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PRELIMINARY CHARACTERIZATION *IN VITRO* OF *Bacillus licheniformis* STRAIN FOR USED AS DIETARY PROBIOTIC

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Abstract

The purpose of this paper was to provide a direction for evaluating the probiotic potential and safety of a microbial strain in order to use in weaning piglets. Bacillus licheniformis ATCC 21424 was analyzed morphologically, culturally, biochemically, for hemolytic activity and enzymatically (amylase and protease screening). In vitro, some probiotic properties were study as: resistance to pH by simulated gastric juice (pH 2 and 3) and bile salts (simulated intestinal fluid). The biochemical characteristics was performed by catalase assay, API 50 CHB Biomerieux strips, apiweb API 50 CHB V 4.0 soft (B. licheniformis, % ID 99.9) and ABIS online (~90.6% similarity). The hemolytic activity was assayed on blood agar medium. The strain was grown in nutrient medium, in two ways: static incubation (37℃, 24 h, 4 x 10^8 CFU/ml) and under constant agitation (37℃, 24 h, 120 rpm, 1.56 x 10^{10} CFU/ml). To screen out, the most favorable carbon source was included in the basal medium (1% w/v, pH = 7) glucose (12.11 ± 0.1), fructose (11.73 ± 0.67), lactose (12.03 ± 0.14) and starch (12.51 ± 0.27). The strain is a Gram-positive, rod-shaped bacteria, arranged in short chains or in small irregular pairs with ability to produce spores. The endospores were central, paracentral and subterminal. The strain growth was aerobic and was non – hemolytic. The enzymatic process was observed by appearance of distinct zones around strain colonies. The strain presents relatively good viability at pH 3 and tolerated oxgall (0.3%). In conclusion, the results suggested that the strain present some probiotic traits and can be further assessed for other probiotic characters as antibacterial activity, induction of local immune response etc.

Key words*: Bacillus licheniformis, biochemical characters, probiotic properties, hemolytic activity, enzymatic screening.*

INTRODUCTION

The administration of live microbial preparations as "dietary supplements" provides a strategy for animal's breeders to raise up meat production, quality and animal health status etc. (Dumitru et al., 2018a; Habeanu et al., 2016; Duc et al., 2004).

Since 2006, the European Union has banned the use of antibiotics in food-producing (European Union, 2006). The probiotics administration which are called direct-fed microbials (DFM) (Chen et al., 2006), have been demonstrated to be useful when are ingested in a sufficient amount and can be an alternative with high positive effects on the animals' health, by maintaining intestinal ecosystem and their performance (Kaewtapee et al., 2017; Dumitru et al., 2018a).

Microorganisms used in animal feed in the EU are mainly bacterial strains of Gram-positive bacteria belonging to the genus *Bacillus, Enterococcus, Lactobacillus, Pedicoccus, Streptococcus* and strains of yeast belonging to the *Saccharomyces* and *Kluveromyces*.

From the all types, *Bacillus* spp. occurred the most attention (Cutting, 2011); spores of these microorganisms can resist to unfavorable conditions, present potential probiotic properties as resistant to heat, radiation, enzymatic degradation during to the animal gastrointestinal tract (GIT) and stomach's acidic medium (pH, acid tolerance, bile resistance etc.) (Lee et al., 2012; Dumitru et al., 2018a). *Bacillus* spp. represent an importance sources for produce various extracellular enzymes that enhance feed digestibility, improving growth performance, feed

conversion ratio and meat quality of animals (Pant et al., 2015).
According to Upadhaya et al. (2015), dietary supplementation of *B. licheniformis* have positive effects on pigs' growth performance. The probiotics activity is influenced by diet composition (Blank et al., 1999); a high protein content in diet can affect the piglet microbiota in the first 14 d after weaning (Wu et al., 2015).
The present study describes *in vitro* some probiotic properties of *B. licheniformis* ATCC 21424 strain as morphological, cultural, biochemical characteristics, hemolytic ability, enzymatic production (amylase and protease screening), viability at pH 2 and 3 and bile resistance by simulated intestinal fluid as a preliminary investigation of probiotic potential in order to use it in piglet nutrition.

