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Original paper

The effect of *Bacillus licheniformis* as direct-fed microbial product on growth performance, gastrointestinal disorders and microflora population in weaning piglets

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Simple Summary

The direct-fed microbial (DFM) supplied as live bacteria represents an alternative to antibiotics for maintaining health and performance in weaning piglets. It was been recognized as probiotic products. Early weaning is a valuable model to get improved efficiency and economical profits in the modern intensive swine industry, but negative factors as diarrhea diminished feed intake, weight loss and gastrointestinal disturbances which can affect the piglet health functions with serious economic costs. To resolve post-weaning complications, probiotics have been used as a good alternative for improving and modulate intestinal health by stimulating the growth of beneficial bacteria that compete with harmful bacteria or some desirable species. In this study, an experiment was conducted to investigate the effects of two levels of *Bacillus licheniformis* ATCC 21424 (*BL*) as DFM on performance growth, gastrointestinal (GI) and fecal microflora population, diarrhea incidence and pH evolution in weaning piglets. Our results indicate that *BL*-DFM has a probiotic potential and can be used as a possible antibiotics substitute to enhance growth rate, favored intestinal microflora, with a high reduction of piglets diarrhea incidence.

Abstract

A total of 60 piglets, 30±3 day-old, with initial average body weight (BW) of 8.53±0.17 kg were randomly distributed to 3 homogeneous groups (C, E1-*BL* 1% and E2-*BL* 3%), 2 replicates/group with 10 piglets/pens, for 16 days of biological trial. The objective was to assess the changes of growth performances, intestinal and feces microflora, and diarrhea incidence by diet supplementation with *BL* low dose (E1-*BL* 1%, 1.6 x 10^9 CFU spores g^{-1} feed), respectively high dose (E2-*BL* 3%, 4.8 x 10^9 CFU spores g^{-1} feed). After the first week (7d), BW and average daily gain (ADG) in piglets fed *BL* diets was increased (P=0.82, P=0.54) compared with the C group, respectively after week 2 (16d, P=0.26; P=0.09). Results showed that *BL* supplementation significantly reduced diarrhea incidence by 40% in E1-*BL* 1% and by 55.5% in E2-*BL* 3%, with a reduction of *E. coli* biotype β-hemolytic from GI content and feces piglets. In the ileum and cecum content, the *BL* diets increased the abundance of *Lactobacillus* spp. (P=0.42, P= 0.02) and *Bacillus* spp. (P= 0.13, P=0.03) with a trend of higher the *Coliforms* and *Enterococcus* counts. After 16d, the presence of *BL*, especially in E2-*BL* 3%, influence positively the GI pH values maintaining the balance of microflora populations with a lower fecal score relative to the C group. These results indicate that *BL*-DFM in piglet diet has a slight effect on growth performance, while health status, fecal, and intestinal beneficial microflora were improved. The diarrhea incidence decreased significantly by 3% *BL* in E2 fed group.

Keywords *Bacillus licheniformis*, probiotic, microbial population, diarrhea incidence, weaning piglets.

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Introduction

Weaning crisis is a critical period in the piglets' life cycle [1] with dramatic modifications, generally associated with infections [2] and GIT disturbances [3]. Multiple aetiological factors as dietary, social, and environmental changes cause a proliferation of opportunistic pathogens bacteria to grow [4, 5], as enterotoxigenic *Escherichia coli* infection [6, 7] which involve mainly colibacillosis diarrhea [8-10]. Considerable attention was occurred by probiotics bacteria known as direct-fed microbial (DFM) [10] used as suitable and effective alternatives to antibiotics [11] to promote the animal growth performance [12, 13], nutriments digestibility [6, 14], feed efficiency [15] and enhancement intestinal microflora of weaning piglets [16]. Probiotics are defined as culture bacteria with potential benefits on gut microflora that are administered to colonize the large intestine of the host [17] and to modify their composition [18]. Added in sufficient amounts, probiotic products are known to confer beneficial effects to the host [19]. Generally, DFM was defined as feed products that are purported to contain life (viable) microorganisms such as bacteria and yeasts [20].

The most commonly used probiotics are Gram-positive bacteria belonging to several species of *Bacillus*, *Lactobacillus*, *Bifidobacterium*, *Enterococcus* and non-bacteria (yeast or fungal) include *Aspergillus oryzae*, *Candida pintolopesii*, *Saccharomyces boulardii*, and *Saccharomyces cerevisiae* [7, 14, 21-23] and can be added to replenish the gut microbial population [14]. The intestinal microflora plays an important role in piglets weaning. Dietary supplementation of *Bacillus* could lead to some positive results on piglets' status health due to their resistance during GIT, and their probiotic and enzymatic action as well [16]. Besides, *Bacillus*-based DFM presents noticeable advantages because are spore-forming bacteria. This characteristic makes them thermostable for long-time storage and feed processing without reducing the viability [23] being a good way to protect themselves against negative environmental factors [24]. The principal criteria such as viability on a large scale, successful survival at low pH in the stomach, bile salts, resistance to heat [21] ability to colonize the intestine [14] and to adhere to the outmost mucus layer or a constituent part of the feed to form a biofilm on their surface [25], antagonize potential pathogens microorganisms [26, 27] or immunity stimulation [27] represents the basic properties for an effective probiotic ensuring the delivery of a viable product to the animals [13].

