Technological Development of Probiotic Supplement for Zootechnical Improvement of Broilers

Magali T. Uono, Sibylle S. Hacker, Catarina V. Manfrinato, Mariana M. Matsuo, Svetoslav D. Todorov, and Cristina S. B. Bogsan
Department of Biochemical-Pharmaceutical Technology, University of São Paulo, São Paulo, Brazil
Email: {magaliuono, sibylle, mariana.matsuo, cris.bogsan}@usp.br, cat.v.m@hotmail.com, slavi310570@abv.bg

***Abstract*—The increasing of the demand for functional foods, which include those supplemented or fermented by probiotic microorganisms, resulted in the advancement of research and development of new strains. The probiotic microorganisms *Bacillus* spp. are attractive for the inherent stability due to their characteristic of spore-forming bacteria. Spores allow prolonged shelf life and increase the ability to survive gastric barriers, which turn out to be an advantage over lactobacilli. Two bacteria were supplied by VitaBridge Company in order to identify which strain would have the best technological performance for the development of a novel functional food with high performance in the zootechnical improvement of broilers. The characterization was performed according to the protocol which included phenotypic and genotypic tests for determining the probiotic potential of the strains. The results of the tests revealed that BVB1 possesses the best technological characteristics and can be used with efficiency in the improvement of the zootechnical performance of broilers.**

***Index Terms*—screening, biochemical tests, MALDI TOF, genome sequencing**

I. Introduction

The fast development of the functional food industry has increased the demand for products which aim the improvement of consumer health, both human and animal. Functional foods include those which are supplemented or fermented with probiotic microorganisms. According to the definition of FAO / WHO (2002), which was revisited by Hill and colleagues (2014), "Probiotics are living microorganisms that when administered in adequate amounts confer benefit to the health of the host" [1], [2].

The antibiotic used as a growth promoter in agricultural and zootechnical development raises concerns about antibiotic resistance. Since 2006, the European Union has banned these practices and the probiotic has been introduced into animal husbandry as an alternative to improving animal growth [3].

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Among the most studied probiotics, the spore-forming bacteria stand out, being the majority belonging to the genus *Bacillus spp*. They are attractive due to the inherent resistance of the spores to the environmental stress [4]. These microorganisms' morphology is in the rod shape, gram-positives, aerobes or facultative aerobes, catalase positive, spore and enzyme producers. Due to the physiological diversity of the vegetative forms, these microorganisms are considered as ubiquitous organisms, being able to be isolated from soil and foodstuffs [5]. They have a bimodal life cycle of growth and sporulation in the environment as well as in the gastrointestinal tract [6].

The fact that it is a spore-former makes bacilli attractive as food additives due to its ability to survive gastric barriers, presenting advantages over non-spore forming microorganisms such as *Lactobacillus* spp. The spore-former characteristic guarantees, in principle, prolonged shelf life without refrigeration [7].

Two putative probiotic bacteria were provided by the VitaBridge Company to identify which of them would have the best probiotic potential and the best technological performance and thus to be used as a functional food in the improvement of the zootechnical performance of broilers.

II. Material and Methods

A. Bacillus Strain

Two strains of *Bacillus* spp., BVB1 and BVB5, were provided by VitaBridge Company (São Paulo, Brazil) for the technological characterization as a potential probiotic strain.

B. Morphological Tests – Phenotypical Chacterization

The colony morphology on the surface of the culture in Petri dishes containing nutrient agar media enriched with 0.3% of yeast extract was observed through an Olympus BX 51 optical microscope. Smears of the 10µl of each fermented broth samples of *Bacillus* spp. were prepared on glass slides for Gram staining and the colored samples were visualized by optical microscopy (OLYMPUS BX51, Japan) by immersion to verify the cell morphologies and dye properties of the strains [8].

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C. Biochemical Tests – Phenotypical Characterization

For the biochemical tests, cultures of 24 h of *Bacillus* spp., striated on Petri dishes containing agar nutrient supplemented with 0.3% of yeast extract and incubated at 37 ℃, were used to perform catalase test, Hydrolysis of starch, Egg-yolk lecithinase, growth in NaCl at 6.5%, Hydrolysis of gelatin, Motility, Formation of indole, Voges-Proskauer, Nitrate reduced to nitrite as described in Bergey and colleagues (1989) Manual of Systematic Bacteriology [8].

