

# The effects of *Bacillus* spp. use as a dietary probiotic in weaning piglets

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## Research article

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# Abstract

**Background:** This study was conducted to evaluate the effects of supplementation with *Bacillus*-based probiotic (*Bacillus subtilis* ATCC 6051a, 1.6x10<sup>9</sup>CFU/ml) on the performance of weaned piglets.

**Methods:** A total of 60 piglets, 30 days±3 days of age, with an initial body weight (BW) of 8.41±0.92 kg were allotted randomly to 3 homogeneous groups (C, E1 and E2, 6 pens/10 piglets, 2 replicates/group) with a supplement of additive (Bs) based on 1% (E1), respectively 3% (E2)/kg feed. The feed was administered ad libitum in the flour form, 2 meals/day. Microbiological analysis and chemical composition of compound feed, the growth performances and diarrhoea incidence were assayed as well.

**Results:** The addition of Bs didn't influence significantly the BW or average daily gain (ADG). After 16 testing days, ADG was 1.13 times higher in E1 compared with C and with 1.04 times higher compared with E2. The feed intake (ADFI) increased with 1.08-fold in C diet compared with E1 diet. Feed efficiency diet was 1.22 times higher in E1 than C and with 1.05 higher than E2 diet.

**Conclusions:** Addition of Bs-1% decreased diarrhoea incidence ( $P>0.05$ ) by 7.6% vs. C and 3.8% vs. Bs-3%. 23.4% piglets had soft faeces, 43.75% score 2 (mild diarrhoea) and 32.81% score 3 (severe diarrhoea), no significant difference was noticed between the group ( $P\geq 0.05$ ).

## Background

Probiotics represent a group of non-pathogenic organisms, which were administered in sufficient amounts, are known to involve beneficial effects of the host health [1, 2]. Probiotics are implied in reducing the number of pathogenic bacteria from the animal gastrointestinal tract (GIT), maintaining and stabilizing a healthy microbiota as a preventive measure during the critical period of weaning [3]. Several probiotic products may contain a single strain or multi-strain from 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*); microscopic fungi such yeast - *Saccharomyces cerevisiae* species and *Kluyveromyces* [4, 5, 6].

Known as a strict aerobe bacterium, *Bacillus subtilis* was noticed to be a possible candidate in monogastric nutrition. The high resistance of the vegetative cells due to the spores presence and the severe environmental conditions make the *Bacillus* species to grow in anaerobe conditions [7]; furthermore, the thermostability is a major property of *Bacillus* which certifies the long-term storage during the pelleting of animal feed.

Studies with dietary supplementation of *Bacillus* spp. product used as a direct-fed microbial (DFM) [8] has reported favorable results in swine diet [9, 10]. Specialty literature described the addition of *Bacillus* spp. as DFM could have some beneficial effects by improving the growth performance and feed efficiency in piglets.

Weaning is a difficult period for piglets due to their incomplete development of the enzymatic system and factors generating stress [11, 34]. Piglets in the weaning crisis must adjust to a solid diet as contrasting to the liquid diet provided by

the sow and their endogenous enzymes will take the same several days to correct it [12]. Early weaning removes piglets from a digestible feed source, and the gastrointestinal tract (GIT) complications are more susceptible to install it [13]. Besides, *Bacillus* spp. was reported to be a possible antibiotic alternative for animals [14].

The objective of this study was to investigate the efficacy of a probiotic containing *Bacillus subtilis* ATCC 6051a on growth performance and diarrhoea in weaning piglets diet feed.

## Methods

The animals were provided by the National Research-Development Institute for Animal Nutrition and Biology Balotesti (INCDBNA) Experimental Farm, Romania. Conducting the experiment, managing weaning and samples collection was in accordance with the research topic presented by the Habeanu et al. [11].