The objective of this study consists on investigation of the effects of *Bacillus licheniformis*-DFM on growth performance, gastrointestinal (GI) and fecal microbial population, diarrhea incidence, and pH evolution in weaning crisis piglets. In this work, we used *Bacillus licheniformis* directly in piglets diet as a fresh liquid culture with the mention that we took into consideration the strain capacity of sporulation during the entire experimental trial.

Materials and Methods

The experiment was performed in compliance with Council Directive 2010/63/EU legislation on the protection of animals used for scientific purposes. No antibiotic treatment was supplied to the animals. The animals were provided by National Research-Development Institute for Animal Nutrition and Biology Balotesti (INCDBNA), Experimental Farm, Romania. Conducting the experiment, supervision weaning, and sample collection were finished in agreement with the protocol approved by the Ethical Committee of the INCDBNA, Romania (protocol number No. 5183).

1. Probiotic Direct-Feed Microbial

Bacillus licheniformis was delivered by American Tissue Culture Collection (ATCC 21424) in the form of freeze-dried. The bacterial strain was grown in a nutrient medium (g/L: tryptone 10; meat extract 5; sodium chloride 5; pH medium 7.2 ± 2 before autoclaving), and incubated in a shaker-incubator, 200 rpm, 37°C for 24 h in aerobic conditions as reported previously Dumitru et al. [21]. The inoculum was analyzed by serial 10-fold dilutions using phosphate buffer saline solution (PBS), and then 1 mL from 10^{-10} to 10^{-12} was placed on nutrient agar medium (g/L: tryptone 5; meat extract 3; bacteriological agar 5; distilled water). The overnight culture contains at least 1×10^{13} CFU mL^{-1}.

To determine the spores-forming, the fresh bacterial culture was inactivated by heating at 80°C for 15 min. The biomass surviving spores were collected by centrifugation (5000 rpm, 10 min., 4°C), washed twice and then resuspended in PBS solution. Before plating on nutrient agar, serial dilutions in PBS were followed, by incubation at 37°C in the aerobic atmosphere for 24 h, and colonies formed were counted and expressed as CFU spores mL^{-1}.

Additionally, *BL* was analyzed for heat-resistance, the overnight culture was diluted in PBS and incubated at 80°C for 0, 30, 90, and 120 min. The survival rate (%) of strain to heat was calculated as following [28]:

$$\text{Survival (\%)} = \frac{\textbf{Log } \boldsymbol{number\ of\ cells\ survived} \left(\frac{\boldsymbol{CFU}}{\boldsymbol{ml}}\right) \boldsymbol{x}\ \mathbf{100}}{\textbf{Log } \boldsymbol{number\ of\ initial\ cells\ inoculated} \left(\frac{\boldsymbol{CFU}}{\boldsymbol{ml}}\right)}$$

The selection criteria of *BL* was based on their viability including growth rate, capacity identification to ferment carbohydrate substrates (API 50 CHB), amylase and protease production, resistance to acid (pH 2.0 and 3.0 during 0, 30, 60, 90 and 120 min at 37°C, 120 rpm), bile salts (0.3% oxgall for 0, 1, 2, 3, and 4 h at 37°C, 120 rpm), respectively the hemolysis activity on Trypticase soy agar medium supplemented with 5% sheep blood (TSA, Sanimed). The strain viability for these probiotic properties was presented in the previous study [21]. Furthermore, to use in piglets diet as DFM, the antibiotic susceptibility of *BL* strain was evaluated using an agar well diffusion method [29]. Ten types of antibiotics (Oxoid) were used: Ampicillin (AMP, 10 µg), Vancomycin (VA, 30 µg), Erythromycin (E, 15 µg), Clindamycin (CM, 2 µg), Gentamycin (GM, 10 µg), Amoxicillin (AMX, 25 µg), Chloramphenicol (C, 30 µg), Ciprofloxacin (CIP, 5 µg), Amikacin (25 µg), Tetracycline (TE, 30 µg), Kanamycin (K, 30 µg). *Bacillus licheniformis*, adjusted to approximatively 1 x 10^8 CFU/mL, until their turbidity becomes equivalent to 0.5 McFarland standard tube, was spread on the nutrient agar plate. Antibiotic-impregnated discs were loaded on agar and the diameter of the inhibition zone was measured after incubation at 37°C for 24 h (data not shown).

