Identification and Characterization of Lactic Acid Bacteria in a Commercial Probiotic Culture

Anita MENCONI1, Gopala KALLAPURA1, Juan D. LATORRE1, Marion J. MORGAN1, Neil R. PUMFORD1, Billy M. HARGIS1 and Guillermo TELLEZ1*

1Department of Poultry Science, University of Arkansas, Fayetteville, AR 72701, USA

Received May 15, 2013; Accepted August 28, 2013

The aim of the present study was to describe the identification and characterization (physiological properties) of two strains of lactic acid bacteria (LAB 18 and 48) present in a commercial probiotic culture, FloraMax®-B11. Isolates were characterized morphologically, and identified biochemically. In addition, the MIDI System ID, the Biolog ID System, and 16S rRNA sequence analyses for identification of LAB 18 and LAB 48 strains were used to compare the identification results. Tolerance and resistance to acidic pH, high osmotic concentration of NaCl, and bile salts were tested in broth medium. In vitro assessment of antimicrobial activity against enteropathogenic bacteria and susceptibility to antibiotics were also tested. The results obtained in this study showed tolerance of LAB 18 and LAB 48 to pH 3.0, 6.5% NaCl and a high bile salt concentration (0.6%). Both strains evaluated showed in vitro antibacterial activity against Salmonella enterica serovar Enteritidis, Escherichia coli (O157:H7), and Campylobacter jejuni. These are important characteristics of lactic acid bacteria that should be evaluated when selecting strains to be used as probiotics. Antimicrobial activity of these effective isolates may contribute to efficacy, possibly by direct antimicrobial activity in vivo.

Key words: lactic acid bacteria, probiotic, identification, characterization, poultry

INTRODUCTION

The use of probiotics in agriculture has increased as potential alternatives to antibiotics used as growth promoters, and in select cases, for control of specific enteric pathogens [1, 2]. For these reasons, the development of effective probiotic products that can be licensed for animal use continues to receive attention [3]. Some characteristics are important for the selection of a successful probiotic, such as being tolerant to gastrointestinal environment, being able to attach to the intestinal mucosa and being exclusively competitive with enteric pathogens [4]. Low pH, gastric enzymes and bile salts are examples of barriers of the gastrointestinal tract that probiotic bacteria need to resist after being ingested [4, 5]. Several years ago, our laboratory worked toward isolation, evaluation and combination of lactic acid bacteria (LAB) to control foodborne pathogens in the digestive tract of poultry [6]. This defined LAB culture has shown accelerated development of normal microflora in chickens and turkeys, providing increased resistance to Salmonella spp. infections under laboratory and field research conditions [7–15]. There have been several reports regarding the efficacy and success of this LAB culture as a poultry probiotic [2], and the purpose of the present study was to describe preliminary and additional data regarding identification and characterization (physiological properties) of the strains present in this commercial probiotic product.

MATERIAL AND METHODS

Bacterial strains

Two lactic acid bacteria present in a commercial probiotic culture identified as LAB 18 and LAB 48 were assessed. This LAB probiotic (FloraMax®-B11) was licensed to a commercial company (Pacific Vet Group-USA, Inc., Fayetteville, Arkansas 72704, USA).

Morphological and biochemical tests

LAB 18 and LAB 48 were cultured aerobically overnight in de Man, Rogosa and Sharpe (MRS, Catalog no. 288110, Becton, Dickinson and Company, Sparks, MD, USA) broth and were tested for Gram stain affinity, catalase and oxidase production. Cell morphology and colonial characteristics were observed on MRS agar.
Comparison between 4 identification schemes

Isolates were sent out for identification, and four identification schemes were carried out by three different laboratories. For the identification of both strains, two private laboratories used the MIDI System ID (MicroTest Laboratories, Inc., Agawam, MA, USA, and Microbial Identification Inc., Newark, DE, USA), and one private laboratory used 16S rRNA Sequence Analyses (Microbial Identification Inc., Newark, DE, USA). Then, a third laboratory (Department of Poultry Science, University of Arkansas) used the Biolog Identification System (Biolog, Inc., Hayward, CA, USA) to compare the identification results obtained.

Resistance to pH, temperature and sodium chloride

A basal MRS medium was used in these series of in vitro studies. An overnight culture of each isolate was used as the inoculum, with the cells being centrifuged and resuspended in 0.9% sterile saline. The suspension (100 µL) was inoculated into 10 mL of MRS broth in each test tube. Two incubation time points, i.e., two and four hours, were evaluated for each of the variables (pH, temperature and sodium chloride - NaCl). The rationale for these two points was mainly based on food matter passage time through the gastrointestinal tract of poultry. The temperatures tested were 15°C and 45°C, and the concentrations of NaCl tested were 3.5 and 6.5% (w/v). The LAB were tested for survivability using two different pHs (2.0 and 3.0). The tubes were incubated with reciprocal shaking at the specific test temperatures or at 37°C for the tests on pH and concentrations of NaCl. At the time points evaluated, each sample was streaked onto MRS agar to determine the presence or absence of growth, which was used to confirm livability of the strains. The turbidity of each tube was also noted as an indication of growth or no growth. Each treatment was tested with triplicate tubes.

