Identification and Characterization of *Bacillus megaterium* as Probiotic Bacteria in Poultry Feed

Mihaela Dumitru[1], Nicoleta Lefter[1], Lavinia Idriceanu[1], Georgeta Ciurescu[1], Mihaela Habeanu[1]

[1] *National Research & Development Institute for Animal Biology and Nutrition (IBNA), Laboratory of Animal Nutrition & Biotechnology, Calea Bucuresti no.1, 077015, Balotesti, Ilfov, Romania.*

Abstract

The present study was planned to identify the *Bacillus* probiotic characteristics from a strain knows as *Bacillus* spp. in with the aim of use as a probiotic candidate in broiler chickens feed. The strain was identified phenotypically and evaluated for their viability through total plate count method, colony morphology, catalase test, hemolysis activity, pH (2 and 3) and bile salts (0.3% w/v) tolerance under simulated gastrointestinal tract (GIT) conditions, rate of survivability (%), spores resistance at high temperature and antibiotics susceptibility tests. Registered under the code IBNA 66, the strain was identified by API 50CHB (ID 99.9%) and ABIS online (91.8% similarity) as *Bacillus megaterium*. The strain presented a viable count of 3 x 10^{11} CFU/mL after 22±2h of incubation at 37°C, 120 rpm with positive catalase, and non-hemolytic activity results. Also, *Bacillus megaterium* showed a significant resistance and survivability at pH 2 (*P <0.0001*, 62.09%) and pH 3 (*P <0.0001*, 77.53%), bile salts (*P <0.0001*, 84.27%), with a high ability to produce spores (after 120 min. at 80°C show 9.47 Log CFU/mL, *P <0.0001*). The antibiotic susceptibility test showed 100.00% resistance of strain to oxacillin (1 µg/mL), with multiple antibiotic resistance indices above 0.5. In conclusion, *Bacillus megaterium* can be an ideal probiotic candidate that can potentially be formulated and applied in the poultry feed for improving performance and modulated GIT microflora.

Keywords: gastrointestinal resistance, *in vitro* properties, probiotic

1. Introduction

Benefits from probiotic microorganisms have been recognized for over 100 years, and as being useful in poultry for more than 50 years [1]. Probiotics are "mono or defined mixed culture of live microorganisms which when applied to animals, beneficially affect the host by improving the properties of the indigenous microbiota"[2]. Added in regular quantities, the use of probiotics in the animal industry can offer a health benefit to the host [3]. Previous studies have shown that dietary supplementation with *Bacillus* spp.-based probiotics could be successfully used in poultry diets and have been shown to have growth-promoting benefits [4].

Specifically from the genus *Bacillus*, probiotics, are evolving as a possible solution does to their capacity to spore formers [2]. Able to survive extreme environmental conditions such as low pH, high heat, bile salts, dry and under adverse nutrition environments [5], afford them several advantages over conventional probiotics [3], which make stable for a long time, *Bacillus* spp. can be a source to enhance the bird's intestinal structure and growth performance [1].

Isolated, generally, from the soil, *Bacillus* spp. present up to 100% survivability in the severe conditions of the animal GIT [3]. *Bacillus* is an

* Corresponding author: Dumitru Mihaela, (+40) 763609262, mihaela.dumitru22@yahoo.com

established bacteria and can be found in the intestinal tracts of the birds, where they can live and increase, could help support overall health. Usually, the GIT is a source of lactic acid bacteria (LAB) with notable functional and technological advantages as a possible source of probiotics [6]. According to the European Food Safety Authority (EFSA, [7]), some of the *Bacillus* species can be used as microbial feed additives include *B. clausii, B. coagulans, B. fusiformis, B. lentus, B. licheniformis, B. megaterium, B. pumilus and Geobacillus stearothermophilus*, as well as species previously or presently classified as *B. subtilis* (*B. amyloliquefaciens, B. atrophaeus, B. mojavensis, B. subtilis* and *B. vallismortis*) [8].

