Preliminary Characterization of the Probiotic Properties of a Bacterial Strain for Used in Monogastric Nutrition

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Bulletin UASVM Animal Science and Biotechnologies 76(2)/ 2019
Print ISSN 1843-5262; Electronic ISSN 1843-536X
DOI:10.15835/buasvmcn-asb: 0015.19

Abstract

This study aimed to evaluate some probiotic properties of *Bacillus subtilis* ATCC 6051a. The phenotypic profile, resistance to pH by simulated gastric juice (pH 2 and 3), bile salts by simulated intestinal fluid, survivability (%), heat and antibiotics tolerance were investigated. The strain is a Gram-positive, rod-shaped bacteria, arranged in short chains or in small irregular pairs with the ability to produce spores. Good viability at pH 2 and 3, with a survival of more than ≥80%, was found. In the presence of bile salts 0.3%, over 4 h, the strain exhibited a survival ≥85%. At 80°C, for 120 min., the strain showed good growth (9.04 log CFU/ml). Results were sensitive to most antibiotics, with a highly susceptible (between 16 – 25 mm) to erythromycin, clindamycin, amoxicillin, chloramphenicol, ciprofloxacin, amikacin and kanamycin. The strain was found to be sensitive to vancomycin, gentamicin, and tetracycline. The present research demonstrated that *Bacillus subtilis* ATCC 6051a can survive under gastrointestinal conditions, which involves them to future *in vitro* and *in vivo* probiotic studies.

Keywords: animal nutrition, *Bacillus* spp., probiotic properties

Introduction

Probiotics are defined as „live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO, 2016). Probiotics can be an alternative to antibiotics in animal nutrition (Nithya and Halami, 2013).

Many strains of some *Bacillus* spp. are currently used as probiotic dietary supplements in animal feed (Bernardeau *et al.*, 2017). The ability of *Bacillus* species to form spores (Dinu *et al.*, 2019) is beneficial and allows for long-time storage without to lose its viability, both at room temperature and under refrigeration conditions (Ritter *et al.*, 2018). The spores production can influence the small intestine to exert their probiotic efficiency providing benefits to the host (Dumitru *et al.*, 2019).

In animal production, probiotics based on *Bacillus* spp. were used as growth-promoting (Cartman *et al.*, 2007). The requirements of a bacteria that could be identified as an effective probiotic include non-pathogenic (Hosseini *et al.*, 2019), resistance through the digestive system (gastric acidity and bile salts, Maruo *et al.*, 2006),

facilitative for the digestion and absorption of nutrients (Gaggìa *et al*., 2010), production of antimicrobial substances, adherence to intestinal epithelium cells (Schillinger *et al*., 2005), sensitiveness to antibiotics (FAO/WHO, 2006), and co-aggregation to form a barrier for prevents the colonization with pathogens (Nithya and Halami, 2013) such as *Salmonella* spp. (Hosseini *et al*., 2019). The bacteria can remain stable in the animal gastrointestinal tract (GIT) and have probiotic beneficial effects (Nicholson, 2002).

As many probiotics, *Bacillus* spp. selection depends on the bacterial capacity to resist acids and bile salts, through the GIT (AlGburi *et al*., 2016; Ionescu *et al*., 2013). According to Merchant *et al*. (2011), the mean gastric pH of pigs was between 2.9-4.4, while in the small intestinal was found in the range of 6.1–6.7. Also, in the digestive tract of pigs a lower pH was registered in the caecum (6.0–6.4), which is similar to human, and colon content (6.1–6.6, Fallingborg *et al*., 1989).

To reflect the survival percentage of strain multiple tests were done. Therefore, the potential of *Bacillus subtilis* ATCC 6051a was evaluated *in vitro* for some probiotic properties in order to use in monogastric nutrition.