MATERIALS AND METHODS

Characterization of bacterial strain, growth media and enumeration of spore counts

Morphological and cultural properties of *B. licheniformis* ATCC 21424 strain was investigated according to the methods described in Bergey's Manual of Systematic Bacteriology (1957) (Ludwig et al., 2012). The strain was grown in nutrient broth and agar medium (Merck), 90 mm in Petri dishes, to evaluate the cultural traits. Serial dilution (1: 10, in 0.85% sterile physiological serum - SPS) was done (10^5 - 10^{10} - fold), for counts number (CFU/ml) in broth culture, incubated static (37°C, 24 h) and under a constant agitation (37°C, 24 h, 120 rpm). An aliquot of 1 ml from each dilution was homogenized and spread on nutrient agar plate. At least three replicas were done for each dilution. The strain was stored at room temperature, 4°C and -80°C with 20% sterile glycerol, until will be establish the preservation viability. Bacteria viability will be assessed every 2 years.
The strain was deposited in the Collection of National Research Development Institute for Biology and Animal Nutrition Baloteşti (INCDBNA), Romania, under the code IBNA 80.

Preservation of bacterial strain

The medium preservation (months) was done by culture in nutrient agar medium tubes. The strain viability was evaluated after 3 and 6 months. Long-time preservation (years) was done at -80^0C, with addition of glycerol 20%. Bacterial viability will be assessed every 2 years (Sorescu et al., 2019).

Biochemical test

The strain was tested for biochemical characters (catalase assay, API 50 CHB Biomerieux strips) and identified by API 50 CHB V4.0 and ABIS online soft (Stoica & Sorescu, 2017).

The catalase test

The catalase test was done according to the protocol described by Dumitru et al. (2018b, 2017). The method consists in highlighting the enzyme catalase from a bacterial culture by using hydrogen peroxide (H_2O_2) in a concentration of 3%. The catalase enzyme facilitates the breakdown of H_2O_2 into oxygen and water ($2H_2O_2$ + Catalase → $2H_2O + O_2$); the reaction was positive when a small inoculum is introduced in the H_2O_2 and visible effervescently bubbles were observed by the rapid elaboration of oxygen.

The API 50 CHB test

API 50 CHB strips were used for evaluated the carbohydrate acidification of *B. licheniformis* ATCC 21424 according to the manufacturer's protocol (BioMerieux) described by Dumitru et al. (2018a). The results are interpreted using database system API 50 CHB V4.0 and ABIS online software.

Hemolysis production

Blood agar plates [Trypticase soy agar (TSA, Sanimed) containing 5% (w/v) sheep blood], were used to test hemolysis activity (Jeon et al., 2018, cited by Dumitru et al., 2018a). Interpretation was followed after incubation at 37°C, for 24 h.

Specific Growth of *B. licheniformis* ATCC 21424 in minimal medium containing different carbon sources

To investigate the effects of various carbon sources, *Bacillus licheniformis* ATCC 21424 strain was grown in nutrient broth flash (pH 7), 24 h at 37°C in a rotary shaker at 120 rpm, with different substrate addition (1% w/v) in the basal medium: glucose, fructose, lactose and starch (Mageshwaran et al., 2014). Loop full of

culture of *B. licheniformis* ATCC 21424 was inoculated into separate flasks, with continuous agitation of 120 rpm, containing nutrient broth with different carbon sources used as substrate. Viable cells were determined by serial dilutions (10^{12}-fold) of the culture in SPS on nutrient agar by plates incubating at 37°C, 24 h. The number of bacteria was calculated according to the standard of ISO 7218 (2007).

Screening of amylase producing bacteria

According to the protocol instructions (Singh et al. 2015, cited by Dumitru et al., 2018), the bacterial strain was screened for amylolytic properties by starch hydrolysis test, on starch (1% w/v) agar plate.

Screening of protease producing bacteria

Bacillus licheniformis ATCC 21424 was screened for proteolytic activity. The bacteria strain was inoculated on the agar plates containing casein (1% w/v) and milk powder (1% w/v), incubated at 37°C, for 48 h. According to Josephine et al. (2012), it was following the protocol and data interpretation.

Acid tolerance test

The resistance of *Bacillus licheniformis* ATCC 21424 strain was investigated under simulated gastric juice (SGJ) by following the method described (Lee et al., 2012) with some modification: 1 ml of culture grown in nutritive broth for 24 h at 37°C, 120 rpm, representing about 10^9 colony forming units (CFU/ml), was transferred to 9 ml of SGJ [0.5% NaCl, 0.3% pepsin (from gastric mucosa, Sigma), 0.1%v peptone (BD Science)], whose pH was adjusted to 2 and 3 with a Portable meter (Waterproof, pH 7+DHS) using HCl 1 N, then incubated for 0, 30, 60, 90 and 120 minutes at 37°C, 120 rpm. Viable cells of the culture were enumerated by plating 10-fold dilutions [1:10, in NaCl 0.85%] on nutrient agar and plates incubating at 37°C, 24 h.