The experimental protocol was adopted by the Ethical Committee of the INCDBNA, Romania. The procedures were in agreement with Council Directive 2010/63/EU legislation for the protection of animals used for scientific purposes. No antibiotic treatment was supplied to the animals during the experiment. The experiment was performed on Topigs hybrid (♂ Large White × Hybrid (Large White × Pietrain) × ♀ Talent, mainly Duroc].

At the end of the study, the piglets were maintained in the Experimental Farm IBNA Balotesti, Romania, until they recorded the weight of  $25 \pm 3$  kg. After that, the animals were sold to the beneficiaries.

## Microbiological analysis of feed

Compounds feed were analyzed microbiologically in the biotechnological laboratory of INCBNA Balotesti. Procedures of sample examination were following the protocol described in STAS 6953–81 (total number of fungi, NTF), SR 13178–1 (total number of germs, TNG), SR 13178–2 (total number of coliforms), SR 13178–2 (*E. coli*), SR 12824 (*Salmonella* spp.). The result was expressed as a logarithm (base 10) of colony-forming units per gram of sample (CFU/g).

## Source of microbial feed additive

The microbial feed additive used in the current study was based on *Bacillus subtilis* ATCC 6051a. The strain's probiotic properties were analyzed *in vitro*, in another study [5].

*Bacillus subtilis* ATCC 6051a strain was grown in nutritive agar medium (Merck), at 37°C, 24h under shaking agitation (150 rpm) and aerobic conditions. The culture strain was prepared in culture form, every

two days and stored in a fridge at 4°C in a sterile bottle. The optical density (OD) of culture was measured and resuspended with physiological saline sterile, for adjusted to  $1.6 \times 10^9$  CFU/ml/kg feed viable spores. Each day, the supplement was mixed manually with the portion of the basal diet, which was previous calculated for piglets feeding.

## Animals and diet

The biological test was conducted on 60 piglets Topigs hybrid [½ Large White × Hybrid (Large White × Pietrain) × ½ Talent, mainly Duroc] weaned at 30 days ± 3 days of ages, the average initial body weight of  $8.41 \pm 0.92$  kg. The piglets

were randomly allotted to 3 groups distributed in 6 pens with 10 piglets, two replicates for each group: a control group (C) and 2 experimental groups with a supplement of *Bacillus subtilis* (*Bs*) bacterial strain based on 1% (E1, *Bs*-1%), respectively 3% (E2, *Bs*-3%). The minimum bacterial concentration of *Bs* was  $1.6 \times 10^9$  UFC/ml/kg feed. Pens measured 2.40 m × 1.80 m × 0.800 m, each with slatted plastic flooring. All pens had one self-feeder and a nipple drinker. Ventilation was delivered by a mechanical system with automatic adjustments. The room temperature was approximately 25°C. The experiment lasted 16 days. The feed was administered *ad libitum* in the flour form, two meals/day with permanent access to water. The intake and refusal of feed were recorded daily. The feed structure is shown in Table 1.

Animals from C group were fed with the classical diet without *Bs*-feed additive. The groups *Bs*-1% and *Bs*-3% were received the same concentration of *Bacillus subtilis* ATCC 6051a, but in different levels of feed addition. The animals were supervised daily to identify the piglets with diarrhoea severity (%). The faeces of every animal were examined visually. A subjective scoring system was used to determine the severity of diarrhoea, ranging from 1 to 3: 1 soft faeces; 2 mild diarrhoea, 3: severe diarrhoea. The incidence of diarrhoea was expressed as the average number of days with diarrhoea related to the total monitoring days (seven days) [11].

Throughout the experimental period we supervised the following parameters: body weight (BW), average daily feed intake (g feed/piglet/day, ADFI), average daily gain (g/piglet/day, ADG), feed conversion ratio (g feed/g gain, G: F).

## Statistical analysis

The data were analyzed with SPSS Statistics version 20.0 (2011) and were expressed as the mean and standard error of the mean (SEM). A probability value of  $P \leq 0.05$  was considered to be statistically significant and tendencies were noted under conditions of  $0.05 < P < 0.10$ .