Materials and methods

Bacterial strain and culture conditions

Bacillus subtilis ATCC 6051a (*BS*) strain previously characterized culturally, morphologically and biochemically by catalase test and API 50 CHB Biomerieux strips (Dumitru *et al*., 2018) was used in this study. The strain was maintained in 20% glycerol (v/v) and stored at -80°C. The bacterial culture can be found in the Collection of National Research Development Institute for Biology and Animal Nutrition Balotești – Romania (INCDBNA), under the code IBNA 74.

Preservation of bacterial strain

The medium preservation (months) was done by culture on nutrient agar medium (Merck). The viability was evaluated from 3 to 3 months (4°C and room temperature) according to Sorescu *et al.*, (2019). Long-time preservation (years) was done at -80°C, with addition of glycerol 20%. Bacterial viability will be assessed every 2 years (Sorescu *et al*., 2019).

Acid tolerance test

The acid resistance of *BS* strain was investigated under simulated gastric juice (SGJ) by following the Lee *et al.,* (2012) method, which was modified by Dumitru *et al*. (2019): 1 ml of 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 minutes 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.

The survival rate was calculated using the formula presented by Ritter *et al*., (2018) and Nithya and Halami (2013):

Survival (%) =

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

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

Bile tolerance test

Resistance of bacteria to bile salts was measured according to Lee *et al*. (2012), respectively by following the Dumitru *et al*. (2019) modifications: 10 mL of culture strain (about 10^{10} CFU/ml) grown in nutritive broth (pH 7.0) for 24h 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). The bacterial growth was monitored 0, 1, 2, 3, and 4h 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, 24h. The survivability was calculated as well.

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 (Chaiyawan *et al*., 2010). 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 24h were done.

Table 1. The viability of *Bacillus subtilis* ATCC 6051a strain preserved at 4°C and room temperature

Strain	Viability at 4°C	Viability at room temperature
Bacillus subtilis ATCC 6051a	+/3 months	+/3 months
	+/6 months	+/6 months
	+/9 months	+/9 months
	+/12 months	+/12 months

+ = positive, - = negative.

Table 2. The effect of synthetic gastric juice (pH 2 and pH 3) on the *Bacillus subtilis* ATCC 6051a viability for 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*
BS ATCC 6051a	pH 3/ 4°C	11.04^{a}	10.49^{a}	10.53^{a}	10.46^{a}	10.59^{a}	0.064	0.0045
	pH 2/ 4°C	10.45^{a}	10.04^{ab}	10.99^{ab}	10.49^{b}	10.61^{b}	0.084	0.0001
	pH 3/ room temperature	10.00^{a}	10.36	10.53^{a}	10.36^{a}	10.48^{a}	0.076	0.1207
	pH 2/ room temperature	9.95^{a}	10.19	10.03	10.31^{ab}	9.88^{b}	0.057	0.0689

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.

Antibiotic susceptibility test

Antibiotic susceptibility of *BS* was analyzed using the disc diffusion method described by European Committee on Antimicrobial Susceptibility Testing (EUCAST 2011). Types of antibiotics disk (Oxoid) tested are presented in Table 4. 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 - S). In another tube with 5 ml of sterile distilled water, are transffered drops from suspension S, until the turbidity becomes equivalent to 0.5 McFarland standard. Antibiotic-impregnated discs were situated on seeded plates within 15 min of swabbing from 5 ml tube, following by incubation at 37°C, 24 h. The results were reading as sensitive (S) and resistance (R) based on the diameter of the inhibition zone (mm).

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 the untitled compact variable.

Results and discussions

Bacterial strain and culture conditions

The phenotypical characterization of *BS* was performed in another study (Dumitru *et al.*, 2018), where the cultural, morphological, biochemical examination, hemolysis, and catalase test were presented. The sporulation capacity of *Bacillus* spp., involves stability and resistance for surviving and development during *in vitro* simulated conditions.

Preservation of bacterial strain

The results of *BS* viability preserved at 4°C and at room temperature are shown in Table 1. To reveal the long time preservation, every 3 months, the *BS* strain was verified, untill the bacterial growth will stop. The number of passages are recorded in a register to confirm the long time preservation. In the present study, our strain exhibited a good viability, more that 12 months, in both situations of preservation. According to Sorescu *et al.* (2019), the strains resistance at 4°C and room temperature is a relevant technical character. To prepare a bacterial probiotic product is very significant to identify the long storage viability, to know how often it needs to be revitalized.

