Isolation of cecal probiotic bacteria and characterization in the M2 competitive exclusion medium

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For the isolation of lactic bacteria (LAB) and *Bacillus spp*. endospores from the cecum of broilers and their characterization in the M2 competitive exclusion medium, five adult and healthy H_2 hybrid chickens were slaughtered at 45 days old. In the initial counting there was a high number of LAB (10^{12} CFU.g^{-1}) with cecal mucus scrape and Bacillus (10^{10} CFU.g^{-1}) endospores in their cecal contents. Inocula were obtained in MRS (Man, Rogosa and Sharpe) medium for LAB and nutrient medium for *Bacillus spp*. endospores. A dynamic microbial and pH counting was made at 0, 6, 12 h during incubation. The M2 medium was based on final molasses, yeasts and inorganic salts hydrolysate and inoculated with MRS and nutrient media, with incubation at 37ºC for 24 h. Growth dynamics, pH, lactic acid production and reducing sugars at 0, 6, 18, 24 h were evaluated, as well as the growth speed and the duplication time. At 24 h of culture, there were lower pH (4.0) and reducing sugars (1.7 g.L^{-1}) values and higher of lactic acid (16.0 g.L^{-1}). The growth speed and the duplication time were less than one hour. There was high LAB and *Bacillus spp*. population. It is considered that the M2 competitive exclusion medium fulfills the characteristics for the growth of probiotic bacteria.

Key words: *probiotic, competitive exclusion, lactic bacteria, bacillus, characterization*

The production of additives and foods by biotechnological procedures is a need nowadays (Mountzouris *et al*. 2007, Sigrid and Green 2009). The use of lactic acid bacteria and *Bacillus spp*. as probiotics is a reality, specifically outstand the competitive exclusion mixtures (CEM), isolated from the cecum of healthy adult birds, which particularly exclude pathogens in recently born chicks, due to the adherence reduction and the colonization of these harmful microorganisms to the epithelial tissue (La Ragione *et al*. 2004). According to López and Domingo (2007), among the probiotics used in modern poultry rearing are these competitive exclusion mixtures which are a complex of beneficial microorganisms that selectively exclude specific genera that colonize the digestive tract and prevent diseases.

It is important that the microorganisms integrating the CEM are beneficial colonizers of the gastrointestinal tract, be in sufficient concentrations and keep viable for long periods. These CEM are natural products that do not leave residues and improve the productivity and health of the host (Pérez *et al*. 2011). In general, all these CEM are formed by different species of the Lactobacillus, Streptococcus, Enterococcus, Bacillus and Saccharomyces genera (Dumonceaux *et al*. 2006 and Sigrid and Green 2009). However, one of the actual problems in the technology for obtaining any probiotic is the establishment of the culture media that will contribute to the growth of the cultures in sufficient cell concentrations allowing to reach the gastrointestinal tract (GIT) and set up in this ecosystem (Lee *et al*. 2005). For Cuban conditions, these mixtures can be obtained from raw materials of low cost and high reproducibility.

The objective of this study was to isolate LAB and *Bacillus spp*. endospores from the cecum of broilers and characterize them in a M2 competitive exclusion medium.

Materials and Methods

Isolation of LAB and Bacillus spp. endospores and inocula obtainment. Five adult and healthy H2 hybrid broilers of 45 d of age from the Institute of Poultry Researches were slaughtered. The slaughtering method was by cervical dislocation (Sánchez 1990 and Stanier 1996). Ten grams of mucus scrape were weighed under sterility conditions; they were introduced in 90 mL of MRS medium (De Mann *et al*. 1960) for LAB isolation. This content was incubated under static conditions at 37ºC for 12 h. The isolation of *Bacillus spp*. endospores was determined from the dilution of 10 g of cecal contents in 90 mL of sterile saline serum (SSS). This dilution was submitted to a thermic treatment at 71ºC for 10 min, to eliminate the vegetative forms. Later, 10 mL of the endospore suspension were inoculated in 90 mL of nutrient medium and incubated for 12 h at 37ºC under sieve conditions at 150 r.p.m. (Pérez 2000).

