces during the

first weeks after weaning and represents one of the major economic problems for the pig industry worldwide (Cai *et al.*, 2015). The current data are in accord with several studies that refer to the addition of *Bs* products as a means of promoting GIT health with stable beneficial bacteria, and enhancing growth performance (Kritas *et al*., 2004; Sun & Kim, 2017; Wu *et al.*, 2018) and reducing the incidence and severity of diarrhoea in weaning piglets (Baker *et al.*, 2013; Pluske, 2013).

Conclusions

The beneficial effects of *Bacillus subtilis* ATCC 6051 were not statistically significant. However, dietary supplementation with *Bs* might improve the health and growth of piglets in the weaning crisis. Experimentation with different concentrations of *Bs* is necessary to establish the beneficial effects of this supplemental probiotic on piglets during weaning.

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Authors' Contributions

MD was responsible for conducting and monitoring the experiment, in vitro testing probiotic properties, which were presented in another study (Dumitru *et al*., 2018a), and preparing bacterial culture based on *Bs*. MH was involved in the feeding trial and performed statistical analysis. IS and CT conducted sample collection and laboratory analysis. All authors read and approved the final manuscript.

Conflict of Interest Declaration

The authors declare that there is no conflict of interest.

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