D. Classification Based on Characteristics of Protein Expression Patterns – Physicochemical Characterization

The MALDI TOF/MS test was performed on the Bruker Daltonik MALDI equipment. The sample used was a 24-hour culture at 37 ℃ of BVB1 in Petri dishes containing nutrient agar enriched with 0.3% of yeast extract. The test consists of identifying the microorganism through mass spectrometry that allows the biomolecule analysis of proteins [9].

E. Sequencing of Full Genome – Genotypical Characterization

Complete sequencing of the BVB1 strain genome was outsourced. Complete sequencing of *Bacillus spp*. strain was performed using the NextSeq500 sequencer from Illumina (USA). DNA extraction from strain BVB1 was followed by the Shotgun analysis (Nextera XT DNA kit), which was submitted to 300 cycles in the high mode of the NextSeq500 v2, being considered a minimum parameter of 1Gb throughput with at least 75% (Q > = 30) and 1000 ng of minimum mass of genomic DNA in a concentration equal to or greater than 50 ng/μL per sample. The integrity of the material was evaluated on agarose gel.

F. Enzymatic Assays of Amylase, Protease and Lipase

For the inoculum, 10μl aliquots of the dilutions of the BVB1 broth in 0.1% peptone water medium were used [10]-[12].

Inoculum for enzymatic activity was prepared inoculating BVB1 in nutrient broth medium supplemented with 0.3% yeast extract and the test tube was taken to the incubator at 37 ° C for 48 hours. The produced broth was serially diluted until 10^{-7}. Aliquots of 10μl of the 10^{-4} to 10^{-7} dilutions were inoculated in Petri dishes containing, amylolytic medium (containing 9.75g of potato agar of Difco, USA and 250ml of distilled water, autoclaved at 121 ℃ for 15min), proteolytic medium (containing 7.38 g of TPB of Difco, USA; 3.25 g of Bacto agar in 230 ml of distilled water; a suspension of 5.0g of Skim Milk of Difco, USA; and 20 ml of distilled water, autoclaved at 121 ℃ for 15 min in distinct flasks and mixed after the temperature decreased to 60 ℃ to avoid denaturation of milk protein by meat extract contained in the nutrient broth medium) and lipolytic medium [containing 2.5g of peptone of Difco, USA, 1.25g of sodium chloride (NaCl), 0.025g of calcium chloride (CaCl2), 3.25g of Bacto agar, 0.5% (v / w) of Tween 80 and 250ml of distilled water was autoclaved at 121 ℃ for 15 min], respectively for the enzyme activity detections. All Petri dishes were taken to the incubator at 37 ℃ for 48h. The revelation of the halo of amylolytic activity was performed by pouring enough lugol to cover the colony formed and around it. The proteolytic activity can be verified by the translucent halo of the culture medium, and the lipolytic activity was verified by a precipitation halo around the colony [10]-[12].

III. Results and Discussion

A. Morphological Tests

The colony morphology on the surface of the culture on Petri dishes observed through an optical microscope, shows that morphological appearance was different between the strains (Fig. 1).

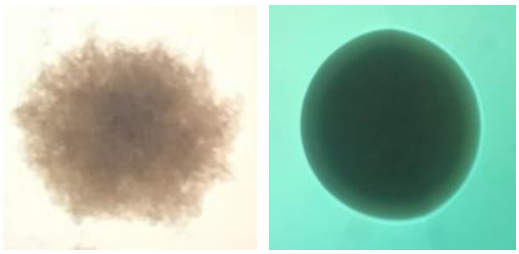

Figure 1. Colony of BVB1 (left) and BVB5 (right)

After that, both samples of *Bacillus spp.* were prepared on glass slides to Gram staining and then they were visualized through an optical microscopy by immersion to verify the cell morphologies and dye properties of the strains. The staining of the Gram test appeared purple and spores could be visualized (Fig. 2).

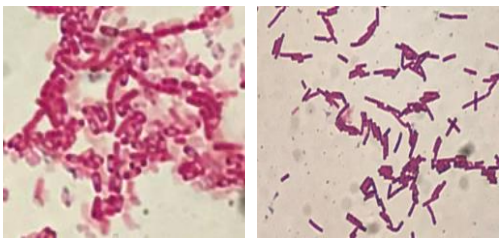

Figure 2. Gram tests of BVB1 (left) and BVB5 (right)

The morphological tests revealed that BVB1 was more suitable to the product development, due to its ability to form more spores than BVB5. This feature is basically important so that the strain can survive in greater numbers by passing through the gastric barriers and reaching the intestine to act as a probiotic. Thus, BVB1 was chosen to proceed with subsequent tests.