The strains cultivation on agar nutrient medium, at low temperature (3-5°C), according to Doneva and Donev (2004), represent the base of

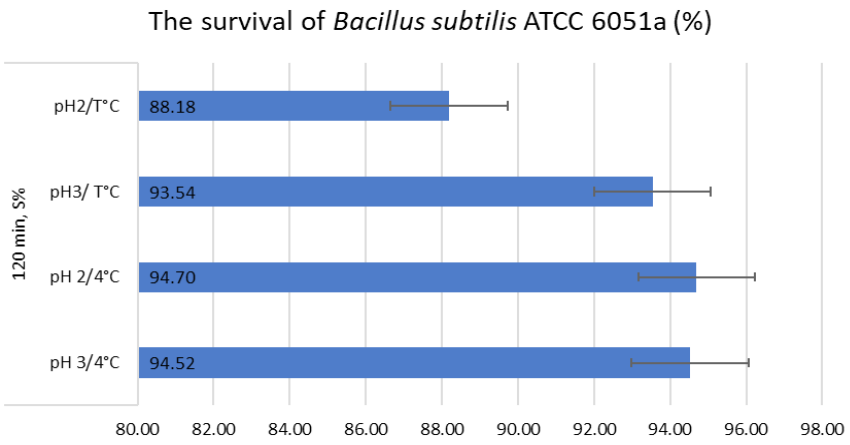

Figure 1. The survival of *Bacillus subtilis* ATCC 6051a for 2 h of incubation

Table 3. The effect of ox gall bile salts on *Bacillus subtilis* ATCC 6051a strain viability for 4 h exposure

Preserved conditions	Viable count (log_{10} CFU/ml) of *Bacillus subtilis* ATCC 6051a						
	0 h	1 h	2 h	3 h	4 h	*SEM*	*P values*
4°C	9.15^{a}	11.02^{ab}	10.26^{abc}	10.57^{acd}	10.35^{abcd}	*0.221*	*0.0001*
Room temperature	9.95^{a}	11.08^{ab}	10.16^{b}	10.46^{abc}	9.98^{abc}	0.126	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.

further preservation by keeping the taxonomical, morphological and biochemical properties. Furthermore, increasing the temperature over 5°C leads to a quick loss of cell viability (Doneva and Donev, 2004).

Acid tolerance test

Bacillus subtilis ATCC 6051a conserved on nutrient agar tubes at 4°C and room temperature (12 months), was tested for resistance to simulated gastric juice (pH 2 and 3), under constant agitation (37°C, 24 h, 120 rpm, Table 2). In Table 2, can be observed the strain resistance when was exposed at low pH value. At 4°C, pH 2, *BS* presented significative different between all times of incubation according to Lee *et al*., (2012).

According to Nithya and Halami (2013), before to use a probiotic strain in animal nutrition, it is very important to know their ability to remain alive during the ingestion process and the environment conditions of GIT (low pH values and bile salts resistance).

The survivability of *BS* at low pH (Figure 1), showed their ability to resist ≥80%, for 2 h of incubation, both at room temperature and 4°C conserved. The pH value of culture medium is an important parameter that influence the bacterial growth; generally in a medium with a low pH, the bacterial evolution is slow or absent (Doneva and Donev, 2004).

Bile tolerance test

Resistance to bile salts is a great status for survival and growth of bacteria in the GIT. Barbosa *et al*., (2005) reported that the presence of *Bacillus* spores as resistance form can be criteria for the selection of an ideal *Bacillus* probiotic. Also, the microorganism with probiotic properties, must not lose their viability after exposure to low pH and bile salts. In our study, the strain was resistant in the presence of oxgall bile salts (Table 3). The results obtained from our study are higher than the scientific data of Lee *et al.* (2012), even after 4 h exposure.

