

Isolation and identification of strains of *Bacillus spp.* in different ecosystems, with probiotic purposes, and their use in animals

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Strains of *Bacillus spp.*, taken from different ecosystems, were isolated and identified. These ecosystems were: fresh and old bovine feces, content of chicken caecum, decayed tomato juice, and a mixture of soil and bovine blood. They were used for producing probiotic biological preparations, which can be used in livestock interest. The strains were previously selected, considering the morphological characteristics of the genre *Bacillus spp.* and their resistance to the main natural barriers of digesta flow (acid pH 1, 2 and 3, and biliary salts 1.5 g L⁻¹ of Ox-Bilis). For identifying strains, different methods were used: traditional methods, fermentation of carbohydrates (API 50 CHB) and polymerase chain reaction (PCR). A total of 100 strains were obtained, 48 expressed characteristics of *Bacillus spp.* genre, and only three showed resistance to biliary salts. The strains corresponded to *Bacillus subtilis*, sub species *subtilis*, which is one of the most used for producing nutritional additives with probiotic effect on livestock interest.

Key words: *isolation, identification, Bacillus spp., nutritional additives*

As a consequence of the growing concern of society about a healthy diet, the Food Agricultural Organization and the World Health Organization (FAO/WHO 2008) established recommendations of biological safety regarding criteria of strain selection for probiotic ends, in which the isolation and intra-specific identification are included, as well as *in vitro* and *in vivo* tests (Vardan 2003 and Lara and Burgos 2012).

Isolation of *Bacillus spp.* strains and selection, according to their ability of producing endospores, are carried out in several laboratories all over the world (Ragazzo *et al.* 2011 and Sosa *et al.* 2011). Molecular methods are also used for identifying strains of *Bacillus spp.*, for a better identification and guarantee of biological safety (Rondón *et al.* 2012 and Vázquez *et al.* 2012).

The objective of this study was to isolate and identify strains of *Bacillus spp.* from different ecosystems for its further use for producing probiotic biological preparations, which can be used in animals of livestock husbandry interest.

Materials and Methods

Collection of samples. Samples were taken from each selected ecosystem (table 1).

Old and fresh bovine feces were taken from different depositions in several production units belonging to the “Empresa Pecuaria Genética de Matanzas”. No antibiotics were applied. For taking the samples of the colonies, the dish was divided into four equal parts that corresponded to vowels, and the number represented the dish.

Food wastes were taken from a flask containing

decayed tomato juice, from the “Empresa de Conservas Planta Libertad”, Colón municipality, Matanzas province. Numbers corresponded to the dishes, and vowels to the parts in which the dishes were divided.

Samples of soil with bovine blood were collected from soil where adult bovines are sacrificed, in areas of the “Combinado Cárnico” from Matanzas province.

Caecum content was taken from five healthy substitute hens (*Gallus gallus domesticus*), of 100 d old, selected at random. Numbers corresponded to dishes and the letter to the place they were taken from (caecal content).

Strain isolation. An amount of 1 g of a sample from the different selected environment was taken and a methodology of serial dilutions (Stanier 1996) was followed. Serial dilutions (1:10) were carried out and the corresponding dilutions, from 10⁻⁷ to 10⁻¹⁰, were taken for the application of a thermal treatment of 71 °C for five minutes, according to the methodology described by Pérez (2000). A total of 0.5 mL was planted in dishes with nutritional agar. Incubation was carried out at 37 °C for 24 h, using an INNOVA NBS incubator, under anaerobiosis conditions.

The isolated colonies were planted in culture tubes. Selection was performed by observing the different morphological characteristics of colonies. After that, they were purified according to the planting technique of exhaustion (Stanier 1996).

Morphological characterization. Each isolated strain received two preliminary tests: dying of Gram and dying of endospores (Harrigan and McCance 1968).

Table 1. Code of isolated strains of *Bacillus spp.* taken from different environments

Bovine feces	Content of fowl caecum	Wastes from food and from other environments
Hfb-3b, Hfb-7b, Hfb-3c, Hfb-3d, Hfb-5b, Hfb-5a, Hfb-5c, Hfb-7a, Hfb-3a, Hfb-4c	C-1, C-2, C-3, C-5, C-8, C-9, C-10, C-12, C-13, C-14, C-15, C-19, C-21, C-23, C-26, C-27,	Ms-7a, Ms-7c, Ms-7b, PL-6b, PL-7b, PL-7a, PL-1b y E-44
Hvb-3b, Hvb-3d, Hvb-4a, Hvb-3 ^a Hvb-5a y Hfb-8a	C-28, C-29, C-30, C-31, C-33, C-34, C-35, y C-36	

Hfb = fresh bovine feces, Hvb = old bovine feces, C= caecum of fowls (the number belongs to the Petri dish, where the colony was taken from), PL= food wastes, E= decayed tomato juice and Ms= soil with bovine blood.