## Results

### Raw material microbiology

The results of the bacteriological examination of the raw materials used in piglet diet are presented in Table 2. The number of aerobic bacteria did not reach  $10^7$  for TNG,  $10^3$  for coliforms, respectively  $10^2$  for *E. coli*. The presence of *Salmonella* spp. according to EN Regulation, is essential to be excluded in 25 g feed materials. Furthermore, TNG defines how many aerobic, mesophilic microorganism colonies, such as bacteria, yeast, and mold fungi will grow for 24–48 hours on an agar nutritive plate at 37°C. Mycological analyses of raw materials were shown in Table 3 and the contamination rate is in normal value, did not exceed  $10^3$  CFU/ml.

Based on the results of microbiological analyses, the raw materials present a low level of contamination and can be used in piglets' diet.

## Growth performance

The growth performance may be linked to diarrhoea incidence, and the diarrhoea appearance of piglets was noted daily in this study. The piglets were weighed individually at the beginning of the experiment ( $30\text{d} \pm 3$ ) and on day 16. The feed offered and refused was weighed daily.

In this study, we noticed some differences between groups caused by treatment. At the beginning of testing the animal had not any sign of illness and were kept in similar environmental conditions.

Table 4 shows the bio-productive performances obtained by supplemented feed with a bacterial preparation-based *Bacillus subtilis*.

The addition of *Bs* did not influence significantly the body weight (BW) or average daily gain (ADG). After 16 testing days, the ADG was 1.13 times higher in the *Bs*-1% group compared to the C group and 1.04 times higher vs. *Bs*-3% group respectively. The feed intake (ADFI) increased

1.08-fold in C diet than the *Bs*-1% diet. Feed efficiency was 1.22 times higher when the diet was supplemented with *Bs*-1% compared to C diet and 1.05 times vs. *Bs*-3%. The values of BW, ADG, G/F are similar with the literature results [15] at the addition of *Bacillus*-based probiotic when a concentration of  $2 \times 10^9$  CFU/kg feed was used, but no significant results were found between treatments.

Addition of *Bs*-1% vs. *Bs*-3% improved BW of piglets weaning in the 1st and 3rd week, but the values were insignificant ( $P > 0.05$ ).

## Diarrhoea incidence

In our study, we did not notice a serious digestive disorder. Higher effectiveness had 1% dietary supplement of *Bs* (Figure 1). Addition of *Bs*-1% decreased diarrhoea incidence by 7.6% compared to the C group ( $P > 0.05$ ) and by 3.8% vs. *Bs*-3% group. 23.4% piglets had soft faeces, 43.75% had score 2 (mild diarrhoea) and 32.81% had score 3 severe diarrhoea; however, no significant difference was noticed between groups.

In the current study, some piglets in all treatment's groups were affected by diarrhoea, the mild score being predominantly. Piglets fed diets supplemented with *Bs*-1% had lower diarrhoea frequency than C and *Bs*-3% group, but no differences were noted ( $P>0.05$ ). The addition of *B. subtilis* ATCC 6051a in piglets feed did not result in any improvement in the diarrhoea frequency.

## Discussion

Weaning is a worrying period in the life cycle of pigs, which is associated with changes in diet, gut environment and gut morphology, and thus may result in low growth percentage, high diarrhoea incidence and imbalanced intestinal microecology [16]. The weaning period is a critical time in whole piglet's life, because the young pigs are separated by the sow, resulting in an abrupt change in diet, a move to a new environment and mixing with unfamiliar animals, generally younger [17, 18]. All raw materials used in the diet must to be safe and suitable for their utilization in animal nutrition. Generally, raw materials are recognized as safe (GRAS) or otherwise deemed acceptable for use in feed [18]. The composition of feedstuff and feeding management are critical issues that influence the health state of piglets after weaning [19]. The animal feed can be exposed to numerous factors (biological, chemical, physical and other agents), which can affect the animal status health and, indirectly, human [18]. Previous studies with supplementation of *Bacillus*-based probiotic had a significant effect on ADG and ADFI of weaned piglets, reducing the F: G [19, 20].