The rata of survival of *BS* was more that >85% at the addition of 0.3% ox gall (Figure 2). It was observed that the strain conserved at room temperature, respectively at 4°C during 4 h, tolerated the bile salts (0.3%) addition; also, at 4°C, the strain exhibited a higher survival rate (92.38%) *vs.* room temperature (89,07%). Balasingham *et al*., (2017), affirmed that an efficient probiotic is necessary to be capable of growing in an acidic environment and at high concentration of bile

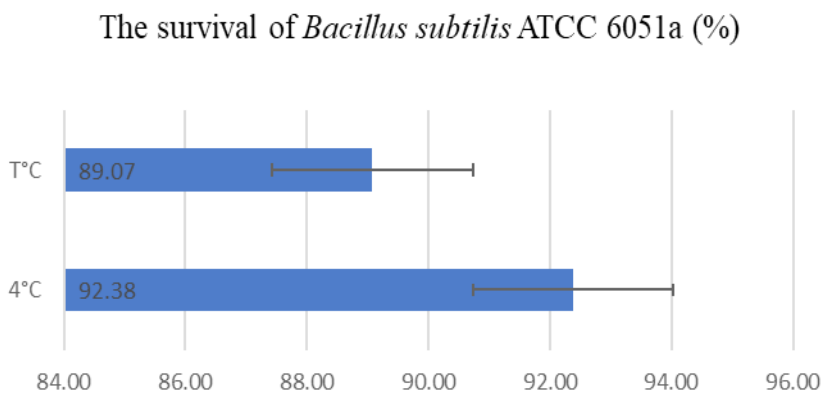

Figure 2. The survival of *Bacillus subtilis* ATCC 6051a during 4 h of incubation

Table 4. The spores resistance of *Bacillus subtilis* ATCC 6051a at 80°C

Tulpina	0 min	30 min	90 min	120 min	*SEM*	*P*
BS	12.72^{ab}	12.26^{c}	11.48^{bcd}	9.04^{cd}	0.437	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.

Table 5. Antibiotic susceptibility of the *Bacillus subtilis* ATCC 6051a

Antibiotic	*Bacillus subtilis* ATCC 6051a
Vancomycin 30 µg	S^{+}
Erythromycin 15 µg	S^{++}
Clindamycin 2 µg	S^{++}
Gentamicin 10 µg	S^{+}
Amoxicillin 25 µg	S^{++}
Chloramphenicol 30 µg	S^{++}
Ciprofloxacin 5 µg	S^{++}
Amikacin 25 µg	S^{++}
Tetracycline 30 µg	S^{+}
Kanamycin 30 µg	S^{++}

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

salts. Vasquez (2016) reported the capacity of *Bacillus* spp. to survive within the GIT, determine the sporulation process, making them commensal bacteria for animals that ingest them.

These results are in agreement with those observed by Zaid (2018), Nithya and Halami (2013), which affirmed that a bacterial strain to be used as a probiotic must to resist under gastrointestinal conditions.

The spores resistance to heat

The temperature resistance is another condition of probiotic until used in animal nutrition. The results obtained confirm the strain viability at 80°C during 120 min. Table 4 shows the resistance of *BS* at high temperature. After 120 min, the *BS* registered good viability. The values obtained differ significantly (P≤0.05) between all incubation times.

The results are consistent with those in the literature (Chaiyawan *et al.,* 2010), which reported the exposure of the *Bacillus* at 80°C by incubation in a water bath; it confirms the resistance of the vegetative cells due to the presence of the spores. The thermostability to high temperatures is a major advantage of *Bacillus* spp., the spores can survive at 113°C (Vazquez, 2016); this property permits the spores incorporation in animal nutrition during the processes of grinding and pelleting of the feed.