B. Biochemical Tests

After 24 h of cultivation, the BVB1 strain were striated on Petri dishes containing agar nutrient supplemented and the tests of catalase, Hydrolisis of starch, Egg-yolk lecithinase, growth in NaCl at 6.5%, Hydrolysis of gelatin, Motility, Formation of indole, Voges-Proskauer, Nitrate reduced to nitrite were performed as described before. The Table I shows the data achievement and agrees with the technological characteristics desired.

TABLE I. BIOCHEMICAL TESTS OF BVB1

Test type	BVB1	Reference [7]
Catalase	+	+
Hydrolysis of starch	+	+
Egg-yolk lecithinase	—	—
Hydrolysis of gelatin	+	+
Motility	+	+
Formation of indole	—	—
Voges-Proskauer	+	+
Growth in nutrient broth	+	+
Growth in 6.5%NaCl	+	+
Nitrate reduced to nitrite	+	+

C. *Enzymatic Tests of Amylase, Protease and Lipase*

All enzymatic tests were positive as expected (Fig. 3, Fig. 4 and Fig. 5). The existence of the halo of amylolytic activity could not be detected unless it was revealed with lugol (Fig. 3, in the middle). In the proteolytic activity, the halo was visible due to the presence of milk in the culture medium (Fig. 4, left). In lipolytic activity the precipitation halo is in the form of a flower crown around the colony of BVB1 (Fig. 5, left and in the middle).

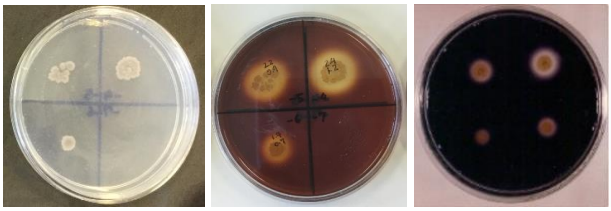

Figure 3. BVB1 amylase tests (left); activity revealed with lugol (middle); reference (right).

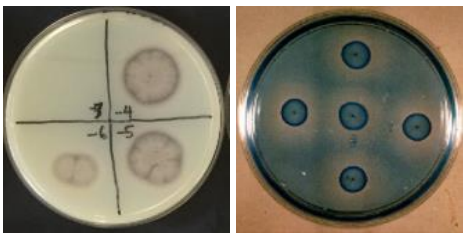

Figure 4. BVB1 protease tests (left); reference (right)

Figure 5. BVB1 lipase tests (left and center); reference (right)

D. *Physicochemical Characterization by Spectrometry*

According to Fig. 6, analysis of MALDI TOF shows that BVB1 is a *Bacillus subtilis* strain

A11 (++)(A)	6	Bacillus subtilis	2.183	Bacillus subtilis	2.093

Figure 6. Result of MALDI TOF / MS spectrometry analysis

The MALDI-TOF technique is not safe for sporulated Gram-positive microorganisms, fungi and yeasts, thus, genomic characterization by sequencing was necessary.

E. *Sequencing of Full Genome*

The Ilumina sequencing technique confirmed (Fig. 7, Fig. 8 and Fig. 9) that the BVB1 strain is *Bacillus subtillis* (Table II).

TABLE II. SCIENTIFIC CLASSIFICATION OF BACILLUS SUBTILIS

Bacillus subtilis scientific classification	
Domain	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Bacillaceae
Genus	Bacillus
Species	B. subtilis

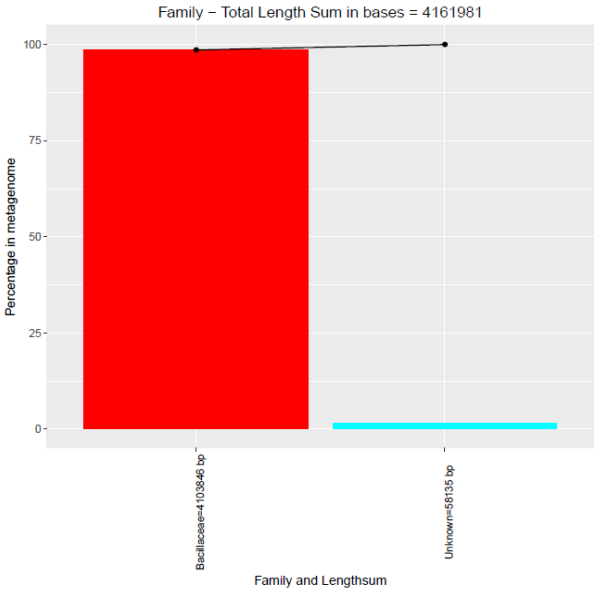

Figure 7. Graphic of family confirmation.