Direct counting of microbial population of the mucus scrape and cecal contents. Simultaneously with the attainment of inoculum, the initial counting was made to confirm LAB population and *Bacillus spp*. endospores from 1 g of mucus scrape and 1 g of cecal contents, respectively. For that, the method of serial dilutions (Stanier 1996) 1:10 (v/v) in peptone water was applied. One mL of the 10^{12} dilution for LAB was

implanted deep in plates with double MRS agar layers to guarantee anaerobiosis. For *Bacillus spp*. endospore counting, the dilutions were realized in SSS of the 10^{10}. Surface implantation of 1 mL was made with Drigalsky spatula to favor growth in aerobiosis. In both cases the incubation temperature was 37ºC for 48 or 24 h, respectively.

Microbial growth and inocula pH. The dynamics of microbial growth was realized by the serial dilution method (Stanier 1996) 1:10 (v/v) in peptone water (0.1%) (OXOID), from 10^{-1} to 10^{-12} for LAB and sterile physiological serum, and from 10^{1} to 10^{10} for Bacillus. Incubation was completed at 37ºC for 12 h. Measurement of pH values was made in a digital pH meter (Basic 20 Crison) at 0, 6 and 12 h of incubation.

Evaluation of LAB and Bacillus spp. growth dynamics in M2 medium. The M2 medium proposed by Laurencio *et al*. (2005) based on final molasses, yeasts and salts hydrolisate contributed with the necessary nutrients for lactic bacteria and bacilli growth as beneficial flora. Pathogen bacteria are excluded.

Next 250 mL of the M2 medium was prepared and inoculated with LAB and Bacillus cultures for 12 h of incubation (5% LAB and 5% *Bacillus spp*.) to form the LAB and *Bacillus spp*. endospore mixture. The incubation was developed in 24 h at 37ºC under static conditions. Fifty mL of samples were taken at 0, 6, 12, 18 and 24 h.

Microorganisms counting. Counts were made from 1 mL of M2 medium sample by the methodology of serial dilutions (Stainer 1996) 1:10 (v/v) in peptone water (0.1% (OXOID), from 10^{1} to 10^{12}, for LAB and sterile physiological serum and from 10^{1} to 10^{10} for Bacillus. Incubation was carried out at 37ºC for 24 h.

Lactic acid determination. Samples of 5 mL were centrifuged at 10 000 r.p.m. for 10 min. One mL was taken from the supernatant and the colorimetry was realized from the technique proposed by Taylor (1995). Firstly a standard curve was prepared with increasing amounts of lactic acid (from 0 to 30 µg) to determine the cotangent. This allowed from the absorbance reading at 570 nm in a spectrophotometer (Ultrospec 2100 pro), to estimate the concentration of lactic acid, once 3 mL of H_2SO_4 (96%), 50 µL CuSO4 (4%) and 100 µL p-phenylphenol in ethanol at 95% (1.5%) were applied.

Determination of reducing sugars. Total reducing sugars (tRS) were determined by the 3-5 dinitro salycilic acid (AOAC 1995).

Determination of the specific growth speed. At hour 24, with results obtained from the viable counting, the growth curves were prepared. Applying the adjustment line, the corresponding polynomials and the values of specific growth speed (µ) were achieved. These procedures were carried out through the Microsoft Excel spreadsheet.

Duplication time. It was calculated by the formula described by Madigán *et al*. (1997): $td = \log 2/\mu$

Statistical processing. For data analysis the INFOSTAT system Version 1 (Belzarini *et al*. 2001) was used. The analyses of variance were made to verify the significant differences between means, with a signification level of $P < 0.05$. Duncan's (1955) multiple range test was applied for mean comparisons.

Results and Discussion

From the obtainment of LAB by mucus scrape of chicken ceca and Bacillus endospores of the cecal content, as well as the dynamics of the microbial growth and pH, the use of both cultures as inocula was considered that should be at 12 h of incubation, since in this time the population is in logarithmic phase, essential for the adequate inoculation of the growth medium, if favorable results are expected (Brizuela 2003 and Rondón 2009).