Bile resistance test

Bile tolerance was determined as previously described (Lee et al., 2012) with the following modifications: 10 ml of culture strain (about 10^9 CFU/ml) grown in nutritive broth (pH 7) for 24 h at 37°C on a rotary shaker (120 rpm), was spun down at 5,000 x g, 20 min, at 4°C. Cell pellets (biomass) were washed with PBS, collected by centrifugation (5,000 x g, 20 min, at 4°C) and resuspended in nutrient broth (pH 7) containing 0.3% oxgall (BD Science). Bacterial suspensions were incubated for 0, 1, 2, 3, and 4 h at 37°C on a rotary shaker at 120 rpm. Viable cells were counted by plating 10-fold dilutions of the culture in SPS on nutrient agar and plates incubating at 37°C, 24 h. The number of bacteria was calculated according to the standard of ISO 7218 (2007).

Data analysis

The analytical data were compared using variance analysis (ANOVA) with STATVIEW for Windows (SAS, version 6.0). The results were expressed as mean values and standard error of the mean (SEM), the differences between means considered statistically significant at $P<0.05$, using Fisher's PLSD test for untitled compact variable.

RESULTS AND DISCUSSIONS

Morphological and biochemical characterization

The taxonomic classification of strain was performed by culturally (aerobic growth), morphologically (Gram-positive, spore forming rods), and biochemically traits (positive catalase test). On nutrient agar after 24 h at 37°C, under aerobic conditions, the *B. licheniformis* ATCC 21424 presents opaque colonies, whitish with rough matte surface, irregular edged (type R) and diameter 0.7-0.9 mm (Figure 1).

Figure 1. Cultural aspect on agar plate for *Bacillus licheniformis* ATCC 21424

After growth in the nutrient medium, the tested strain at microscopic observation appeared as Gram positive rods shaped, arranged in diploid form, in long chains (nutrient agar) or in small irregular pairs (nutrient broth) (Figure 2 a, b).

The sporulation capacity of genus *Bacillus,* determine them a high stability to survive at low pH; the strain produced oval endospores located central, paracentral or subterminal without distorting the vegetative cell.

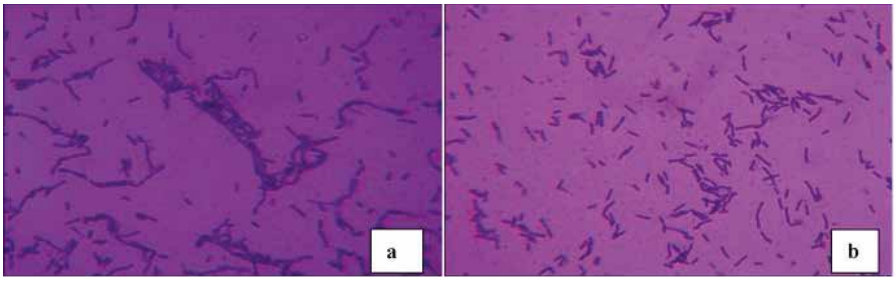

Figure 2. Microscopic observation of *Bacillus licheniformis* ATCC 21424 strain (1000x): a. culture on agar plate; b. culture from nutrient broth

Bernardeau et al. (2017) reported that the feed supplementation with specific *Bacillus* strains can provide numerous benefits including improvement in digestibility, the gut microbiota, immune modulation and growth performance.

These positive effects can be sustained by the ability of bacilli to produce spores, considering a direction in a long storage of processing feed, involving resistant to survive at environmental conditions. More *Bacillus* strains can remain viable for hundreds of years (Liao & Nyachoti, 2017).

The strain sporulation is necessary to regain after entering in the host, the metabolism is reactivated and benefits positive effects will be observed (Cutting, 2011).

The strain was catalase positive, gas bubbles was observed at addition of 3% H_2O_2; this is a characteristic that differentiates *Bacillus* spp. from the anaerobic spore-forming, for example *Clostridium* spp. (Barbosa et al., 2005). The catalase production can stimulate, according to Hosoi et al. (2011), the growth and viability of *Lactobacillus* spp. from GIT.

Results obtained from the API 50 CHB tests indicated that used test was able to confirm *B. licheniformis* ATCC 21424 around 99.9% ID (very good percentage identification) and ABIS online (~90.6% similarity, Figure 3).

The fermentation capacity of carbohydrate was observed by the discoloration of the basal medium, from red to yellow, as positive answer (Figure 4).