Bacillus megaterium is a Gram positive, a nonpathogenic bacteria, spore-forming, mainly aerobically, isolated generaly from soil environment. Recently studies, have focused on isolating *Bacillus* strain from poultry GIT and their utilisation as source of probiotic [9, 10]. Barbosa et al. [11] affirmed that *Bacillus megaterium* is a comensal bacteria which was isolated and identified in chicken intestinal tract. In another study, Nguyen et al. [12] reported that *Bacillus megaterium* was isolated from poultry faces.

The aim of the present study was to characterize the probiotic qualities of *Bacillus* spp. and evaluated it prior to possible application as supplements in poultry nutrition.

2. Materials and methods

Bacterial strain, culture medium and growth conditions

A bacterial strain isolated from soil and conserved in lyophilized form as *Bacillus* spp., was revitalized 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. The inoculum was analyzed by serial 10-fold dilutions using phosphate buffer saline solution (PBS), and then 1 mL from 10^{-10} to 10^{-14} was placed on nutrient agar medium (g/L: tryptone 5; meat extract 3; bacteriological agar 5; distilled water).

Determination of colony-forming units

The growth rate of *Bacillus* spp. was determined from overnight culture and the result was expressed as $\log_{10}$ CFU per mL. The strain contains at least 5×10^{11} CFU mL^{-1}.

Strain identification and characterization

The overnight culture was analyzed morphologically by Gram staining according to Bergey's Manual of Systematic Bacteriology [13] and biochemically by API 50 CHB strips according to manufacturer's instructions (Biomerieux, France). The attained results are interpreted using database system API 50 CHB V4.0 and ABIS online software [14].

Catalase test

The catalase activity was detected by resuspending the culture in a 3% solution of hydrogen peroxide (H_2O_2).

Hemolysis production

Blood agar plates [Trypticase soy agar (TSA, Sanimed) containing 5% (w/v) sheep blood], were used to test hemolysis activity [15]. The interpretation was followed after incubation at 37°C, for 24 h.

Preservation of bacterial strain

The strain was stored at -80°C with 20% sterile glycerol according to Sorescu et al. [16] and deposited in the Collection of National Research Development Institute for Biology and Animal Nutrition Balotești (INCDBNA), Romania, under the code IBNA 66. The research was carried out at Laboratory of Biotechnology of (INCDBNA), Romania.

Characterization for probiotic properties

The new strain identified was evaluated *in vitro* for some of the probiotic properties.

Acid tolerance test

The acid resistance of strain was investigated under simulated gastric juice (SGJ) by following the Lee et al. [17] method, which was modified by Dumitru et al. [18]: 1 mL of a culture grown in nutritive broth for 24h at 37°C, 120 rpm, representing about 10^{10} colony forming units (CFU/mL), was transferred to 9 mL of SGJ [0.5% NaCl, 0.3% pepsin (from gastric mucosa, Sigma), 0.1% peptone (BD Science)], whose pH was adjusted to 2 and 3 with a Portable meter (Waterproof, pH 7.0+DHS) using HCl 1 N, then incubated for 0, 30,

60, 90 and 120 min. at 37°C, 120 rpm. Viable cells of the culture were enumerated by plating 10-fold dilutions [1:10, in the phosphate-saline buffer (PBS at pH 7.2)] on nutrient agar and plates incubating at 37°C, 24 h.

Bile salts tolerance

The resistance of bacteria to bile salts was assayed as described Lee et al. [17], respectively following the modifications by Dumitru et al [18]: 10 mL of culture strain (about 10^{10} CFU/mL) grown in nutritive broth (pH 7.0) for 24 h at 37°C on a rotary shaker (120 rpm), was centrifuged at 5.000 rpm, 20 min, at 4°C. Cell pellets were washed with PBS, collected by centrifugation (5.000 rpm, 20 min, at 4°C), and resuspended in nutrient broth (pH 7.0) containing 0.3% bile salts (w/v, Oxoid, Basingstoke, Hampshire, UK). The bacterial growth was monitored 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 in the PBS (1:10, in the PBS at pH 7.2), on nutrient agar at 37°C, 24 h. The survivability was calculated as well.