2. Animals and Diets

The experiment was performed on Topigs hybrid [♀Large White × Hybrid (Large White × Pietrain) × ♂ Talent, mainly Duroc]. A total of 60 weaned piglets, 30 ± 3 days old, with an average initial body weight of 8.53 ± 0.17 kg, were assigned completely randomized for 16 days into 3 treatments distributed in 6 pens with 10 piglets per pen, two replicates for each group. The 3 dietary treatments included: 1) a negative control diet (C diet) that contains on a basal diet shown in Table 1; 2) E1-*BL* 1% diet that consists on C diet + a low dose of *Bacillus licheniformis* ATCC 214241 (6 x 10^9 CFU viable spores g^{-1} feed); 3) E2-*BL* 3% diet, that consists on C diet + a high dose of *Bacillus licheniformis* ATCC 21424 (4.8 x 10^9 CFU viable spores g^{-1} feed). Feed was administered *ad libitum* in the flour form, two meals/day at 08:00 and 14:00 h with permanent access to water throughout the experimental period.

Pens measured 2.40 m × 1.80 m x 0.800 m, each with the slatted plastic floor, in an environmentally controlled isolation. 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.

Table 1. Nutrient and chemical composition of the diet

Items %	*BL* inclusion		
	Control	E1-*BL* 1%	E2-*BL* 3%
Maize	33.48	33.48	33.48
Sorghum	25	25	25
Peas	17	17	17
Soybean meal	13	13	13
Corn gluten meal	3.0	3.0	3.0
Milk powder, skimmed	5.0	5.0	5.0
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	1.0	1.0	1.0
BL*-DFM*	**0.0**	**1.0**	**3.0**
Chemical composition %			
Dry matter	88.16	88.16	88.16
Metabolizable energy * (EM, MJ/Kg)	13.54	13.54	13.54
Crude protein (CP)	18.23	18.23	18.23
Lysine	1.2	1.2	1.2
Digestible Lysine	0.95	0.95	0.95
Methionine + Cystine	0.59	0.59	0.59
Digestible Methionine + Cystine	0.61	0.61	0.61
Ether extract	2.2	2.2	2.2
Ca	0.9	0.9	0.9
P	0.7	0.7	0.7
Digestible P	0.42	0.42	0.42
Cellulose	3.02	3.02	3.02

*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.
*******BL*, *Bacillus licheniformis* ATCC 21424: C, basal diet; E1-*BL* 1%, basal diet + low dose of *BL* (1.6 x 10^9 CFU spores g^{-1} feed); E2-*BL* 3%, basal diet + high dose of *BL* (4.8 x 10^9 CFU spores g^{-1} feed).

3. Growth Performance Study

The individual BW, ADG, ADFI, G: F were recorded every week. The intake and refusal feed were noted daily and overall were calculated.

4. Sample Collections and Preparation

At the end of the trial, two piglets per group were selected and slaughtered by electrical stunned according to Council Regulation (EC) No. 1099/2009. Following exsanguination, the carcasses were dehaired and eviscerated under the supervision of a veterinarian. The abdominal piglet cavity is opened in order to collect the GIT. Intestinal content from ileum and cecum was removed immediately after killing and aseptically collected in sterile plastic bags and quickly transferred on ice to the laboratory. Similarly, fresh fecal samples were randomly collected from each pean in the same conditions in 1d, 8d, and 16d of the biological trial until bacterial analyses were done (no more three months).

5. Microbial Populations

From GIT content, 1 g of sample (ileum and cecum) per capita from two piglets per group were homogenized with 7 ml BHI (Brain Heart Infusion, Oxoid) broth with 2 ml glycerol, and immediately stored at -20°C until testing [30]. The fecal samples stored conditions were similar.