Figure 3. Strain identification by ABIS online (www.tgw1916.net)

Figure 4. API 50 CHB strips inoculated with *Bacillus licheniformis* ATCC 21424, before and after incubation (24 h, 37°C)

The results by API 50 CHB were registered as final interpretation after 48 h, at 37°C (Table 1). From the analysis of Table 1, is observed that, the strain fermented esculin, glycerol, salicin, D-cellobiose, D-arabinose, D-maltose, L-arabinose, D-lactose, D-ribose, D-melibiose, D-xylose, D-saccharose (sucrose), D-trehalose, D-raffinose, starch, D-glucose, D-fructose,

glycogen, D-mannose, D-turanose, inositol, D-tagatose, D-mannitol, D-sorbitol, methyl-αD-glucopyranoside, amygdalin and arbutin. The strain did not ferment of erythritol, D-arabinose, L-arabinose, D-lactose, D-melibiose, L-xylose, D-adonitol, inulin, methyl-βD-xylopyranoside, D-galactose, D-melezitose, xylitol, L-sorbose, gentibiose, dulcitol, D-lyxose, D-fucose, L-fucose, methyl-αD-mannopyranoside, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5 ketogluconate and N-acetylglucosamine.

Table 1. The results obtained with API 50 CHB for *Bacillus licheniformis* ATCC 21424

Biochemical tests	Interpretation				
	24h	48h		24h	48h
Control	-	-	Esculin	+	+
Glycerol	+	+	Salicin	+	+
Erythritol	-	-	D-cellobiose	+	+
D-arabinose	-	-	D-maltose	+	+
L-arabinose	+	+	D-lactose	-	+
D-ribose	+	+	D-melibiose	-	+
D-xylose	?	+	D-saccharose (sucrose)	+	+
L-xylose	-	-	D-trehalose	+	+
D-adonitol	-	-	Inulin	-	-
Methyl-βD-xylopyranoside	-	-	D-melezitose	-	-
D-galactose	-	-	D-raffinose	-	+
D-glucose	+	+	Starch	+	+
D-fructose	+	+	Glycogen	+	+
D-mannose	+	+	Xylitol	-	-
L-sorbose	-	-	Gentibiose	?	-
L-rhamnose	-	+	D-turanose	-	?/+
Dulcitol	-	-	D-lyxose	-	-
Inositol	+	+	D-tagatose	+	+
D-mannitol	+	+	D-fucose	-	-
D-sorbitol	+	+	L-fucose	-	-
Methyl-αD-mannopyranoside	-	-	D-arabitol	-	-
Methyl-αD-glucopyranoside	+	+	L-arabitol	-	-
N-acetylglucosamine	-	-	Potassium gluconate	-	-
Amygdalin	+	+	Potassium 2-ketogluconate	-	-
Arbutin	+	+	Potassium 5-ketogluconate	-	-

- = negative; + = positive; ? = doubtful, weakly positive.

The API 50 CHB test gives information about the strain capacity to act on the substrate and to fermented it; also, this analyze represents an alternative to understand the strain's enzymatic equipment and can be observed by discoloration of the basal medium.

Hemolysis production

The hemolytic evaluation was determined on TSA agar plates supplemented with 5% sheep blood. The assay is based on the ability of strain to lyse blood cells of culture medium.

The safety of *B. licheniformis* ATCC 21424 to be used as a possible probiotic in piglets' feed was confirmed by non-hemolytic activity on 5% sheep blood agar plate (γ-hemolysis, Figure 5). A clear zone around colonies on TSA medium indicate a complete hydrolysis (β-hemolysis), the strain must to be eliminated for utilization as a probiotic in animal nutrition. According to Prieto et al. (2012) and Seker (2010), non-hemolysis (γ-hemolysis) and α-hemolysis (a green zone around colony) are considered to be safety.

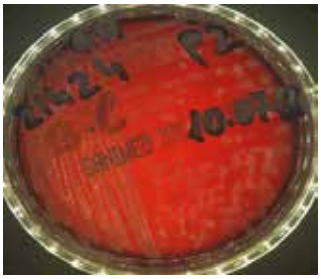

Figure 5. Hemolysis assay of *Bacillus licheniformis* ATCC 21424, at 37°C, 24 h

Growth of the bacterial strain

The growth of *B. licheniformis* was investigated after 24 h incubation at 37°C under static conditions and under a constant agitation, by incubating culture flasks at 150 rpm. After static incubation number cells of *B. licheniformis* were approximatively 4 x 10^8 (CFU/ml), while it in case of agitation were amounted 1.56 x 10^{10} (CFU/ml).

The experimental results, in Figure 6, showed that agitation is a better parameter for growing bacteria, compared with the static incubation. The result was expressed as logarithm of colony forming units/ml (log_{10} CFU/ml).