Strain survivability percentage

The survival percentage for pH and bile salt resistance was determined using the method presented by Ritter et al. [19], following the formula:

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

The spores resistance to heat

Strain's ability to resist a high temperature was carried out at 80°C, a specific temperature used for the pelleting process in the animal feed industry [20]. The suspension of vegetative cells or spores was heated up on a water bath at 80°C for 120 min. Viable cells were determined at 0, 30, 60, 90 and 120 min. 10-fold dilutions of the culture in the PBS (pH 7.2), on nutrient agar medium at 37°C, for 24 h were done.

Antibiotic susceptibility

The antibiotic susceptibility of culture strain was tested using disk diffusin method [21]. Eleven kinds of antibiotics (Oxoid, Basingstoke, Hampshire, UK) were used: Tetracycline (TE, 30 μg), Vancomycin (VA, 30 μg), Amoxicillin (AMX, 25 μg), Clindamycin (CLI, 2 μg), Erythromycin (E, 15 μg), Ciprofloxacin (CIP, 5 μg), Gentamicin (CN, 10 μg), Amikacin (AMK, 25 μg), Chloramphenicol (C, 30 μg), Kanamycin (K, 30 μg) and Oxacillin (OXA, 1 μg)The bacteria was grown on nutrient agar and incubated at 37°C, 120 rpm, 24 h. Cells from 24±2 h-old culture by using a sterile swab were suspended in a tube containing 2 mL of sterile distilled water (heavy suspension - H). In another tube with 5 mL of sterile distilled water, are transferred drops from suspension H, until the turbidity becomes equivalent to 0.5 McFarland standard. Culture adjusted to approximately 1 x 10^8 CFU/mL, was spread on nutrient agar plates. Antibiotic-impregnated discs were situated onto agar plates within 15 min of swabbing from 5 mL tube, following by incubation at 37°C, 24 h. The inhibition zone diameter was measured with a ruler as mm. The results were reading as sensitive (S) and resistance (R) based on the diameter of the inhibition zone (mm).

Statistical 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 Tukey LSD test for untitled compact variable.

3. Results and discussion

Bacterial strain, culture medium and growth conditions

Morphologically, on agar medium, *Bacillus* strain generated small colonies, with a diameter of 1-1.3 mm, reaching up to 1.5 mm at 72 h of incubation, type S, round, with regular edges, opaque, whitish, unpigmented, with creamy consistency. In nutrient broth medium, at 24 h, the culture registered a reduced turbidity, with a weak deposit, homogeneous on stirring, without surface formations; at 72 h the strain tends to form a deposit.

Microscopically, it appeared rod-shaped Gram-positive *Bacillus.* Arranged in diploid form, bacilli appear with rounded ends, short chains, with spore located centrally. The sporulation process takes place in aerobiosis conditions. In nutrient broth medium, the culture appears rarely (Figure 1, A), compared to the agar medium where the grade of

sporulation is much more accentuated (Figure 1, B).