Determination of resistance to acid pH and biliary salts. It was performed according to the methodology of Refugio (2002).

Identification. Traditional microbiological methods were applied, which included biochemical and morphological tests. For determining the species of *Bacillus*, the identification code of Fields (1978) was used.

Determination of fermentation profiles of carbohydrates. The API 50 CHB Medium and the Galleries API 50 CH (BioMérieux S.A., France) were used. Identification of strains was carried out using Apilab Plus Software, Version 3.3.3.

Polymerase chain reaction (PCR). It was applied to the selected strains (C-31, C-34 and E-44). For that purpose, the strains were cultured by exhaustion in nutrient agar, enriched at 37 °C, under conditions of aerobiosis for 24 h. After obtaining the pure colonies, the DNA was extracted, amplified (methodology of Greisen *et al.* 1994), purified and sequenced. The molecular marker Gene Ruler™ 100 bp DNA ladder Plus, MBI Fermentas was used.

DNA sequencing and amplification. The intergenic region of low variability 16S rDNA was used. Each strain was amplified with primers for *Bacillus spp.* (Goto *et al.* 2000):

- Direct primers: 5'-TGT AAA ACG ACG GCC AGT GCC TAA TAC ATG CAA GTC GAG CG-3'
- Reverse primers: 5'-CAG GAAACAGCTATG ACC ACT GCT GCC TCC CGT AGG AGT- 3'

Samples were sequenced with an automatic sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystem). A fragment of DNA was taken, which was amplified by PCR using the genomic DNA of the evaluated strains. Sequences were compared to the international databases of the collection of non redundant nucleotides (nr/nt) from the GenBank+EMBL+DDBJ+sequences PDB GenBank using the BLASTN 2.2. 19+ software (Stephen *et al.* 1997). This program is available on the web of the National Center of Biological Information (NCBI). Later, the sequences were changed to the FASTA format for proceeding to their alignment. The alignment of obtained sequences was performed with

the CLUSTALW program.

Results and Discussion

A total of 100 strains were isolated: 50 % from the GIT, 33.3 % from bovine feces and 16.66 % from decayed tomato juice and samples of soil with blood. Out of the isolated strains, only 48 showed characteristics of the *Bacillus spp.* All of them presented cells of bacillary shape, Gram positive. The presence of endospores was confirmed. These characteristics coincide with the described by Shanka *et al.* (2011) and Anne and Kolter (2011).

The 48 strains of *Bacillus spp.*, evaluated with the three acid pH, simulated *in vitro* for three hours, endured pH-1 (33.3 %), pH-2 (55 %) and pH-3 (96 %) of survival. This result is important because when using a microbial culture as probiotic, orally, it has to go through the action of the acid barrier of the stomach, where the pH can reach values of 2 (Overland *et al.* 2003, Rondón *et al.* 2012 and Rondón *et al.* 2013). If the microorganisms survive to this chemical barrier, they possibly can reach the lower parts of the digestive tract, where they develop their probiotic action. These results are similar to those obtained by Reiss *et al.* (2011), who evaluated the strains of *Bacillus subtilis* and *Bacillus licheniformes*, and demonstrated the ability of these strains to endure the conditions of acid pH. The results also coincide with those obtained by Sánchez *et al.* (2011), who evaluated strains of *Lactobacillus spp.*, candidates to probiotics.

Out of the 48 strains of *Bacillus spp.* evaluated, only three (C-31, C-34 and E-44) showed ability to survive the effect of biliary salts. The remaining strains showed low or non survival. According to the results obtained in this study, and to those informed in the literature (Xiaohong *et al.* 2011 and Siele *et al.* 2012), it is evident that the strains of *Bacillus spp.* can be sensitive, between a 20 and 25 %, to simulated biliary fluids.

The strains C-31, C-34 and E-44 have long bacilli shape and were positive to Gram staining. All the strains evidenced the presence of endospores, with oval shape and centrally located. Colonies showed a beige color, undulated edges, dull internal structure, convex elevation

and irregular shape.

The three strains were positive to the biochemical studies (catalase production, casein hydrolysis, starch hydrolysis, indole production, reduction of nitrates, test of Voges Proskauer and citrate use). They grew at different temperature levels: 45, 50 and 60 °C and with pH of 6.0, 7.0, 7.5 and 8.0. They showed growth in NaCl at 2, 4 and 7 %. They were all negative to growth under anaerobiosis conditions.