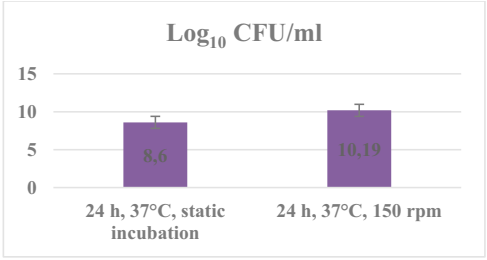

Figure 6. The viability of *B. licheniformis* ATCC 21424 at different conditions

Specific Growth of strain in minimal medium containing different carbon sources

To screen out, the most favorable carbon source was included in the basal medium: glucose, fructose, lactose and starch (1%). The bacterial growth on different substrates was registered in Table 2.

Table 2. Log_{10} CFU/ml of *B. licheniformis* ATCC 21424 on minimal medium containing different carbon sources

Carbon sources	Log_{10} CFU/ml
Control	12.11 ± 0.16^{b}
Starch	12.08 ± 0.14^{a}
Fructose	11.73 ± 0.67^{ab}
Glucose	12.11 ± 0.1
Lactose	12.03 ± 0.022

Results represent the mean ± standard deviation of three experiments (n=3).
[a, b] Values which differ significantly at P <0.05.

The maximum growth rate of *B. licheniformis* was registered in agar medium containing glucose, following by starch, lactose and fructose (Figure 7). The medium with starch addition was selected to put in evidence the enzymatic capacity of strain.

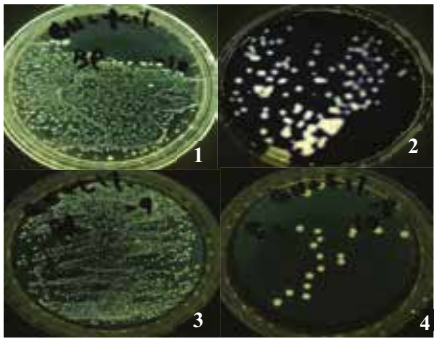

Figure 7. Growth of *B. licheniformis* ATCC 21424 in nutrient agar medium containing different carbon sources (1: glucose, 2: starch, 3: lactose, 4: fructose)

Amylases break down starch; by increasing starch digestibility, amylases potentially permit pigs to extract more energy from the feed, which can be efficiently converted into meat production. In young pig diets, amylases provide benefits by supplementing an immature digestive system where low feed intake post-weaning is associated with a slow maturation of amylase secretion. In addition, amylase also tolerates the use of less cooked grain in the diet, with benefits in feed cost reduction, without compromising young pig performance after weaning (Barletta, 2011).

Screening of amylase enzyme

The amylase method, as qualitative assay, was based on the reduction in blue color intensity resulting from enzyme hydrolysis of different quantity of starch addition in the basal medium (1%, 2% and 3%, w/v). Production of this enzyme was studied after 24 h of incubation at 37°C and pH 7. After addition of Lugol solution, a clear zone of hydrolysis on starch was observed on agar plates (Figure 8). On the plate with 1% starch addition was registered the maximum zone of hydrolysis and this concentration will be selected for other investigations.

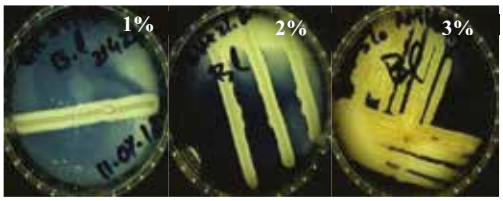

Figure 8. Screening of hydrolysis amylase of *Bacillus licheniformis* ATCC 21424:
1%, 2%, 3%: the quantity of starch addition in the basal medium

The capacity of selected *Bacillus* strains to produce and secrete large quantities of extracellular enzymes has placed them the most important industrial enzyme producers (Manabe et al., 2013). Dumitru et al. (2018a), reported similar data about the screening for amylase producing by a strain from *Bacillus* spp.

The addition of microbial enzymes as DFM, can aiding the digestion process of young animals (piglets, broilers etc.), whose enzymatic system is incomplete development.

Improving feed efficiency, nutrients supplementation, palatability, health in the stressful piglet period by decrease the numbers of pathogenic bacteria from GIT and increase the benefic bacteria colonization, DFM with enzymatic action represent a systematic role in young animal life (Van der Aar et al., 2016).

Screening of protease enzyme

Bacillus licheniformis ATCC 21424 strain was screened for extracellular protease production,

on agar plates containing casein (1% w/v, left photo) and milk powder (1% w/v, right photo). The strain hydrolysis capacity can be observed by appearance of a clear zone around bacteria growth, at addition of TCA 25% (Figure 9).