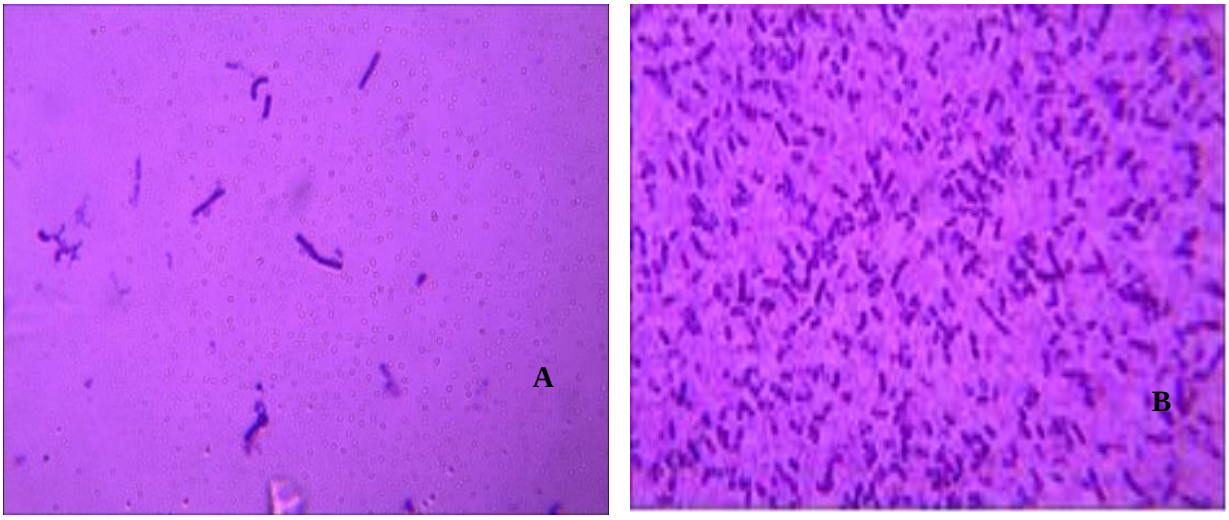

Figure 1. *Bacillus* spp. strain aerobe culture in broth medium (A)/ on agar medium (B) (Gram staining, x 1000)

Simple sub-culturing actions on nutrient agar medium are generally used to obtain individual *Bacillus* cultures, followed by quick verification techniques such as microscopic observation of spores, Gram stain, catalase reaction, and other *in vitro* tests applied for evaluation of a bacterial probiotic with application in the animal industry [22]. In a large scale of fermentation, *Bacillus* spp. confer certain advantages due to their capacity to be cultivated at high cell density and tolerance to a varied range of process conditions, with high sporulation productivities on low cost substrates [23].

Strain identification and characterization

The strain's ability to ferment and degrade the carbohydrates from API 50 CHB strips are detailed in Table 1. After 48 h, the soft results of *Bacillus* by API and Abis online test are presented in Figure 2. The percentage of identification (ID), respectively their similarity (SIM for ABIS online) is shown in Table 2. Furthermore, after taxonomical identification, according to the manufacture protocol API Biomeriuex (France), strains were registered in the IBNA Bacterial Collection as *Bacillus megaterium* IBNA 66.

Table 1. The API 50 CHB for *Bacillus* spp.

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	+	+
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

GOOD IDENTIFICATION

Strip	API 50 CHB V4.0
Profile	- + - - - + - - - - + + + - - - - + + - - - - - - - + + + + + + + - + + + + - - - - - - - + - - - -
Note	

Significant taxa	% ID	T	Tests against			
Bacillus megaterium	99.9	0.34	LARA 87%	DXYL 76%	NAG 87%	ARB 80%
			ESC 97%	GEN 81%	DARL 11%	

Next taxon	% ID	T	Tests against			
Bacillus lentus	0.1	0.19	MNE 95%	INO 1%	NAG 82%	ARB 82%
			ESC 98%	DARL 4%		