6. Microflora of Digesta (Plate Culture Techniques)

After defrost, one mL from the BHI sample (ileum and cecum) tube was diluted with 9 mL of Phosphate-Buffered Saline (PBS; Oxoid LTD, England) and then homogenized. Decimal dilutions were performed for Lactic Acid Bacteria (LAB) as well as *Escherichia coli* (*E. coli*; biotype β-hemolytic), *Salmonella* spp., *Clostridium* spp., Coliforms, *Bacillus* spp., and *Enterococcus* spp. The viable counts of LAB were conducted by plating serial 10-fold dilutions (10^{-1}-10^{-10}) on de Man, Rogosa and Sharpe agar medium (MRS; Oxoid CM0361) incubated under anaerobic conditions at 37°C for 48 h (Oxoid jar with Anaerogen 2.5 L). Coliforms were cultured on MacConkey agar (Oxoid CM0007) incubated aerobically at 37 °C for 24 h as red specifically colonies. *E. coli* biotype β-hemolytic was analyzed as reported by Dumitru et al. [21]. Briefly, was inoculated 0.01 mL from 10^{-1} dilution on sheep blood agar [Trypticase soy agar (TSA) 5% (w/v)], and incubated at 37°C for 24 h in aerobic conditions; presumptive identification of *E. coli* consists in a clear zone around colonies on TSA medium which indicate complete hydrolysis (β-hemolysis), respectively confirmation with the biochemical test [Triple sugar iron agar (TSI, Sanimed 10902) based on the ability to reduce sulfur and ferment carbohydrates; Motility Indole Urea agar (MIU, Sanimed 11652) based on the presence/absence of motile microorganisms and production of cherry red reagent layer after the introduction of Kovac's reagent in MIU medium that indicates an Indole positive reaction; Simmons Citrate agar (Sanimed 10842), based on the ability to ferment citrate as the only source of carbon based on a profound blue coloration of the medium as a positive answer) [31]. *Clostridium* spp. were cultured on Reinforced Clostridial Agar (Oxoid CM0151) incubated anaerobically at 37°C for 48 h. *Enterococcus* spp. were

enumerated on Slanetz-Bartley agar (Oxoid CM0377) incubated at 37°C for 48 h in anaerobic conditions, according to the method of Mountzouris et al. [32], modified by Sorescu et al. [30]. *Bacillus* spp. were counted on nutritive agar medium and *Salmonella* spp. on *Salmonella-Shigella* agar (Oxoid CM0099) respectively, incubated aerobically at 37°C for 24 h.

The samples microbial plates were repeated 3 times. The intestinal microflora enumerations were expressed as log10 CFU per gram.

7. Fecal Bacterial Enumeration

Fecal samples were collected from piglets on the 1st, 8th, and 16th day of the experiment for microbial analysis. After defrosted, the fecal was performed by enumeration method of *Lactobacillus* spp., *Coliforms*, *Clostridium* spp., *Enterococcus* spp., *E. coli* β-hemolytic, *Bacillus* spp. and *Salmonella* spp. following the same methods from intestinal microbial populations' analysis.

8. Digesta pH Assessment

To measure the pH, 1 g of the ileum and cecum content of each piglet was collected and transferred into 9 mL distilled water (1:10 dilution); then the pH value was measured (mean of 3 readings) using a portable pH meter (pH 7 + DHS, XS Instruments, Italy). After each pH measurement, the pH probes was carefully washed with water and calibrated between each animal.

9. Assessment of Incidence of the Diarrhea

The animals were supervised daily to identify the piglets with diarrhea severity (%). The feces consistency of every piglet were monitored daily at 08:00 and 15:00 h throughout the study. A subjective scoring system was used to determine the severity of diarrhea, ranging from 1 to 3: 1 soft feces; 2 mild diarrhea, 3: severe diarrhea. The incidence of diarrhea as an effect of dietary addition of *BL* strain was evaluated in the first seven days of the biological trial and was calculated as the average number of days with diarrhea related to the total monitoring days [33].

10. Statistical Analyses

All results were statistically performed for each parameter of piglets. The data generated were compared by analysis of variance (one-way ANOVA) as a completely randomized design, using the GLM procedure of SPSS program, version 20.0 (SPSS Inc., Chicago, IL, USA, 2011). Inclusion of different levels of *BL*-DFM was the fixed effect and block was the random factor in the statistical model. The results of this experiment are expressed as mean values and standard error of the mean (SEM). Means were compared using Tukey's HSD test at a significant level of $p \leq 0.05$ and tendencies were noted under conditions of $0.05 < p < 0.10$. Sex was not included in the statistical model. This study was carried out in similar conditions at experimental level and piglet sex is not considered a decision factor.

Results

1. Effect of Bacillus licheniformis-DFM on Growth Performance of Piglets

The effects of *BL* on the growth performance of weaned piglets are described in Table 2. The results showed that *BL* strain used in different concentrations affects BW, ADG, ADFI, feed consumption, respectively F: G in piglets relative to C group. During the week I (0 to 7 d) and week II (0 to 16 d) too, the piglets' BW was improved by *BL* supplementation, whatever the dose used, the effect being more pronounced in *BL* 3% fed group (higher with 0.9% and 7.15% in the week I and week II respectively compared with C diet, $P>0.05$). A trend to be influenced was noticed in *BL* 3% fed group for ADG. While ADG increased, we noticed a decrease in ADFI in E2-*BL* 3% although the gain to feed ratio increased ($P>0.05$).