In our study, soybean meal was included 13% (w/w) as main protein resource for animal production [21] and the addition of *Bacillus subtilis* can ferment it, as affirmed Kiers et al. [24], due to the hydrolysis of protein to amino acids and peptides [22]. *Bacillus subtilis* strain concentration is in concordance with EFSA Guide,,Panel on Additives and products or substances used in Animal Feed" which affirms that the minimum inclusion level in piglets feeding stuffs is  $1 \times 10^9$  CFU/kg feed [23].

The effect of a probiotic could be affected by strain composition and inclusion levels, but rendering to the results of previous reports, it should be noted that the positive effects of probiotics on growth performance were always observed in the early period after weaning [24].

The dramatic fluctuations in GIT after weaning and the gut needs time to adapt to the new feed, may cause a gastrointestinal infection, mostly colibacillosis diarrhoea, which producing an extensive morbidity and/or mortality (around 17% of piglets born in Europe) in the most serious of affected cases [25].

*Bacillus* spp. is not a principal member of the normal animal microbiota and could not colonize the intestine for long periods; it consumes oxygen rapidly and reduces the pH value, which this parameter favors Lactobacilli number and inhibits pathogens like *E. coli* and *Salmonella* spp. [27]. As a Gram-positive bacterium and the ability to form endospores, *Bacillus subtilis* can endure the high temperatures of animal feed pelleting and also, to be stable for long time storage [26].

Post-weaning diarrhoea (PWD) is a condition in weaned piglets characterized by the frequent ejection of soggy faces during the first 2 weeks after weaning and continues to represent one of the major economic

problems for the pig industry worldwide [27]. Several studies with the addition of *B. subtilis* as DFM products may be a means for GIT health by beneficial bacteria stability, reducing diarrhoea incidence and enhancing the growth performance [28, 29].

The results from previous studies [30, 31] found that *Bacillus* supplementation reduced the incidence and severity of diarrhoea in weaning piglets.

## Conclusions

The results of this study indicate that dietary supplementation with *Bacillus subtilis* ATCC 6051a (1.6 x 10<sup>9</sup>UFC/kg feed), did not influence significantly the body weight gain, feed intake while feed conversion ratio in the weaned piglets.

The results suggest that the addition of *Bacillus*-as DFM did not ameliorate the incidence of diarrhoea of piglets during experimental design.

Further experiment will be performed with different concentrations of analyzed strain, until it will establish if it can be considerate a possible probiotic for piglets weaning.

## Abbreviations

BW: body weight; *Bs*: *Bacillus subtilis* ATCC 6051a; ADG: average daily gain; ADFI: feed intake; *vs.*: vis-à-vis; DFM: direct-fed microbial; OD: optical density; GIT: gastrointestinal tract; PWD: post-weaning diarrhea; TNG: total numbers of germs; TNF: total numbers of fungi.

## Declarations

### Ethical approval and consent to participate

The experimental protocol was carried out in accordance with the ethical guideline for the Care and Use of Laboratory Animals and approved by the Research Committee of INCDBNA Balotesti, Romania (No. 5183).

### Consent for publication

„Not applicable”.

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

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## Authors' contributions

MD\* was responsible for conducting and monitoring of the experiment, *in vitro* testing of probiotic properties which are presented in another study [2], preparing of bacterial culture based on *Bs*. MH involved the feeding trial and performed statistical analysis. IS and CT conducted sample collection and laboratory analysis. The all authors read and approved the final manuscript.