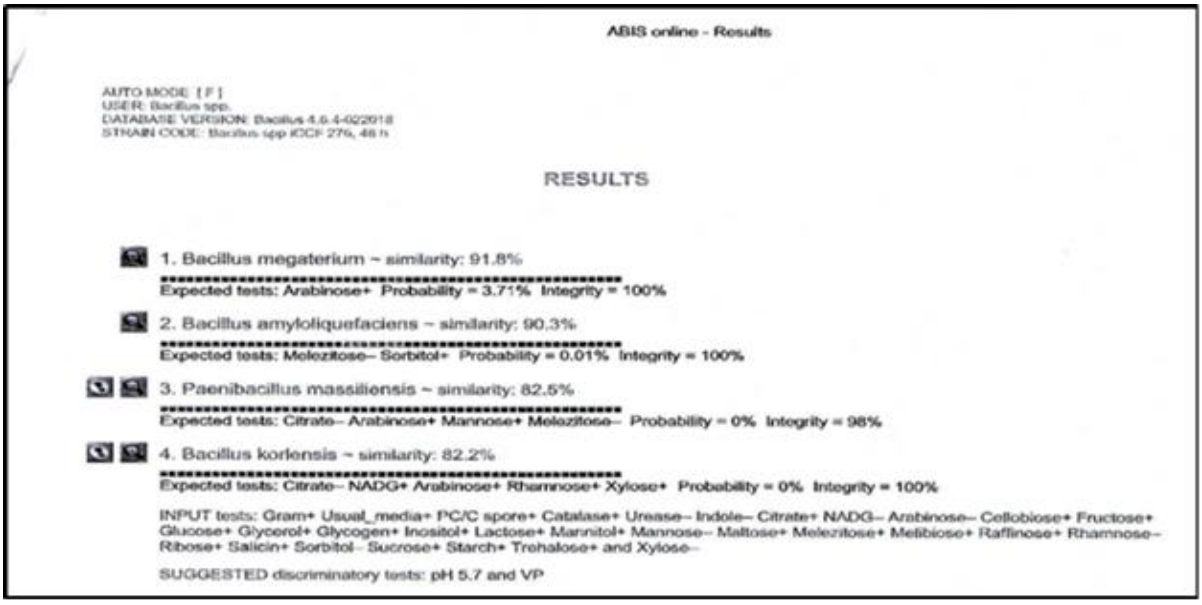

Figure 2. API Test CHB 50 and Abis online results of *B. megaterium* IBNA 66

Table 2. *Bacillus* spp. identification by API 50 CHB and ABIS online

No	Strains	Code	Source	Identification	
				API 50 CHB (ID)	ABIS online (SM)
1	*Bacillus* spp.	IBNA 66	soil	*B. megaterium*, 99.9% (GI)	*B. megaterium* ~91.8%

ID-identification; GI- good identification; SM-similarity

Catalase test

The strain was catalase-positive, formed gas bubbles after the addition of 3% solution H_2O_2.

Hemolysis production

The safety of strain identified as *Bacillus megaterium* was confirmed by non-hemolytic activity on 5% sheep blood agar plate (γ-hemolysis) which means a no reaction (Figure 2).

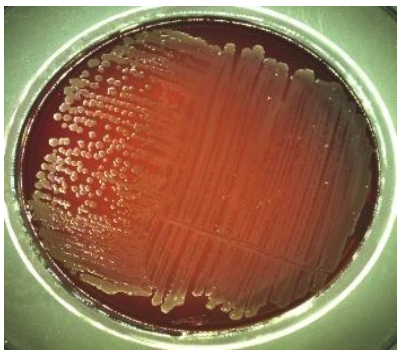

Figure 2. Haemolysis assay of *Bacillus megaterium* IBNA 66

Our strain had no clear transparent or greenish zone surrounding colonies on TSA agar. The results are similar to Kavitha et al. [24] and Dumitru et al. [15].

Preservation of bacterial strain

The results of *B. megaterium* IBNA 66 viability preserved at 4°C and at room temperature are shown in Table 3. To reveal the long time preservation, every 3 months, the viability of *B. megaterium* IBNA 66 was verified until the bacterial growth will stop. The number of passages is recorded in a register to confirm the long time preservation. In the present study, our strain exhibited good viability, more than 12 months, in both situations of preservation.

Table 3. The viability of *Bacillus megaterium* IBNA 66 at 4°C and room temperature

Strains	Viability	
	4°C	**Room T°C**
B. megaterium IBNA 66	≤ 12 months	< 9 months

To prepare a bacterial probiotic product is very significant to identify the long storage viability, to know how often it needs to be revitalized. *Bacillus* group comparative to all species of *Lactobacillus*, do to the presence of spores involve several advandages as the capability to survive at low pH of the gastric barrier, respectively to be stored at room temperature without losing on their viability. For the production of probiotics products based on bacteria such as *Bacillus* spp., it is essential to have a stable culture that is appropriately preserved. In contradiction to vegetative non-sporulation cells [16], *Bacillus* spp. can be stored in spore form with better stability and capability to grow [25].

Acid tolerance test

The ability to tolerate high acid concentrations present in an animal stomach is one of the relevant criteria for selecting a good probiotic product.