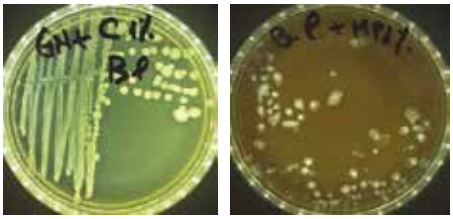

Figure 9. Screening of hydrolysis protease of *Bacillus licheniformis* ATCC 21424

Strains from the genus *Bacillus* produce a huge variety of extracellular enzymes, proteases occurring an important role in animal nutrition. Feed supplementation with specific enzymes improves the nutritional value of raw compound's feed, increasing the efficiency of digestion. Using bacterial strains as sources of extracellular proteases, provide a serial of benefits by break down the proteins from various raw materials, releasing bound energy that can be digested by the animal body (Barletta, 2011).

According to Merchant et al. (2011) the *Bacillus* spp. can be used as DFM in animal nutrition because the pH value in the small intestine is 6 to 7, which is optimal for spores to germinate, grow and produce enzymes and, also, to resist of the enzymatic degradation and low pH of the gastric barrier (Barbosa et al., 2005).

Preservation of bacterial strain

In Table 3 are presented the results of strain viability test which was preserved at 4°C and at room temperature.

Table 3. Testing the viability of *Bacillus licheniformis* ATCC 21424 strain preserved at 4°C and room temperature

Strain	Viability at 4°C	Viability at room temperature
Bacillus licheniformis ATCC 21424	+/3 months; +/6 months	+/3 months; +/6 months

+ = positive, - = negative.

The strain viability will be tested at 9 months, until will be establish the long-time preservation.

Resistance to pH

Bacillus licheniformis ATCC 21424 strain, conserved on nutrient agar tubes at 4°C and room temperature (6 months), was tested for resistance to simulated gastric juice (pH 2 and 3), under a constant agitation (37°C, 24 h, 120 rpm, Table 4).

The strain preserved in different conditions, presents a best survival rate, during 120 min incubation at pH 2 and 3. In Table 4, can be observed the strain resistance when was exposed to simulated gastric juice.

The strain preserved at 4°C, pH 2, present significative different between all times of incubation according to Lee et al. (2012) method. The pH 2 reduced slowly the cell numbers at 4°C and room temperature preservation, compared with pH 3 at 4°C, where the survivability did not differ significantly between the all incubation times.

Table 4. The effect of synthetic gastric juice (pH 2 and pH 3) on the *Bacillus licheniformis* ATCC 21424 viability during 120 min under constant agitation exposure

Strain	pH of synthetic gastric juice	0 min	30 min	60 min	90 min	120 min	SEM	P value
B l ATCC 21424	pH 2/4°C	10.803^{a}	8.393^{ab}	10.15^{bc}	8.593^{ac}	8.393^{ac}	0.283	<0.0001
	pH 3/4°C	10.353	10.16	10.067	10.1	10.047	0.060	<0.5548
	pH 2/room temperature	10.487^{a}	10.16^{ab}	10.083^{ac}	9.177^{abcd}	8.697^{abcd}	0.182	<0.0001
	pH 3/room temperature	11.533^{a}	9.913^{ab}	9.6^{ac}	8.687^{abc}	8.45^{abc}	0.301	<0.0001

Viable counts (log_{10} CFU/ml) of strain at 30, 60, 90 and 120 min was compared with counts at 0 min.
Results represent the mean of three experiments (n = 3). $^{a, b, c, d}$Means in the same row differ significantly at P <0.05.

In addition, Lee et al. (2012), reported low values of analyzed strains from *Bacillus* spp. during 30 min incubation at pH 2. The *Bacillus licheniformis* strain can be a possible probiotic candidate in piglet nutrition, because it maintained high viability during 120 min incubation, at 2 and 3 pH.

According to Nhi and Huong (2016), the numbers of *Bacillus* spp. strains have reduced prominently, comparative with our result, where the strain is capable to survival at low pH (2 and 3).

Bile resistance test

The strain was resistant to oxgall bile salt (Table 5). During incubation at 4 h in medium with 0.3% bill salt, the strain presents a high level of bile tolerance with a slow loss of viability.

Table 5. Effect of oxgall bile salt on the viability of *Bacillus licheniformis* ATCC 21424 strain during 4 hours exposure

Preserved conditions	Viable count (log_{10} CFU/ml) of *Bacillus licheniformis* ATCC 21424						
	0 h	1 h	2 h	3 h	4 h	SEM	P values
4°C	9.187^{a}	9.06^{b}	9.043^{c}	9.437^{d}	8.44^{abcd}	0.112	0.0332
Room temperature	10.153^{a}	10.347^{b}	9.527^{abc}	8.91^{abcd}	8.28^{abcd}	0.213	0.0001

Viable counts (log_{10} CFU/ml) of strain at 1, 2, 3 and 4 h was compared with counts at 0 h. Results represent the mean of three experiments (n=3). [a, b, c, d] Means in the same row differ significantly at P <0.05.