Antibiotic resistance assay

Bacillus subtilis ATCC 6051a strain was evaluated for antibiotic resistance by using discs impregnated with vancomycin (30 µg), erythromycin (15 µg), clindamycin (2 µg), gentamicin (10 µg), amoxicillin (25 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), amikacin (25 µg), tetracycline (30 µg) and kanamycin (30 µg). The results of antibiotic sensitivity test of bacterial strain are shown in Table 5. *BS* was highly susceptible (between 16 – 25 mm of zone of inhibition) to antibiotics as erythromycin, clindamycin, amoxicillin, chloramphenicol, ciprofloxacin, amikacin and kanamycin. The strain was found to be sensitive to vancomycin, gentamicin, and tetracycline.

The antibiotics utilization can improve the zootechnical parameters, their administration can be given as a protection for animals' health, controlling as well, the gastrointestinal infections and microbiota modification (Mehdi *et al*., 2018). Given that, the European Union has banned the use of antibiotics in food-production (European Union, 2006; Dumitru *et al*., 2019) and an alternative of these can be occurred by probiotics (Chiang *et al*., 2015; Dumitru *et al*., 2018).

Conclusion

Our results indicate the resistance of Bacillus subtilis ATCC 6051a in the presence of bile salts and low pH values, with high survivability (%). These probiotic properties will help the strain to reach the harsh gastrointestinal tract conditions and to contribute in the balance of intestinal microbiota. Besides, the bacterial culture was sensitive to the antibiotics, more of these with applications in animal nutrition, which are used only to treat clinical disease. Based on our *in vitro* results, *Bacillus subtilis* ATCC 6051a presented notable probiotic criteria and can be selected as a possible candidate for further investigations.

Acknowledgments. This study was funded by Romanian Ministry of Research and Innovation through Program 1 – Development National Research-Development, Sub-program 1.2 – Institutional Performance - Projects funding excellence in R & D, Contract no. 17 PFE and PN 19 09 01 04.

References

1. AlGburi A, Volski A, Cugini C, Walsh EM, Chistyakov VA, Mazanko MS, Chikindas ML (2016). Safety properties and probiotic potential of *Bacillus subtilis* KATMIRA1933 and *Bacillus amyloliquefaciens* B-1895. Advances in Microbiology, 06(06): 432–452. doi:10.4236/aim.2016.66043
2. Barbosa TM, Serra CR, La Ragione RM, Woodward MJ, Henriques AO (2005). Screening for *Bacillus* isolates in the broiler gastrointestinal tract. Applied and Environmental Microbiology, 71(2): 968–978. https://doi.org/10.1128/AEM.71.2.968-978.2005
3. Balasingham K, Valli C, Radhakrishnan L, Balasuramanyam D, (2017). Probiotic characterization of lactic acid bacteria isolated from swine intestine. Veterinary World, 10(7): 825-829.
4. Bernardeau M, Lehtinen MJ, Forssten SD, Nurminen P (2017). Importance of the gastrointestinal life cycle of *Bacillus* for probiotic functionality. Journal of Food Science and Technology, 54(8): 2570–2584. https://doi.org/10.1007/s13197-017-2688-3
5. Cartman M, Ragione L, Woodward MJ (2007). Bacterial spore formers as probiotics for poultry. Food Sci Technol Bull, 4: 21–30.
6. Chaiyawan N, Taveeteptaikul P, Wannissorn B (2010). Characterization and probiotic properties of *Bacillus* strains isolated from broiler. Thai J. Vet. Med., 40(2): 207–214.
7. Chiang ML, Chen HC, Chen KN, Lin YC, Lin YT, Chen MJ (2015). Optimizing production of two potential probiotic lactobacilli strains isolated from piglet feces as feed additives for weaned piglets. Asian-Australas J Anim Sci, 28(8): 1163–1170. https://doi.org/10.5713/ajas.14.0780
8. Dinu S, Boiu-Sicuia OA, Constantinescu F (2019). Influence of *Bacillus* spp. Based bioproducts on potato plant growth and control of *Rhizoctonia solani*. Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies, 76(1): 29. doi:10.15835/buasvmcn-asb:2018-0022
9. Doneva TU, Donev T, 2005. Anabiosis and conservation of microorganisms. J of Culture collections, 4: 17-28.
10. Dumitru M, Sorescu I, Habeanu M, Tabuc C, Jurcoane S (2019). Preliminary characterization *in vitro* of *Bacillus licheniformis* strain for used as dietary probiotic. Scientific Bulletin. Series F. Biotechnologies, XXIII: 164-172.
11. Dumitru M, Sorescu I, Habeanu M, Tabuc C, Idriceanu L, Jurcoane S (2018). Preliminary characterisation of *Bacillus*

subtilis strain use as a dietary probiotic bio-additive in weaning piglet. Food and Feed Research, 45(7): 203–211. https://doi.org/10.5937/FFR1802203D