Table 2. Effects of different concentrations of *B. licheniformis* – DFM on growth performance of piglets

Parameters[A]	Treatment[B]			SEM	*P*-value
	C	E1-*BL* 1%	E2-*BL* 3%		
Initial BW	8.44	8.72	8.42	0.12	0.58
BW – 7d	9.66	9.88	9.75	0.14	0.82
BW - 16d	12.16	12.59	13.03	0.22	0.26
ADG – 7d	0.202	0.192	0.222	0.01	0.54
ADG – 16d	0.233	0.241	0.288	0.01	0.09
ADFI	0.521	0.481	0.476	0.02	0.56
gain:feed	0.51	0.43	0.60	0.02	0.38

[A]BW, body weight gain (kg/piglet); ADG, average daily gain (kg/piglet/day); ADFI, average daily feed intake (kg/piglet/day); F: G (kg feed: kg gain), feed efficiency; [B]C, basal diet; E1-*BL* 1%, basal diet + low dose of *BL* (1.6×10^9 CFU spores g^{-1} feed); E2-*BL* 3%, basal diet + high dose of *BL* (4.8×10^9 CFU spores g^{-1} feed); DFM, direct fed microbial; *BL*, *Bacillus licheniformis* ATCC 21424; Standard error of the Means ($P< 0.0001$ highly significant difference; $P < 0.05$ significant difference; $P < 0.10$ tendency of influence; $P > 0.10$ not significant).

2. Effect of Bacillus licheniformis-DFM on Piglets Microflora Populations

The effects of *BL*-DFM on feces piglet's microflora were presented in Table 3. The number of *Bacillus* spp. had the highest abundance at 16thd both E1-*BL* 1% and E2-*BL* 3% relative to the Control group ($P < 0.05$). Unexpectedly, we noticed an insignificant descrease of *Bacillus* spp. at 8thd for E1. Although insignificant, the LABs from feces were not affected by *BL* treatments ($P > 0.05$).

The count of *Coliforms* and *Clostridium* were increased significantly at 16thd, especially in the E1 group ($P < 0.05$), but we must take into consideration that in 1st d, considering as reference, the number of these strains were much higher. On 8th d the descrease of *Coliform*s and *Clostridium* number were not significantly affected.

A trend to be influenced was noticed at 16thd for *Enterococcus* counts for groups that were treated with *BL*, regardless of the dose used.

E. coli number through plate culture on TSA medium was lower in E1-*BL* 1% and expressly in E2-*BL* 3% than that of the Control group (P >0.05).

Table 3. Effect of *BL*-DFM supplementation on piglets feces

Item	Days	Control	*BL* inclusion		^{3}SEM	*P*-values
			^{1}E1-*BL* 1%	^{2}E2-*BL* 3%		
LABs, log_{10} CFU/g	1st	8.20	8.64	8.95	0.16	0.18
	8th	9.01	8.15	8.24	0.20	0.16
	16th	8.44	8.15	8.24	0.18	0.81
Coliforms, log_{10} CFU /g	1st	6.23^{a}	6.73^{b}	7.31^{c}	0.13	0.0003
	8th	6.12	5.89	4.97	0.34	0.36
	16th	6.01^{a}	6.94^{b}	6.50^{c}	0.15	0.04
Clostridium spp., log_{10} CFU /g	1st	6.33^{a}	7.28^{bc}	7.30^{c}	0.18	0.03
	8th	5.93^{a}	6.36	6.96^{c}	0.19	0.07
	16th	6.05^{a}	6.76^{bc}	6.29^{c}	0.13	0.09
Enterococcus spp., log_{10} CFU /g	1st	5.71^{a}	7.21^{b}	7.52^{c}	0.22	0.0001
	8th	5.33	4.93	4.17	0.29	0.26
	16th	5.41^{a}	6.19^{b}	6.30^{c}	0.16	0.03
Bacillus spp., log_{10} CFU /g	1st	5.69^{a}	4.95^{b}	5.23	0.12	0.03
	8th	4.45	4.62	5.29	0.17	0.11
	16th	4.18^{a}	4.95^{b}	5.53^{bc}	0.17	0.0008
E. coli, biotype β-hemolytic, log_{10} CFU /g	1st	absent	absent	absent	na	na
	8th	3.78	3.80	3.49	0.18	0.77
	16th	5.36	5.01	4.89	0.11	0.22
Salmonella spp., log_{10} CFU /g	1st	absent	absent	absent	na	na
	8th	absent	absent	absent	na	na
	16th	absent	absent	absent	na	na

BL, *Bacillus licheniformis* ATCC 21424, ^{1}E1, basal diet + low dose of *BL* (1.6 x 10^{9} CFU spores g^{-1} feed); ^{2}E2, basal diet + high dose of *BL* (4.8 x 10^{9} CFU spores g^{-1} feed); na: not applied; ^{3}SEM, standard error of the mean; ^{a,b,c}Means with different superscripts in a row differ significantly (P < 0.0001 highly significant difference; P < 0.05 significant difference; P < 0.10 tendency of influence; P > 0.10 not significant); 1st d of the trial (30 ± 3 d); 8th d of the trial (38 ± 3 d); 16th d of the trial (46 ± 3 d).