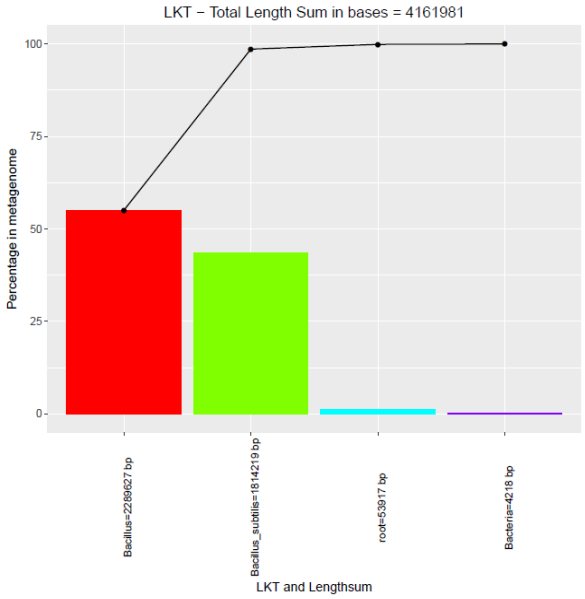

Figure 8. Graphic of specie confirmation

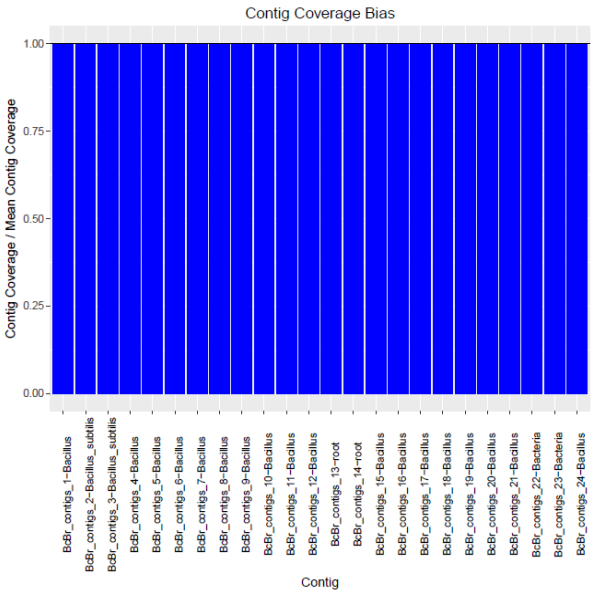

Figure 9. Graphic of specie confirmation

Previous studies have shown the efficacy of *Bacillus subtillis* bacteria in improving zootechnical performance in broilers. In this way, the possibility of also using this strain as a zootechnical enhancer was evaluated. For this, the presence of virulence genes, antimicrobial resistance genes and pathogenicity of the strain were investigated.

BVB1 strain does not present plasmids (Fig. 10), according to the safety specification for the development of new probiotic strains [1].

Plasmids

Contig	PlasmidOri	HITCOVERAGE	PCTIDENTITY	COVoverMEAN
none	0	0	0	0

Contigs with plasmid origins

Figure 10. Analysis of plasmids

Chromosomal resistance genes were found (Fig. 11) for tetracycline tet (L) and for streptomycin (aadK). The tet (L) gene represents the mechanism of resistance by efflux pump, so that being a chromosomal gene does not imply risk to the zootechnical use.

Resistance genes

Contig	ResGene	HITCOVERAGE	PCTIDENTITY	Replicon	COVoverMEAN
BcBr_contigs_3	aadK	100	100	Unknown	1
BcBr_contigs_1	tet(L)	100	100	Unknown	1

Significant hits to known resistance genes

Figure 11. Analysis of resistance genes

The possible virulence factors (4206 CDSs), only 0.02% (1 factor in 4206 CDSs) showed toxicity and 8.11% (341 factors of 4206) were confirmed (Fig. 12 and Fig. 13) as non-pathogenic.

Statistic	Value
Total number of CDSs	4206
Number of Pathogenic CDSs	1
Percentage of Pathogenic CDSs	0.02 %

Figure 12. Analysis of toxicity

Statistic	Value
Total number of CDSs	4206
Number of NonPathogenic CDSs	341
Percentage of NonPathogenic CDSs	8.11 %

Figure 13. Analysis of pathogenicity

Further studies are needed to ensure the safety of this strain.

IV. Conclusion

The results of the tests revealed that BVB1 possesses characteristics of high probiotic potential and can be used with efficiency in the improvement of the zootechnical performance of broilers. Further studies are needed to ensure the safety of this strain [13], [14].