Direct microbial counting of *Bacillus spp*. endospores of the cecal contents and lactic acid bacteria were 10^{12} CFU.g^{-1} and 10^{10} CFU.g^{-1}, respectively. Different researchers studied the microbial ecology of the gastrointestinal tract of broilers (Barbosa *et al*. 2005 and Dumonceaux *et al*. 2006). Among the aspects most analyzed are the microorganisms found and their physiological activity, the relationships established between them and the host animal, as well as the factors affecting the microbial population of this ecosystem.

Diverse authors refer the presence of LAB, as essential part of the microbial ecosystem of chickens' GIT (Shome *et al*. 2001, Guan *et al*. 2003 and Dumonceaux *et al* 2006). In a lower rank appear bacteria of *Bacillus spp*. genus, mainly in the form of endospores (Gálvez 2004 and Milián 2009). These results coincide with those presented in this study, since LAB were found in higher concentration in the animal cecum regarding the number of *Bacillus spp*. in that ecosystem.

Souza *et al*. (2007) found that in the cecum of adult healthy birds predominated species of Lactobacillus genus and, to a lesser extent, Enterococcus. Outdoors chickens showed a high presence of *Lactobacillus acidophilus*, while Lactobacillus routeri and *Lactobacillus johnsonii* were found more frequently in commercial chickens. *Lactobacillus crispatus* was only isolated from commercial chickens, not being so *Lactobacillus vaginalis* and *Lactobacillus agilis*, which were only observed in extensively reared birds. *Lactobacillus salivarius* was localized in the cecum of all animals, regardless their rearing conditions.

The previous information reveals that LAB are predominant in the gastrointestinal ecosystem of birds, and that *Bacillus spp*. endospores are transit microorganisms in this complex microbiota.

In figures 1 and 2 are shown the dynamics of the

viable BAL and *Bacillus spp*. counts in MRS and nutrient media, respectively. Also, pH performance during 12 h of incubation for inocula preparation is illustrated. BAL grew more than 10^{12} CFU.mL^{-1} and the pH decreased gradually until attaining values of 4.6 at hour 12. *Bacillus spp*. reached growths of 1010 CFU.mL^{-1} and the pH fell until values of 6.4 at hour 12.

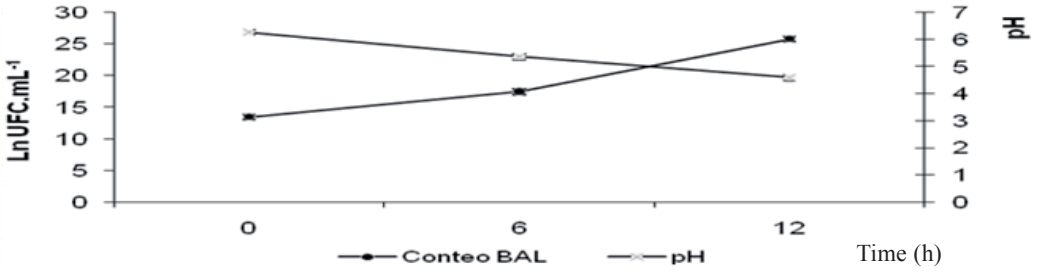
BAL during their growth release short chain fatty acids and lactic acid (Rondón 2009 and Pérez *et al*. 2011), which contribute to pH decrease. BAL are the microorganisms most used as probiotics. The homofermentative bacteria are herein included. In BAL prevail lactic acid production, together with *Bacillus spp*. endospores. This acid could have a bearing on the undesirable bacteria in the gastrointestinal tract (Milián *et al*. 2008 and Pérez *et al*. 2011). The *Bacillus spp*. culture showed good growth of 10^{10} CFU.mL^{-1}, although poor variation in the pH (6.3). This is due to the fact that this group of microorganisms is characterized by having an oxidative metabolism. That is, uses oxygen for its respiration, thus, does not produce substantial concentrations of acid that could acidify the medium (Madigan *et al*. 1997 and Milián *et al* 2008).