The effects of *BL* on ileal and cecal piglet's microflora were presented in Table 4. On 16d, dietary *BL* affected positively the ileum numbers of *Bacillus* spp. (higher with 37.42% in E1-*BL* 1%, respectively > 33.23% in E2-*BL* 3%, P < 0.05) and LAB spp. More pronounced was the effect in the cecum were both *Bacillus* and LAB counts were significantly altered. The bacterial strain of *Coliforms*, *Clostridium* spp. and *Enterococcus* were not affected significantly by *BL* dietary supplement. Piglets diets supplemented with different levels of *BL* had lower ileal *E. coli* abundance than piglets fed with basal diet.

Table 4. Effect of *BL*-DFM supplementation on intestinal microflora and pH evolution of weaned piglets

Item	Control	*BL* inclusion		^{3}SEM	*P*-values
		^{1}E1-*BL* 1%	^{2}E2-*BL* 3%		
Ileum, 16th d					
pH	6.75	7.64	5.72	0.450	0.24
Bacillus spp., log_{10} CFU /g	3.34	4.59	4.45	0.291	0.13
LAB, log_{10} CFU /g	7.74	8.33	8.85	0.306	0.42
Coliforms, log_{10} CFU /g	6.17	6.96	5.79	0.387	0.56
Clostridium spp., log_{10} CFU /g	6.50	6.02	6.29	0.175	0.66
Enterococcus spp., log_{10} CFU /g	5.50	5.99	5.92	0.163	0.51
E. coli, biotype β-hemolytic, log_{10} CFU /g	>5.17	4.87	4.30	0.220	0.31
Salmonella spp., log_{10} CFU /g	absent	absent	absent	na	na
Cecum, 16th d					
pH	6.46	6.20	6.20	0.219	0.11
Bacillus spp., log_{10} CFU /g	2.99^{a}	4.69	5.65^{b}	0.522	0.03
LAB, log_{10} CFU /g	7.15^{a}	8.36	9.09^{b}	0.376	0.02
Coliforms, log_{10} CFU /g	6.60	7.13	6.77	0.346	0.88
Clostridium spp., log_{10} CFU /g	6.50	6.50	7.24	0.241	0.44
Enterococcus spp., log_{10} CFU /g	5.99	6.00	6.73	0.247	0.47
E. coli, biotype β-hemolytic, log_{10} CFU /g	>5.74	4.89	4.35	0.296	0.13
Salmonella spp., log_{10} CFU /g	absent	absent	absent	na	na

BL, *Bacillus licheniformis* ATCC 21424, ^{1}E1-*BL* 1%, basal diet + low dose of *BL* (1.6 x 10^{9} CFU spores g^{-1} feed); ^{2}E2-*BL* 3%, basal diet + high dose of *BL* (4.8 x 10^{9} CFU spores g^{-1} feed); na: not applied; ^{3}SEM, standard error of the mean; ^{a,b,c}Means with different superscripts in a row differ significantly (P < 0.0001 highly significant difference; P < 0.05 significant difference; P < 0.10 tendency of influence; P > 0.10 not significant).

As shown in Table 4, the ileal pH of piglets fed with the high dose of *BL* was lower compared to the C group ($P > 0.05$). The *BL* levels decreased the cecal pH ($P > 0.05$). The highest pH values were founded in piglets fed with basal diet, in both sampling piglets locations, respectively ileum and cecum segment.

3. Effect B. licheniformis-DFM on Diarrhea Incidence in Piglets in the Weaning Crisis

The effect of *BL*-DFM on fecal scores of weaned piglets is shown in Table 5. Piglets feed with high dose of *BL* had a lower fecal score 3 (P =0.05) with a lower incidence of diarrhea compared with the group. The addition of *BL* reduced the diarrhea incidence by 40% when was used a low dose of *BL* inclusion, respectively 55.5% for E2 when was used a high dose of *BL* relative to the piglets feed with basal diet.

Discussion

The present study investigated the influence of *Bacillus licheniformis* on performance, GI and fecal microbial population, diarrhea incidence, and pH evolution of piglets in the weaning crisis.

As is known, weaning period is a critical part from piglets life, known as an important time of transition and stress animals [34] due to various nutritional (derived from plant based-diets with various antinutritional factors), social environmental conditions [35] and potentially pathogenic microorganisms [36].