Bile salt tolerance

The method of Gilliland et al. [16], with some modifications, was used to determine bile salt tolerance. MRS broth containing 0%, 0.4%, 0.5% or 0.6% of bile salts No. 3 (Catalog no. 213010, Becton, Dickinson and Company, Sparks, MD, USA) was inoculated with 10⁷ cfu/ml of each probiotic strain from their respective overnight growth cultures after they were centrifuged at 3,000 g for 15 minutes and washed three times. Samples were incubated for 24 hr at 37°C with shaking at 100 rpm. Control (no bile salts) and test cultures were evaluated at 2, 4 and 24 hr for the presence or absence of growth by streaking samples onto MRS agar.

In vitro assessment of antimicrobial activity against enteropathogenic bacteria

The lactic acid isolates were screened for in vitro antimicrobial activity against Salmonella enterica serovar Enteritidis phage type 13A (SE), Escherichia coli (O157:H7) (EC) and Campylobacter jejuni (CJ). Ten microliters of lactic acid isolates 18 and 48 in FloraMax®-B11 were placed in the center of MRS plates. After 24 hr of incubation at 37°C, the plated samples were overlaid with TSA (Tryptic Soy Agar, catalog no. 211822, Becton, Dickinson and Company, Sparks, MD, USA) containing 10⁷ cfu/mL of SE or EC. After 24 hr of incubation at 37°C, plates were evaluated, and those colonies that produced zones of inhibition were selected. A similar overlay method as described above was used for CJ, in which 10⁶ cfu/mL of CJ was inoculated in TSA containing 0.2 g of sodium thioglycolate as a reducing agent and overlaid over the solid agar. Plates were incubated in a microaerophilic environment for 48 hr at 42°C. Colonies that produced zones of inhibition were selected.

RESULTS AND DISCUSSION

Morphological, biochemical and genotypic identification

Both phenotypic and genotypic identifications are part of the first step in the selection of potential probiotic bacteria [4]. Table 1 summarizes the morphological and biochemical tests of LAB 18 and 48. Both strains tested Gram positive and catalase and oxidase negative. However, LAB 18 showed a coccal morphology, whereas LAB 48 showed a rod-shaped morphology. Genotypic systems are becoming valuable tools for use in a wide range of microorganisms [2, 4]. Genotypic 16S rRNA identification of microorganisms from probiotic cultures may be more consistent than the current standard microbial techniques [2]. On the other hand, this method has been shown to have issues and limitations. Speciation relies on the closest match with previously identified species in the database because the identification is based on specific sequence homology compared with a known database generated from previously identified organisms through conventional methodologies [2, 4]. Because databases have been constantly changing and increasing, the same sequence may match other taxons with greater homology. Therefore, at this moment, it is nearly impossible to confidently know the speciation of LAB except with very highly characterized isolates [2]. Thus, while 16s RNA sequencing can positively identify one LAB isolate as unique among several, true accuracy of homology comparisons is somewhat subjective.
IDENTIFICATION AND CHARACTERIZATION OF PROBIOTIC BACTERIA

Even with many new experimental molecular identification techniques and with the known problem of database accuracy and consistency over time, sequence analysis of 16S rRNA is the major molecular technology presently available for microbial identification [17]. Table 2 shows the identification scheme for LAB 18 and 48 using the MIDI System Identification (from two laboratories), the Biolog Identification System and 16S rRNA Sequence Analyses. The results showed that identification of these strains is difficult; nevertheless, the use of defined cultures for probiotic use is still safer than undefined cultures.

Resistance to pH, temperature, and sodium chloride

The first host factors that may affect commercial probiotics are the high acidity in the proventriculus and ventriculus and the high concentration of bile components in the proximal intestine [5, 18]. Therefore, being tolerant to acidic conditions is an important criterion to be considered during the selection of potential probiotic isolates to assure their viability and functionality. Moreover, probiotic bacteria show variable resistance to acidic conditions, and this characteristic is species and strain dependent [4]. LAB 18 and 48 did not survive an incubation period of 2 or 4 hr at pH 2.0. However, at a pH of 3.0, both strains were resistant after 2 and 4 hr of incubation (Table 3). As reported by Fontana et al. [4], Lactobacillus spp. isolates have been shown to be very resistant to low pH, with high survival rates at pH 3.0 for 1 hr. On the other hand, studies have shown that Bifidobacterium spp. isolates are very sensitive to pH 2.0 and pH 3.0 [4]. Lactic acid bacteria are acidophilic, which means they are tolerant to low pH. However, this needs to be differentiated from a condition of high concentration of free acids (H+), because the free acids may cause growth inhibition [19]. Probiotic bacteria need to survive passage through the stomach, where the pH can be as low as 1.5 to 2.0 [20], and stay alive for 4 hr or more [5] before they move to the intestinal tract. However, the feed passage rate for birds is faster than for other animals, especially mammals; therefore, bacterial acid tolerance is not as critical in chickens as it is in other animals [21].