It was considered that the utilization of both cultures as inoculum should include 12 h of incubation, since in this time the population is in logarithmic phase, which is essential to inoculate the growth medium and to attain favorable results (Brizuela 2003 and Rondón 2009).

In figure 3 the performance of the BAL and *Bacillus spp*. viable counts in the competitive exclusion M2 medium is shown. The growth of both microbial groups in higher ($P < 0.05$) from 18 h, an increase is observed.

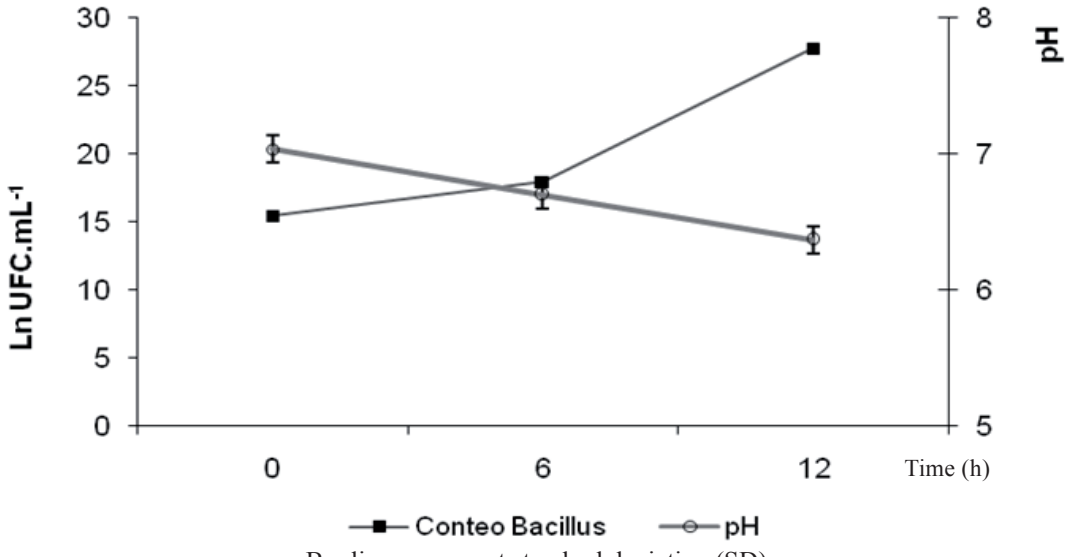
Table 1 shows the values of the specific growth speed (μ) and the duplication time (td) of the competitive exclusion mixture in the M2 medium. Values of the specific growth speed reached are approximately found at 0.6 h^{-1}, with duplication times of 0.5 h^{-1}. This agrees with what was indicated by Gorbach (1991) and Garriga *et al*. (1998), as regards to the strains used for the preparation of probiotic products that for considering them efficient they must have duplication times of one hour, or less.

The values of growth speed are related to the nutrient composition in the culture medium and the contribution of energy, nitrogen, vitamins and mineral compounds. It could be inferred that the levels of reducing sugars and nitrogen, incorporated as carbon and nitrogen sources,