Table 5. The effect of *BL*-DFM on fecal scores of weaned piglets

Item	Control	[1]E1-*BL* 1%	[2]E2-*BL* 3%	*P*-values
Fecal[1]	0.52	0.33	0.23	0.02*
Fecal score 1	0.15	0.20	0.15	0.11
Fecal score 2	0.50	0.50	0.40	0.16
Fecal score 3	0.90	0.33	0.15	0.05
Diarrhea incidence	4.2	3.0	2.7	0.37

BL, *Bacillus licheniformis* ATCC 21424; [1]E1-*BL* 1%, basal diet + low dose of *BL* (1.6 x 10^9 CFU spores g^{-1} feed); [2]E2-*BL* 3%, basal diet + high dose of *BL* (4.8 x 10^9 CFU spores g^{-1} feed); *Means with different superscripts in a row differ significantly ($P < 0.0001$ highly significant difference; $P < 0.05$ significant difference; $P < 0.10$ tendency of influence; $P > 0.10$ not significant).

The first two weeks post-weaning piglets is the most worrying period during animal progress characterized by the change from a liquid (milk) to a solid diet [37], which may cause digestive instabilities, reduced feed intake, and underdeveloped growth for newly piglets [38]. The probiotic action and their viability throughout animal GIT are the main ones aspects we take into consideration for using as feed additive [7].

The weaning stress factors such as change of type of diet can determine to the piglets an incapacity to secret enough digestive enzymes. On the other hand, it is known that decrease feed intake, the digestive tract is affected and the absorption capacity is reduced [39,40]. Enzyme maturation starts at this stage [1]. Heo et al. [41] affirmed that a high level of protein in piglets diets from 28 to 70 d of age, affects the gut microflora and can influence the proliferation and the colonization with *Escherichia coli* biotype *β*-hemolytic, a pathogenic agent of GIT, responsible for diarrhea, causing a poor growth rate, low feed conversion and higher feed costs [4]. It is known that *Bacillus licheniformis* used as DFM, can synthesize enzymes as protease and amylase (screening using casein and starch plate [23], thus improving the piglet's digestive process). Furthermore, the soybean meal used as a protein source in piglets diet, in the presence of *Bacillus* spp. it will be more easily fermented [42]. Due to the enzymatic system, vegetative cells of *Bacillus* can secrete extracellular carbohydrate-, lipid-, and protein-degrading enzymes [43] that will start to activate the digestive and fermentative process of piglets by breaking down the polysaccharides into smaller polysaccharides or monosaccharides components from feed [8] improving the growth performance [44, 45].

In this research, we demonstrated that the diet administration of different levels of *Bacillus licheniformis* determines a slight effect on the piglet's growth, however, the influence was not significant. Our positive results can be confirmed by the resistance of *Bacillus* spores studied in many publications for pH, bile salts, high temperatures [21, 23, 46-47]. Besides, how we have shown previously, the present strain registered high viability at pH and bile salts [23].

Many studies confirm our results [6, 11, 48-49]. Contrary, Balasubramanian et al. [11], suggested that a combination of *Bacillu*s spp. as probiotic (0%, 0.01%, and 0.02%) with a basal diet, improved significantly ADG and G: F, but without effects on ADGI in growing-finishing pigs. Another study [50], shows that a probiotic based on *Clostridium butyricum* in combination with *Bacillus licheniformis* improved growth performance, and decreased the incidence of post-weaning diarrhea providing an alternative for diminishing the ZnO and antibiotics concentrations from piglets diets. In agreement with Giang et al. [51], *Bacillus subtilis* H4 improved feed digestibility with an increase of piglet growth. Addition of *Bacillus*-based probiotics to the diet may improve feed efficiency and/or ADG of growing-finishing pigs [52]. Other researchers also described that *Bacillus subtilis* and *Bacillus licheniformis* to the diet improved the piglets weight significantly [44, 53-55]. The effect of *Bacillus* on performance in animal

nutrition is highly unpredictable, perhaps because of different diet compositions, strains or dose levels used [55, 56]. Generally, the association between two or more strain is known as being more effective. Although we used one single strain of bacteria in the current study, we noticed a positive effect on performance especially at a high level of *BL*.

Bacterial colonization of piglets GIT begins at birth and especially appear from the sow and the growth environment of suckling piglet [57]. The diversification of the large intestinal microflora starts at weaning due to abrupt separation from sows [58]. We intended was to investigate also in this work the effects of one single strain of *Bacillus* on microflora and piglets diarrhea incidence. The animal answer at our probiotic treatment was characterized by an individual variation. The effects of the probiotics could vary due to the difference in intestinal microflora composition [59]. As Gram-positive spore-forming bacteria, *Bacillus* spp. have the capacity to germinate but not to proliferate in piglets GIT [57]. Once they started to germinate, Bacilli spores block pathogenic bacteria to go to the intestinal epithelium.