12. Dumitru M, Tabuc C, Jurcoane S (2018). Obtaining a feed additive based of *Lactobacillus plantarum* strain. Scientific Paper Series A. Agronomy, 61(2): 115–122.
13. Hosseini GN, Modarressi MH, Mousavi SN, Ebrahimi TM (2019). Evaluation of novel probiotic *Bacillus* strains based on enzyme production and protective activity against salmonellosis. Journal of the Hellenic Veterinary Medical Society, 69(4): 1205. doi:10.12681/jhvms.16089
14. Fallingborg J, Christensen LA, Ingeman-Nielsen M, Jacobsen BA, Abildgaard K, Rasmussen HH (1989). pH-profile and regional transit times of the normal gut measured by a radiotelemetry device. Aliment. Pharmacol. Ther., 3 (6): 605–613.
15. FAO. (2016). Probiotics in animal nutrition – Production, impact and regulation. FAO.
16. FAO/WHO (2006) Probiotics in food. Health and nutritional properties and guidelines for evaluation. FAO Food and Nutrition, Paper 85, ftp://ftp.fao.org/docrep/fao/009/a0512e/a0512e00.pdf
17. Gaggìa F, Mattarelli P, Biavati B (2010). Probiotics and prebiotics in animal feeding for safe food production. Int. J. Food Microbiol, 141:S15-28.
18. Ionescu AD, Casarica A, Boca E, Nita S, Rasit I, Vamanu A, Vamanu E (2013). Studies concerning the characterisation of a probiotic preparation, containing a mixture of *Lactobacillus* pure biomass and media with pollen and honey. Agrolife Scientific Journal, 2(1): 113-116.
19. Maruo T, Sakamoto M, Toda T, Benno Y (2006) Monitoring the cell number of *Lactococcus lactis* subsp. cremoris FC in human feces by real-time PCR with strain-specific primers designed using the RAPD technique. Int. J. Food Microbiol., 110: 69– 76.
20. Mehdi Y, Létourneau-Montminy MP, Gaucher M Lou, Chorfi Y, Suresh G, Rouissi T, Godbout S (2018). Use of antibiotics in broiler production: Global impacts and alternatives. Animal Nutrition, 4(2): 170–178. https://doi.org/10.1016/j.aninu.2018.03.002
21. Nithya V, Halami PM (2013). Evaluation of the probiotic characteristics of *Bacillus* species isolated from different food sources. Annals of Microbiology, 63(1): 129–137. https://doi.org/10.1007/s13213-012-0453-4
22. Ritter AC, Paula A, Correa F, Veras FF, Brandelli A (2018). Characterization of *Bacillus subtilis* available as probiotics, Journal of Microbiology Research, 8(2): 23–32. https://doi.org/10.5923/j.microbiology.20180802.01
23. Schillinger U, Guigas C, Holzapfel WH (2005). *In vitro* adherence and other properties of lactobacilli used in probiotic yogurt-like products. Int Dairy J., 15: 1289–1297.
24. Vasquez AP (2016). *Bacillus* species are superior probiotic feed-additives for poultry. Journal of Bacteriology & Mycology, 2(3): 57-59. DOI: 10.15406/jbmoa.2016.02.00023
25. Zaid AA (2018). Study the effect of probiotic bacteria isolated from foods on pathogens. Biomedical Research (India), 29(12): 2509–2515. https://doi.org/10.4066/biomedicalresearch.29-17-3692