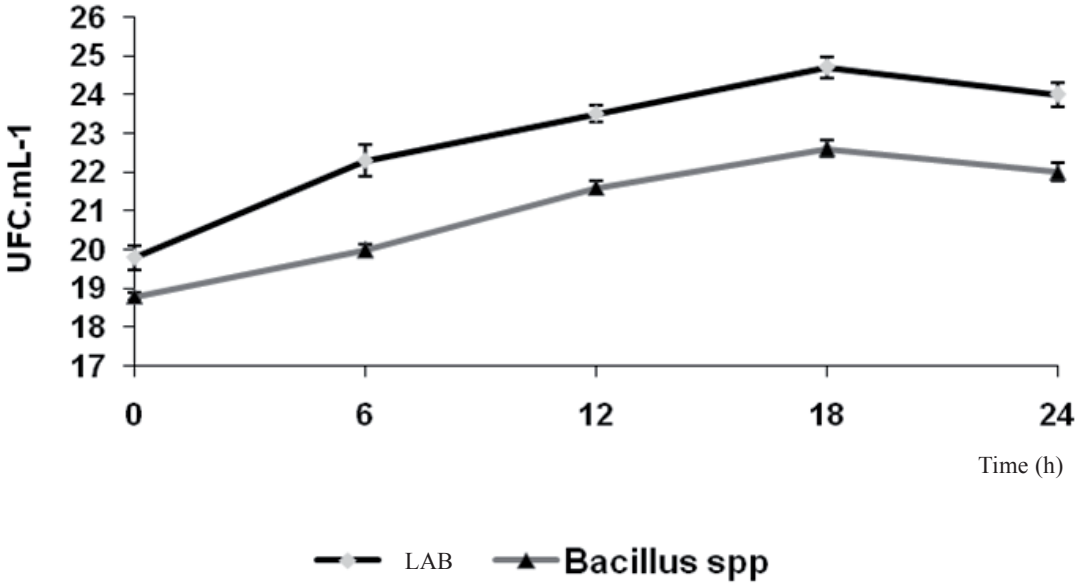
Bar lines represent standard deviation (SD)

Figure 1. Dynamics of viable and pH counts of LAB in MRS medium used as inoculum



Bar lines represent standard deviation (SD)

Figure 2. Dynamics of viable and pH counts of *Bacillus spp*. in nutrient medium used as inoculum



Bar lines represent standard deviation (SD)
LAB: P <0. 05 SE ± 2.08
Bacillus: P < 0.05 SE ± 1.04

Figure 3. Dynamics of LAB and *Bacillus spp*. counts in M2 medium during incubation

Table 1. Specific growth speed and duplication time of LAB and *Bacillus spp*. in M2 medium

Culture media	Microbial groups	Specific growth speed μ (h^{-1})	SE ± Sig	Duplication time, h	SE ± Sig
M2	LAB	0.5892[a]	0.01	0.5092b	0.03
	Bacillus spp.	0.6895[b]	0.02	0.4352a	0.04

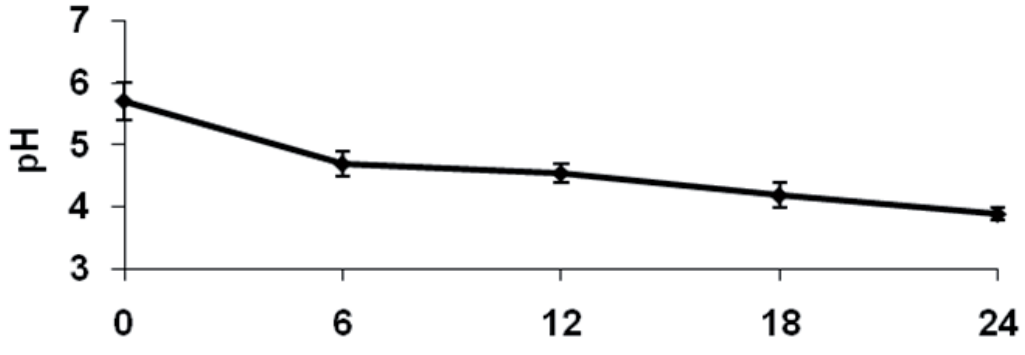
[a,b]Means with different letters within the same row differ at P < 0.05 (Duncan 1955)
*P 0.05

respectively, supply to the cells the necessary nutrients for their growth and metabolic functions.

Mayea *et al*. (1997) indicate that in a medium rich in nutrients the growth speed is faster. Results obtained, regarding the LAB growth speed in the M2 medium, indicate that is superior to that developed by *Bacillus spp*. population, but is acceptable under growth conditions. This points out that the M2 medium can be considered nutritionally complete.

pH results in M2 medium are shown in figure 4. Its decrease can be observed as time elapses, with minimum value of 4.0 at 24 hours of incubation.