The *Bacillus licheniformis* ATCC 21424 pre-servation at 4°C differ significantly compared with room temperature preservation, suggesting that the spores are able to germinate without to be inhibited by the presence of bile salt in the small intestine (Guo et al., 2006).

CONCLUSIONS

The results suggested that the *Bacillus licheniformis* ATCC 21424 strain presents some probiotic properties. This strain has the capacity to secret amylase and protease enzymes, resists 6 months at 4°C and room temperature, does not show hemolytic activity (γ- hemolysis) on TSA medium confirming that is not pathogenic.

The strain spores were able to tolerate bile salt and survives at low pH in the conditions of stimulate gastric juice after 120 minutes.

This *in vitro* study can represent an alternative to improve animal health by increasing nutrition digestibility. Further experiments on animals are necessary to confirm the probiotic potential and to validate the importance of bacterial strain as source of feed additive.

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REFERENCES

Barbosa, T. M., Serra, C. R., La Regione, R. M., Woodward, M. J., Henriques, A. O. (2005). Screening for *Bacillus* isolates in the broiler gastrointestinal tract. *Applied and Environmental Microbiology*, *71*(2), 968–978.

Barletta, A. (2011). Introduction: Current market and expected developments. Chapter I from *Enzymes in farm animal nutrition, 2nd Edition*, 3.

Blank, R., Mosenthin, R., Sauer, W. C., Huang, S. (1999). Effect of fumaric acid and dietary buffering capacity on ileal and fecal amino acid digestibilities in early-weaned pigs. *Journal of Animal Science*. https://doi.org/10.2527/1999.77112974x.

Chen, Y. J., Min, B. J., Cho, J. H., Kwon, O. S., Son, K. S., Kim, H. J., Kim, I. H. (2006). Effects of dietary *Bacillus*-based probiotic on growth performance, nutrients digestibility, blood characteristics and fecal noxious gas content in finishing pigs. *Asian Australis J. of Anim. Sci.*, 19, 587–592.

Cutting, S. M. (2011). *Bacillus* probiotics. *Food Microbiol.*, *28*(2), 214–220.

Duc, L. H., Hong, H. A., Barbosa, T. M., Henriques, A. O, Cutting, S. M. (2004). Characterization of *Bacillus* probiotics available for human use. *Appl. Environ. Microbiol.*, *70*, 2161–2171.

Dumitru, M., Sorescu, I., Habeanu, M., Tabuc, C., Idriceanu, L., Jurcoane, S. (2018a). Preliminary characterisation of *Bacillus subtilis* strain use as a dietary probiotic bio-additive in weaning piglet. *Food and Feed Research*, *45*(2), 203–211.

Dumitru, M., Tabuc, C., Jurcoane, S. (2018b). Obtaining a feed additive based of *Lactobacillus plantarum*

strain. *Scientific Papers. Series A. Agronomy*, *61*(2), 115–122.

European Union (2005). Ban on antibiotics as growth promoters in animal feed enters into effect (1831/2003/EC).

Guo, X., Li, D., Lu, W., Piao, X., Hen, X. (2006). Screening of *Bacillus* strains as potential probiotics and subsequent confirmation of the *in vivo* effectiveness of *Bacillus subtilis* MA 139 in pigs. *Antonie van Leeuwenhoek*, *90*, 139–146.

Hăbeanu, M., Tabuc, C., Gheorghe, A., Ropota, M., Dumitru, M., Călin, L., Mihalcea, T., Palade, M. (2016). Preliminary study on the interrelation between sow milk quality and litter performance in relation to their health. *Scientific Papers-Animal Science Series*, *66*, 35–40.

Hosoi, T., Ametani, A., Kiuchi, K., Kaminogawa, S. (2011). Improved growth and viability of lactobacilli in the presence of *Bacillus subtilis* (natto), catalase or subtilisin. *Canadian Journal of Microbiology*, *46*, 892–897.

International Organization for Standardization: ISO 7218 (2007). *Food Microbiology-General Requirements and Recommendations*, *66*.

Jha, R., Berrocoso, J. F. D. (2015). Dietary fiber and protein fermentation in the intestine of swine and their interactive effects on gut health and on the environment: A review. *Animal Feed Science and Technology*, *212*, 18–26.

Jeon, H. L., Yang, S. J., Son, S. H., Kim, W. S., Lee, N. K., Paik, H. D. (2018). Evaluation of probiotic *Bacillus subtilis* P229 isolated from cheonggukjang and its application in soybean fermentation. *Food Science and Technology*, *97*, 94–99.