## Authors' information

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## Tables

**Table 1.** Compound feed formula and chemical composition used for hybrid Topigs piglets during weaning crisis

Items %*/	Control	E1 Bs-1%	E2 Bs- 3%
Maize	33.48	33.48	33.48
Sorghum	25	25	25
Peas	17	17	17
Soybean meal	13	13	13
Maize gluten	3	3	3
Milk replacer	5	5	5
DL methionine	0.1	0.1	0.1
L- Lysine	0.21	0.21	0.21
Calcium carbonate	1.6	1.6	1.6
Phytase	0.01	0.01	0.01
Monocalcium phosphate	0.4	0.4	0.4
Salt	0.1	0.1	0.1
Premix choline	0.1	0.1	0.1
Vitamin-mineral premix	1	1	1
Bacterial additive (Bs)**	-	1	3
<b>Chemical composition % (g/kg feed)</b>			
Metabolizable energy (EM, Kcal/ Kg)	3237.31	3237.31	3237.31
Crude protein (CP)	18.23	18.23	18.23
Lysine	1.2	1.2	1.2
Methionine + Cystine	0.59	0.59	0.59
Ether extract	2.2	2.2	2.2
Ca	0.9	0.9	0.9
P	0.7	0.7	0.7
Cellulose	4.11	4.11	4.11

\*ME was calculated based on feed composition and theoretical coefficients. The vitamin-mineral premix contained (/kg feed): 10 000 IU vitamin A; 2 000 IU vitamin D3; 30 IU vitamin E; 3 mg vitamin K3; 2 mg vitamin B1; 6 mg vitamin B2; 20 mg vitamin B3; 13.5 mg vitamin B5; 3 mg vitamin B6; 0.06 mg vitamin B7; 0.8 mg vitamin B9; 0.05 mg vitamin B12; 10 mg vitamin C; 30 mg Mn; 110 mg Fe; 25 mg Cu; 100 mg Zn; 0.38 mg I; 0.36 mg Se; 0.3 mg Co; 60 mg antioxidant. \*\*Bs-*Bacillus subtilis*

Source: compound feed formula calculated, Mihaela Habeanu, INCDBNA Balotesti

**Table 2.** Bacteriological analysis of the compound feeds

Raw materials		TNG	Total Coliforms	<i>Escherichia coli</i>	<i>Salmonella</i> spp.
		SR 13178-1	SR 13178-2	SR 13178-2	SR EN 12824
Maize		6.95 x 10 <sup>3</sup>	2.5	2.5	N
Sorghum		3.4 x 10 <sup>3</sup>	0	0	N
Peas		3.6 x 10 <sup>3</sup>	0	0	N
Soybean meal		3.6 x 10 <sup>3</sup>	0	0	N
Gluten		8.5 x 10 <sup>4</sup>	0	0	N
Milk replacer		6.6 x 10 <sup>3</sup>	0	0	N
Feed 01 B	Batch 1	9.0 x 10 <sup>3</sup>	0.5	0	N
	Batch 2	4.2 x 10 <sup>4</sup>	16.5	9.5	N

Allowed maximal limits: (MO 362 bis/2003): TNG: maximum 1.5 x 10<sup>7</sup> CFU/g; Total coliforms: maximum 3 x 10<sup>3</sup> CFU/g; *E. coli*: maximum 1 x 10<sup>2</sup> CFU/g; *Salmonella* spp.: 0 CFU/g (N = negative). Where: SR= Romanian standard; SR EN= European standards

**Table 3.** Mycological analysis of raw materials

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

Allowed maximal limits: (MO 362 bis/2003): TNF - total numbers of fungi; Raw materials: 5 x 10<sup>3</sup> CFU/g; Feed 5 x 10<sup>4</sup> CFU/g. Fungal species known to produce mycotoxins 5 x 10<sup>2</sup> CFU/g. Where: STAS = State standards