Previous results are related to the established environment for the fermentation under static conditions. Lactic acid bacteria grow better under anaerobiosis or microaerobiosis conditions (Rosmini



Bar lines represent standard deviation (SD)

Figure 4. pH performance in M2 medium during incubation

et al. 204 and Savino *et al*. 2011). On the other hand, the representatives of the *Bacillus spp*. genus have better growth in the presence of oxygen (Gusils *et al*. 2008). The decrease of the pH of the medium is attributed to the increase of the acids produced by LAB, main characteristic to exhibit the development of enteropathogens, as *Salmonella spp*., *Escherichia coli* and Campylocater spp. These pH conditions could have also contribute to *Bacillus spp*. growth inhibition in the medium. Barbosa *et al*. (2005) and Milián (2009) cultivated *Bacillus spp*. in the presence of acid pH and they observed growth decrease under these conditions. The most common is that the members of this genus develop their sporulated form (Mayea *et al*. 1997 and Overland *et al*. 2003).

In figure 5 is shown the variation of total reducing sugars and lactic acid during BAL and *Bacillus spp*. growth in M2 medium. There was a decrease of tRS, with a minimum of 1.7 $g.L^{-1}$, since these microbial groups use sugars as carbon source for their growth and energy production (Naheeda 1997). The gradual increase of lactic acid production evidenced a maximum of 16 $g.L^{-1}$, produced mainly by LAB (Naheeda 1997, Ferreira *et al*. 2003 and Brizuela 2003), at 24 h, respectively.

The lactic acid concentration levels increase because these microbial groups are acid producers, mainly BAL (Rondón 2009 and Riboulet-Bisson *et al*. 2012). In the literature BAL are referred as the microorganisms mostly used as biological additives, due to the high production of short chain fatty acids (FASC) and of lactic acid that they develop (Brisbin *et al*. 2010). These results coincide with Higgins *et al*. (2008), Milián (2009) and Rondón (2009), who state that from the fermentative point of view, these microorganisms develop in a wide pH range, which facilitates their manipulation in function of their application.

It is concluded that the microbial groups isolated from the cecum of broilers belong to LAB and *Bacillus spp*. endospores. Thus, they represent at 12 h incubation an appropriate inoculum for the M2 medium, which presents a good pH performance, lactic acid and reducing sugars production, growth speed and duplication time for considering it as a potential competitive exclusion product.

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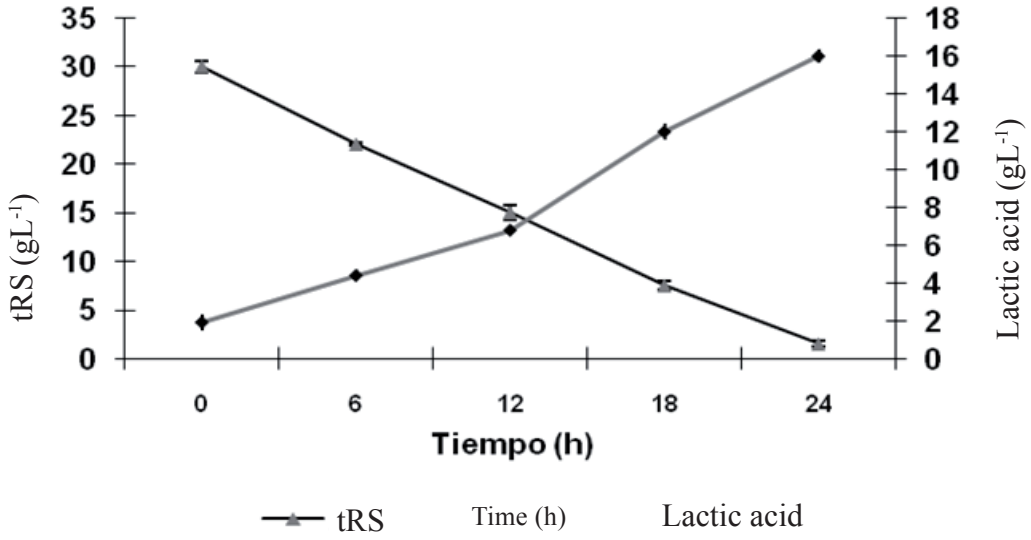

Bar lines represent standard deviation (SD)

Figure 5. Lactic acid and tRS production during fermentation in M2 medium.

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