The addition of *B. licheniformis* did not disturbance the *LAB* spp. from fecal piglets. However, Lactobacilli counts decline dramatically immediately after weaning, resulting in microflora imbalance, digestive disturbance, and poor performance of piglets. *Clostridium* spp. were the most predominant spore-forming bacteria which is associated with pathogenicity [40]. At 16st day of the trial, the fecal *Clostridium* concentration was highest in treated groups, a statement that is also confirmed by Guevarra et al. [34]. Comparative to the 1st day of the trial, the *Coliforms* number in 7th and 16th d, decreased at the addition of a high dose of *BL* in the diet. *Enterococcus* counts from piglets feces in the presence of *BL* were modified relatively. The addition of *BL* accelerated the feces microflora, determining an increase in *Bacillus* concentration in the treatment group compared to the C group, as shown in Table 3. Besides, compared to the basal diet, it was observed that *BL* groups registered a decrease of *E. coli β*-hemolytic proportion. Similar results were found by Balasubramanian et al. [11], who reported that growing pigs fed with *Bacillus*-based probiotic influenced the *LAB* counts and decreased fecal *E. coli.*

Under natural environment conditions, harmful microorganisms can pass in piglet GIT and colonize this area. As we know, *E. coli* is one of the major intestinal pathogens for the swine industry [48] with a negative impact like low performance and increased mortality in the postweaning period [60, 61]. Previous studies on piglets intestinal microflora confirmed that *E. coli* level increased while numbers of LAB declined [62]. The defensive barrier in the stomach can modify the microflora populations. Assay experimental groups, the *B. licheniformis* influenced the intestinal microflora with a beneficial effect on *LAB* counts in ileum and cecum content, inhibiting the *E. coli* development in the gut. As is known, lactic acid bacteria are Gram-positive, non-spores forming with the capacity to secret lactic acid as the main metabolic product. Within the porcine GIT, LAB populations were estimated to reach 10^{11} per gram of tissue, 10^{10} per gram of luminal contents, and 10^{10} per gram of feces [63].

In our study, inside piglets gut, LABs were confirmed in ileum around 10^7 (basal diet) to 10^8 (*BL* groups), comparatively to cecum content were number ranged from 10^7 (piglets feed without *BL*) respectively 10^8 (law dose of *BL*) to 10^9 (high dose of *BL*). In our case, the inclusion of the *BL* in piglets feed shows an improvement of *Bacillus* cecum counts around 57% (low dose of *BL*) to 89% (high dose of *BL*) compared to the Control would reflect that most of the administered spores were viable. It appears that *B. licheniformis* administrated in piglets diet induced the increase of *Enterococcus* spp. in the cecum segment. A significant reduction in *E. coli* concentration was observed in the piglet's groups that received the feed with *BL*. Another positive effect was found that the diarrhea incidence was significantly reduced by *Bacillus licheniformis*, and 4.8 x 10^9 CFU dose had a better effect on piglet health relative to a low dose of DFM during the entire trial period. This finding is in agreement with the report shown by Alexopoulos et al. [53] where it has been shown that *B. licheniformis* and *B. subtilis* are involved to decrease morbidity, mortality, and post-weaning diarrhea. The beneficial effect of our probiotic bacterial product can be clarified by pathogens decrease in the intestinal gut, which did not allow the growth of harmful bacteria.

According to Merchant et al. [64] one of the arguments for using *Bacillus* spp. as DFM in animal nutrition is due to pH value in the small intestine that is 6 to 7. These values are optimal for spores to germinate, grow and produce enzymes and, also, to resist the enzymatic degradation and low pH of the gastric barrier [65]. *Bacillus* spp. used as probiotics, can stimulate the growth of LAB [66]. As an oxygen consumer, *Bacillus* spp. diminished the gut pH, favoring LAB populations to produce more lactic acid, actions that can inhibit *E. coli* proliferation [67-69].

In summary, the results of the present study suggest that the administration of *Bacillus licheniformis* ATCC 21424 can be an alternative to antibiotic growth promoters in weaned piglets, the more so as the antibiotics determine a high level of microbial resistance which led to an increase in interest to study the optimal alternative. Although we used a single strain of *Bacillus licheniformis* as DFM in piglets in a critical period of development the results were confirming the hypothesis from which we initiated this study.

Conclusions

In conclusion, our study indicated that dietary *B. licheniformis*-DFM increased growth performance in weaning piglets. The ADG for the entire experiment was positively influenced. While ADG increased we noticed a descrease in ADFI *B. licheniformis* worked strongly at a dose of 4.8 x 10^9 CFU viable spores in piglets feed, with a significant increase of *Lactobacillus* counts and *Bacillus* spp. in the ileum and cecum content with a trend of higher the *Coliforms* and *Enterococcus* spp. from piglets feces. The addition of a high dose of *BL*, influence positively

the GI pH values maintaining the balance of microbial populations with a lower fecal score. The results of the present study suggest that supplementation with *Bacillus licheniformis* ATCC 21424 improves the growth, intestinal microflora, with significant positive changes in diarrhea incidence of weaning piglets.

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Conflicts of Interest

The authors declare no conflict of interest.

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