**Table 4.** Effects of *Bacillus subtilis* ATCC 6051a on BW, ADG, ADFI and G: F

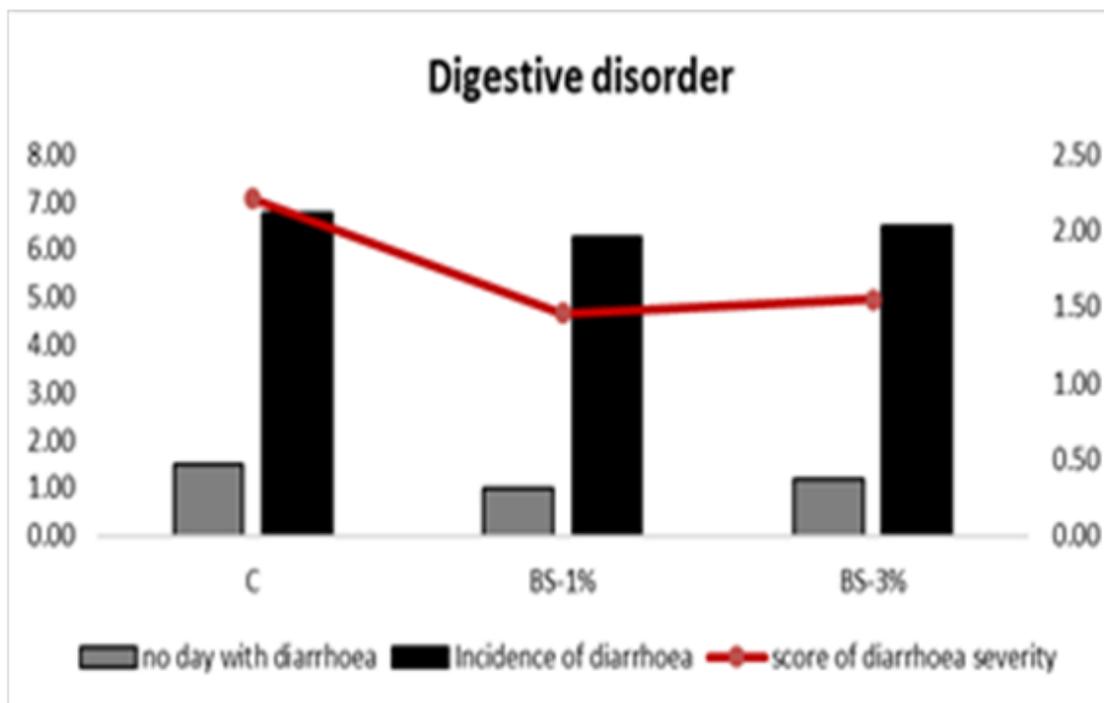
on piglets weaning

Item*	Control	Bs-1%	Bs-3%	SEM	P- value**
BW at weaning (30d ± 3)	8.44	8.42	8.38	0.14	NS
BW -1 <sup>st</sup> w	9.80	9.76	9.70	0.15	NS
BW- w 3	12.72	13.02	12.63	0.23	NS
ADG 16 d	0.260	0.293	0.282	0.23	NS
ADG 1 <sup>st</sup> w	0.20	0.18	0.22	0.01	NS
ADFI (Kg/head/ day)	0.520	0.481	0.452		
G: F	0.50	0.61	0.58		

\*BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; G: F (Gain: feed) = feed conversion ratio; \*\*NS = insignificant; T = a tendency to be influence by the treatment; Bs-1%: basal

diet + *Bacillus subtilis* ATCC 6051a 1%,  $1.6 \times 10^9$  UFC/ml/kg feed; Bs-3%: basal diet + *Bacillus subtilis* ATCC 6051a 3%,  $1.6 \times 10^9$  UFC/ml/kg feed.

## Figures



**Figure 1**

Diarrhoea and score of diarrhoea severity. The animals were monitored daily. Incidence of diarrhoea = average number of days with diarrhoea related to the total monitoring days