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Magali Thiyomi Uono was born in São Paulo, Brazil in 1963. She has undergraduate in Chemistry by Institute of Chemistry of USP (1987). Has experience in the area of Science and Technology of the Food, with emphasis on Food Technology. She is coauthor of 3 scientific papers published on international and national journal. She has got presentation in international and national congresses. She has 1 patent in the field of food. Some of her publications are:
IMAFUKU, K., ARADONO, K., UONO, M., OGAWA, K., & MATSUSHITA, Y. I. (1992). Synthesis and Biological Activities of 1-[2-(Dimethylamino) ethyl]-and 1-[3-(Dimethylamino) propyl]-substituted 3-Methyl-1, 8-dihydrocycloheptapyrazol-8-ones and Related Compounds. Chemical and pharmaceutical bulletin, 40(6), 1606-1609.
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Sibylle Sophie Hacker was born in São Paulo, Brazil in 1971. She received her Business Bachelor degree in 1994 and Chemistry Bachelor in 2002, Business MS in 2002. Now participates in the PhD degree program in Biochemical Pharmaceutical Technology of University of São Paulo,, Brazil (finishes in 2018). Her research area is in Pharmaceutical Biotechnologies of Polysaccharides Vaccine. She is author and coauthor of 01 scientific papers published on national journal. She has got presentations in national congresses. Her publications are: The strategic focus on supply chain management: analysis and application of conceptual aspects in the Gradina case. *Associação Nacional dos Programas de Pós Graduação Em Administração*, Rio de Janeiro, p. 259-260, 2002.

Catarina Vezetiv Manfrinato was born in São Paulo, Brazil in 1994. She studies Nutrition in the School of Applied Science – State University of Campinas (finishes in 2018). She is currently in the program of Scientific Initiation by PIBIC at the University of Campinas. She is active participating in national food nutrition congresses.

Mariana Mitiko Matsuo was born in São Paulo, Brazil in 1997. She studies Nutrition in the São Paulo University. She is currently in the program of Scholarships in Brazil - Scientific Initiation at the Faculty of Pharmaceutical Sciences in the University of São Paulo with the project: Comparative study of influence of fermented milk and fermented bufallo's milk by *Bifidobacterium animalis* subsp. lactis HN019 on cellular immunity of male Swiss mice mucosa. She is active participating in national food nutrition congresses.

Svetoslav Dimitrov Todorov was born in Bulgaria and he is Doctorate at Sofiiski Universitet 'Kliment Ohridsky', Bulgaria. He has a bachelor's degree in biotechnology and industrial microbiology - Sofiiski Universitet Kliment Ohridsky (1994), a master's degree in biotechnology and industrial microbiology - Sofiiski Universitet Kliment Ohridsky (1995), a master's degree in international relations - Sofiiski Universitet Kliment Ohridsky Universitet Kliment Ohridsky / Enitiaa, FR (2000) and postdoctoral degree in microbiology - Stellenbosch University, South Africa. From 2008 to 2013, he served as visiting professor at the University of São Paulo. He has experience in Microbiology, with emphasis on applied microbiology, working mainly on the following topics: lactic acid bacteria, bacteriocins, *Lactobacillus plantarum*, optimization and fermented products. He is author and coauthor of scientific papers published on international and national journal. Some of his publications are: Unravelling the mechanism of action of bacteriocins produced by lactic acid bacteria against Listeria monocytogenes in foods

Cristina Stewart Bittencourt Bogsan, professor of Food Technology, Department of Biochemical Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, University of São Paulo and researcher from Technology of Dairy Functional Food and Analogues Lab - has graduated at Pharmacy and Biochemistry from Universidade Paulista in 1999, obtained her master degree at Immunology and Microbiology from Universidade Federal de São Paulo in 2002 and has completed her PhD from Universidade de São Paulo in 2012Has experience in Microbiology and Immunology and Food Science and Technology, focusing on Immunology and Science and Technology of the Food, acting on the following subjects: B-1 cell, Foreign-body giant cell, inflammation, Fermented milk, Probiotics, interaction between food matrix-probiotic-mucosal. Development and Characterization of probiotic functional food: "in vitro" and "in vivo" assays, development of new functional food. Regular Research Grants at the project: Study of probiotic fermented buffalo's milk biogenic compounds in the modulation of adaptive and innate mucosal immune response. She is author and coauthor of scientific papers published on international and national journal. She has got presentation in international and national congresses. Some of her publications are:
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