Kaewtapee, C., Burbach, K., Tomforde, G., Hartinger, T., Silva, A. C., Heinritz, S., Seifert, J., Wiltafsky, M., Mosenthin, R. F., Rosenfelder-Kuon, P. (2017). Effect *of Bacillus subtilis* and *Bacillus licheniformis* supplementation in diets with low- and high-protein content on ileal crude protein and amino acid digestibility and intestinal microbiota composition of growing pigs. *Journal of Animal Science and Biotechnology*, *8*(37), 1–15.

Lee, J., Park, I., Choi, Y., Cho, J. (2012). *Bacillus* strain as feed additive: in vitro evaluation of its potential probiotic properties. *Rev. Colombiana de Ciencias Pecuarias*, *25*, 577–585.

Lyao, S. F., Nyachoti, M. (2017). Using probiotics to improve swine gut health and nutrient utilization. *Animal Nutrition*, *3*, 331–343.

Liao, S. F., Nyachoti, M. (2017). Using probiotics to improve swine gut health and nutrient utilization. *Animal Nutrition*, *3*(4), 331–343,
https://doi.org/10.1016/j.aninu.2017.06.007.

Ludwig, W., Euzéby, J., Whitman, W. B. (2012). Taxonomic outline of the phylum Actinobacteria. In *Bergey's Manual of Systematic Bacteriology*. https://doi.org/10.1007/978-0-387-68233-4_2.

Manabe, K., Kageyama, Y., Morimoto, T., Shimizu, E., Takahashi, H., Kanaya, S., Ara, K., Ozaki, K., and Ogasawara, N. (2013). Improved production of secreted heterologous enzyme in *Bacillus subtilis* strain mgb874 via modification of glutamate metabolism and growth conditions. *Microbial Cell Factories*, *12*, 18. http://dx.doi.org/10.1186/1475-2859-12-18.

Merchant, H. A., McConnell, E. L., Liu, F., Ramaswamy, C., Kulkarni, R. P., Basit, A. W., Murdan, S. (2011). Assessment of gastrointestinal pH, fluid and lymphoid tissue in the Guinea pig, rabbit and pig and implications for their use in drug development. *European Journal of Pharmaceutical Science*, *42*, 3–10.

Nhi, H. T. H., Huong, T. (2016). Examining some probiotics activities of *Bacillus subtilis* Natto. *International Journal of Modern Eng. Research*, *6*(5), 33–37.

Pant, G., Prakash, A., Pavani, J. V. P., Bera, S., Deviram, G. V. N. S., Kumar, A., Panchpuri, M., Prasun, R. (2015). Production, optimization and partial purification of protease from *Bacillus subtilis*. *J. of Taibah University for Science*, *9*, 50–55.

Prieto, M. L., O'Sullivan, L., Tan, S. P., McLoughlin, P., Hughes, H., Gutierrez, M., Jonathan, A., Lane, J., Hickey, R. M. G., Lawlor, P. G., Gardiner, G. E. (2014). *In vitro* assessment of marine *Bacillus* for use as livestock probiotics. *Mar. Drugs*, *12*, 2422–2445.

Seker E. (2010). Identification of Candida species isolated from bovine mastitis milk and their in vitro hemolytic activity in Western Turkey. *Mycopathologia, 169*, 303–308.

Stoica, C., Sorescu, I. (2017). ABIS online - Advanced Bacterial Identification Software, an original tool for phenotypic bacterial identification. Regnum Prokaryotae, www.tgw1916.net.

Sorescu, I., Dumitru, M., Ciurescu, G. (2019). *Lactobacillus* spp. and *Enterococcus faecium* strains isolation, identification, preservation and quantitative determinations from turkey gut content. *Romanian Biotechnological Letters*, *24*(1), 41–49.

Upadhaya, S. D., Kim, S. C., Valientes, R. A., Kim, I. H. (2015). The effect of *Bacillus*-based feed additive on growth performance, nutrient digestibility, fecal gas emission, and pen cleanup characteristics of growing-finishing pigs. *Asian Australas J. Anim. Sci*., *28*, 999–1005.

Van der Aar Molist, F., Van der Klis, J. D. (2016). The central role of intestinal health on the effect of feed additives on feed intake in swine and poultry. *Animal Feed Science and Technology, 233*, 64–75.

Wu, Y. P., Jiang, Z. Y., Zheng, C. T., Wang, L., Zhu, C., Yang, X. F.,Wen, X., Ma, X. (2015). Effects of protein sources and levels in antibiotic-free diets on diarrhea, intestinal morphology and expression of tight junctions in weaned piglets. *Anim. Nutrition*, *1*(3), 170–17.