In Table 4, can be observed the strain viability identified as *Bacillus megaterium*, when was exposed at low pH during 120 min under constant agitation. A decrease of cell viability was registered when the strain was exposed to pH 3 (P <0.05) and pH 2 (P <0.05). Significantly very high resistance of *Bacillus megaterium* IBNA 66 was registered at pH 3.

Table 4. The effect of synthetic gastric juice (pH 2 and pH 3) on the *Bacillus megaterium* IBNA 66 viability for 120 min under constant agitation exposure.

Preserved conditions	pH 7	0 min	30 min	60 min	120 min	24 h/37°C	*SEM*	*P*
pH 2	11.66^{a}	10.24^{b}	9.09^{c}	8.04^{d}	8.50^{e}	7.24	0.43	0.0001
pH 3	11.66^{a}	11.27^{b}	11.07	8.65^{d}	9.19^{e}	9.04	0.40	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). $^{a, b, c, d, e}$Means in the same row differ significantly at P <0.05.

A benefic strain, before to use as a probiotic, is necessary to remain alive during the gastrointestinal tract which includes the acidic condition of the stomach and bile salts [26]. Probiotics show highly variable resistance to acid and bile salts, and this characteristic is both species and strain dependent [27]. Sharma et al. [28] affirmed that a good probiotic should survive at least pH 3.0.

Barbosa et al. [11] affirmed that a selected probiotic from *Bacillus* group is necessary to sporulate efficiently in the lab, for resist to gastrointestinal tract conditions.

Bile salts tolerance

In order to determine the strain survival rate, the viability percentage of cells with and without 0.3% bile salt addition was carried out for 0-h to 4-h exposure (Table 5). *Bacillus megaterium* IBNA 66 was able to survive up to 5 x 10^{8} CFU/mL after 4 h exposure in the presence of bile salts with highly significant differences (*P<0.001*). Our strain exhibited the ability to survive in 0.3% bile salts, te results being similar to Mingmongkolchai and Panbangred [29]. Tolerance to bile is an important feature that enables probiotic bacteria to involve in gastrointestinal conditions.

Table 5. The effect of bile salts on *Bacillus megaterium* IBNA 66

Preserved conditions	***Bacillus megaterium* IBNA 66**						
	pH 7	1 h	2 h	3 h	4 h	*SEM*	*P*
Bile salts (0.3% oxgall)	13.50[a]	10.73[b]	9.41[c]	8.85[d]	8.63[e]	0.48	*0.0001*

Viable counts (log10 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, e]Means in the same row differ significantly at $P < 0.05$.

Strain survivability percentage

The survivability of *Bacillus megaterium* IBNA 66 in the simulated gastric juice at 37°C for 3h depends on their ability to tolerate the low pH. The strain was found to exhibiting up to 62.09% of survivability at pH 2 after 3 h of incubation, whereas at pH 3 the survival rate increased around 77.53%. Similarly, the viability percentage of *B. megaterium* IBNA 66 decreased with 15.73% at the addition of bile salts in broth selective medium. The tested strain was able to grow better with bile salts comparatively with low pH values, recording a high percentage of survival cells (Table 6). Furthermore, the presence of spores can retain approximately 85% viability during bile salts harvesting process.

Table 6. The strain survivability during *in vitro* gastric intestinal condition

Strain	**Survivability, %**		
B. megaterium IBNA 66	**pH 2.0**	**pH 3.0**	**0.3% Bile salts**
	62.09	77.53	84.27

The spores resistance to heat

The viability rate of *B. megaterium* IBNA 66 at heat 80°C exposure during 2h is shown in Table 7. The level of spores during heat conditions involves a highest-stability ($P<0.0001$). Moreover, the strain spores remain stable at a concentration of 3 x 10^9 spores/mL.