When analyzing the results of all tests and using the method of Fields (1978), it was determined that the three strains belong to the species *Bacillus subtilis* (Blackwood). This species is a microorganism considered as safe (GRAS), according to the criterion stated by the Association of American Feed Control Officials (AAFCO) and the Food and Drug Administration (FDA) (Anon 1998).

The results of the characterization of carbohydrate fermentation profiles for *Bacillus* genre demonstrated that the strains have equal profile of fermentation before 24 sugars (glycerol, L-arabinose, ribose, D-xylose, D-glucose, D-fructose, D-mannose, inositol, mannitol, sorbitol, α -methyl-D-glucoside, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, D-raffinose, starch and glycogen).

The analysis of results, using the computer system that accompanies the API 50 CH gallery test, demonstrated that all belong to *Bacillus* genre. The strains C-31, C-34 and E-44 belong to the species *Bacillus subtilis* (Blackwood), with an identification reliability of 99.7, 99.5 and 99.3 %, respectively.

The identification results, using the API 50 CH gallery test, coincide to those informed when the traditional microbiological methods for the taxonomical characterization of strains were used, which demonstrates the validity of these methods.

According to the identification using PCR (Polymerase Chain Reaction), the strains C-31, C-34 and E-44 had a similar performance in their sequences, which allow to

identify them as *Bacillus subtilis* sub-species *subtilis*, with a 100 % of identity. The biological safety of these strains and their possible use in the production of probiotic biological preparations for animals of livestock husbandry interest were confirmed (Milián *et al.* 2012, Hernández 2012 and Silva 2013).

Guillen *et al.* (2006) and Scott and Hayley (2011) isolated and identified strains of *Bacillus spp.* and demonstrated that they can be classified in different groups, regarding their metabolic and biochemical characteristics, and in homology with other rDNA 16S sequences. Species of *B. licheniformis*, like NC006270-3, NC000964-2 and NC006322-1, are similar to the identified strains (figure 1). It is also reported the use of these species for enzyme production, products with antimicrobial activity and probiotics, used in animal feeding (Pérez *et al.* 2007, Olmos *et al.* 2011, Pérez *et al.* 2011 and Pérez *et al.* 2012).

The identified strains were provided and their effect on indicators of productivity and health of livestock interest (fowls and calves) was evaluated. These indicators belong to different institutions from Matanzas territory. Very good results were obtained regarding the increase of weight gain, increase the eggs number, decrease of deaths due to diarrheic diseases and mortality reduction (Díaz 2012, González 2012, Hernández 2012 and Silva 2013).

A total of 100 strains were isolated as a result from the study of isolation and identification of strains of *Bacillus spp.* from different ecosystems. Out of them, C-31 and C-34, isolated from the caecal content of fowls, and the strain E-44, from decayed tomato juice, resisted the *in vitro* biliary and acid barriers, and showed genetic, physiological and morphologic characteristics that coincide with the species *Bacillus subtilis* sub-species *subtilis*. These strains showed potential characteristics, which allow their use for preparing products with probiotic activity that can be used as nutritional additives for livestock interest.

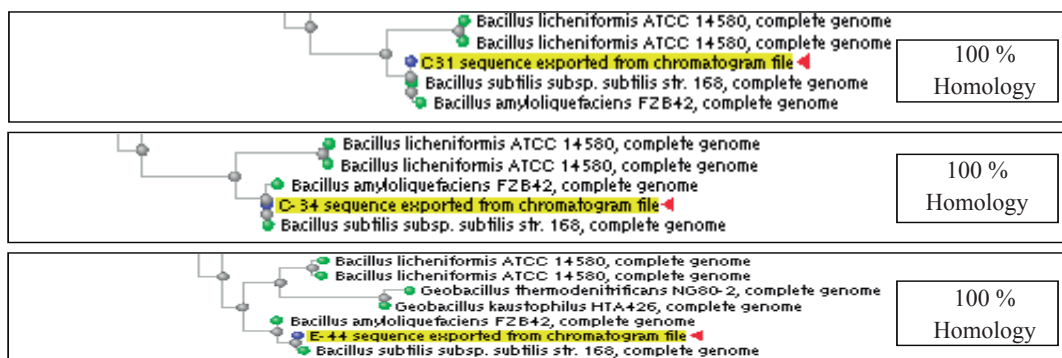


Figura 1. Trees that show relation between isolation of *Bacillus subtilis*, identified with other sequences based on alignments of the ribosomal gen 16S (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

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