The formation of a spore determines a type of cell that can survive for long periods with few or no nutrients, but is poised to return to life if nutrients become accessible [30]. A stress treatment as heat can inactivate some component of the spore's germination but may still be reactivated, for example at 37°C, optimal temperature of incubation. Generally, spores are resistant to approximately 40°C [30]. Grant et al. [31] affirmed that *Bacillus* bacteria as a spore-forming facultative anaerobe, it can survive at temperatures up to 113°C approx. 8 min, which makes it easier to use and increases its probability of surviving feed processing steps. As affirmed by Ramlucken et al. [22], spores can retain approximately 90% viability during the probiotic harvesting process. For example, a desiccation process or other stress factors like pH and bile salts did not decrease significantly the resistance of spores [1].

Table 7. The spores resistance of *Bacillus megaterium* IBNA 66 at 80°C

Strain	**0 min**	**30 min**	**90 min**	**120 min**	***SEM***	***P***
B. megaterium IBNA 66	13.50[a]	10.84[b]	9.23[c]	9.47[d]	0.51	0.0001

Results represent the mean of three experiments (n=3). [a, b, c, d]Means in the same row differ significantly at $P < 0.05$.

It is essential to determine the bacterial spores capacity until to use them as live microbial products in poultry feed. This property is useful for spore-based products with applications in the animal industry, offering a long-term life and hold their sustainability during distribution and storage [32]. *Bacillus* interferes with intestinal colonization of enteric pathogens and has value as probiotics [31, 33]. Knarreborg et al. [34] showed that the addition of *Bacillus* spores in broiler chicken feed increased the microbial diversity in the ileum and increased the growth of lactic acid bacteria in the birds fed *Bacillus* organisms.

Antibiotic susceptibility

The results of antibiotic sensitivity test of *B. megaterium* IBNA 66 are exposed in Table 8. *B. megaterium* IBNA 66 was found to be susceptible to all antibiotics test. A highly susceptible (more than 16 mm of the zone of inhibition) to tetracycline, vancomycin, amoxicillin, clindamycin, gentamicin, amikacin and kanamycin was registered. *B. megaterium* IBNA 66 involves a resistance to oxacillin (*P<0.0001*), with a high susceptibility to erytromycin, ciprofloxacin and chloramphenicol (*P<0.0001*).

Table 8. Antibiotic susceptibility of *Bacillus megaterium* IBNA 66

Item	Antibiotics	*B. megaterium* IBNA 66	Interpretation
1	Tetracycline 30 µg	2.46^{a}	S^{++}
2	Vancomycin 30 µg	2.36^{ab}	S^{++}
3	Amoxicillin 25 µg	2.30^{c}	S^{++}
4	Clindamycin 2 µg	2.13^{cd}	S^{++}
5	Erytromycin 15 µg	2.86^{e}	S^{+++}
6	Ciprofloxacin 5 µg	2.83^{ef}	S^{+++}
7	Gentamicin 10 µg	1.83^{g}	S^{+}-S^{++}
8	Amikacin 25 µg	2.23^{bcdh}	S^{++}
9	Chloramphenicol 30 µg	2.96^{efi}	S^{+++}
10	Kanamycin 30 µg	2.36^{abchj}	S^{++}
11	Oxacillin 1 µg	0^{k}	R
	SEM	0.138	na
	P	0.0001	na

Resistance (R): 0–5 mm; Sensitive (S+): 6–15 mm; Susceptible (S++): 16–25 mm; More susceptible (S+++): 26–35 mm.

As growth promoters, antibiotics have been used in livestock practice for over five decades. However, rising socio-political concerns with their use has prompted a quest for alternative methods of disease intervention and optimization of growth promotion in commercial poultry farming [35].

4. Conclusion

Our results indicate the resistance of *Bacillus megaterium* IBNA 66 in the presence of bile salts and low pH values, with a high percentage of survivability. This organism is nonpathogen and can survive to production feed processing and harsh conditions of the animal gastrointestinal tract. Besides, the bacterial culture was sensitive to antibiotics, more of these with applications in poultry nutrition, which are used only to treat clinical disease. Based on *in vitro* results, *Bacillus megaterium* IBNA 66 presented notable probiotic criteria and can be selected as a viable probiotic product for application in chickens broiler feed.

Conflict of interest statement

We declare that we have no conflict of interest

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