Both strains grew at 15 and 45°C at 2 and 4 hr of incubation (Table 3). Wouters et al. [22] demonstrated reduced glycolytic activity leading to reduced production of lactic acid in Lactococcus lactis at low temperature. According to Ibourahema et al. [23], the bacterial capability to grow at high temperature is a good characteristic, as it could be interpreted as indicating an increased rate of growth and lactic acid production. Moreover, a high fermentation temperature decreases contamination by other microorganisms [23]. Both strains were also able to tolerate high osmotic concentrations of NaCl (Table 3). This examination gave an indication of the osmotolerance level of the LAB strains. According to Ibourahema et al. [23], bacterial cells cultured with a high
salt concentration could show a loss of turgor pressure, which would then affect their physiology, enzyme activity, water activity and metabolism. According to Adnan and Tan [24], high osmotolerance would be a requirement of LAB strains to be used as commercial strains, because when lactic acid is produced by the strain, alkali would be pumped into the broth to prevent an excessive reduction in pH, and the free acid would be converted to its salt form, increasing the osmotic pressure on the bacterial cells.

**Bile salt tolerance**

In general, tolerance to bile salts has been considered a condition for colonization and metabolic activity of bacteria in the host’s intestine [25], bile salts can influence the intestinal microflora by acting as antimicrobial molecules [4]. Consequently, when evaluating the potential use of LAB as a probiotic, it is usually important to evaluate their ability to tolerate bile salts [26]. Table 4 shows the results of bile tolerance of the strains evaluated. LAB 18 and LAB 48 were able to grow when cultured at bile salt concentrations of 0.4%, 0.5% and 0.6% at 2, 4 and 24 hr of incubation. The average concentration of bile salts in the small intestine is around 0.2% to 0.3%, and it may go up to 2% (w/v), depending upon the individual and the type and amount of food ingested [5, 27]. According to Xanthopoulos et al. [28], the ability to tolerate bile salts varies a lot among the LAB species and between strains themselves. Bile resistance of some isolates is related to the enzyme activity of bile salt hydrolase (BSH) that helps to hydrolyze conjugated bile, reducing its toxic effect [29]. BSH activity has most often been found in microorganisms isolated from the intestines or feces of animals [30].

**In vitro assessment of antimicrobial activity against enteropathogenic bacteria**

Both strains evaluated showed in vitro antibacterial activity against the three enteropathogenic bacteria (Table 5). The inhibitory activity of LAB has been previously reported and is mainly due to the accumulation of primary metabolites such as lactic acid, ethanol, and carbon dioxide and to the production of other antimicrobial compounds such as bacteriocins [31]. The production levels and proportions among these compounds depend on the biochemical properties of the strains used and physical and chemical conditions of growth [32].

**CONCLUSION**

Characterization and identification of beneficial enteric lactic acid bacterial isolates is highly dependent upon methodology. The bile and salt resistances of enteric resident microflora are high, with tolerances expected from resident microflora. Antimicrobial activity of these effective isolates may contribute to efficacy, possibly by direct antimicrobial activity in vivo. Alternatively, localized production of volatile fatty acids, and possibly bacteriocins, may contribute to the colonization ability of these isolates, enabling them to compete locally and colonize within the gastrointestinal tract. Importantly, previous [33] and unpublished research from our laboratory indicates very rapid induction of specific host-gene expression pathways, which are associated with reductions in enteric colonization with *Salmonella*. While many mechanisms of action have been proposed for the observed efficacy, precise modalities have not been completely described for this highly effective culture.

**REFERENCES**

1. Anadón A, Larrañaga RMM, Martínez AM. 2006. Probiotics for animal nutrition in the European union, regulation and safety assessment. Regul Toxicol Pharmacol 45: 91–95. [Medline] [CrossRef]
2. Tellez G, Pixley C, Wolfenden RE, Layton SL, Hargis BM. 2012. Probiotics/direct fed microbials
for Salmonella control in poultry. Food Res Int 45: 628–633. [CrossRef]

3. Patterson JA, Burkholder KM. 2003. Application of prebiotics and probiotics in poultry production. Poult Sci 82: 627–631. [Medline] [CrossRef]

4. Fontana L, Brito MB, Diaz JP, Quezada SM, Gil A. 2013. Sources, isolation, characterisation and evaluation of probiotics. Br J Nutr 109: S35–S50. [Medline] [CrossRef]

5. Bakari D, Tatsadjieu NL, Mbawala A, Mbofung CM. 2006. Digestive physiology and the role of microorganisms. J Appl Poult Res 15: 136–144.

6. Higgins SE, Vicente JL, Wolfenden AD. 2007. Temporal effects of lactic acid bacteria probiotic culture on Salmonella in neonatal broilers. Poult Sci 86: 1662–1666. [Medline]

7. Higgins JP, Vicente JL, Wolfenden AD, Tellez G, Hargis BM. 2007. Effect of a defined competitive exclusion culture for prophylaxis and reduction of horizontal transmission of Salmonella enteritidis in broiler chickens. Int J Poult Sci 6: 489–492. [CrossRef]

8. Higgins SE, Vicente JL, Wolfenden AD, Henderson SN, Torres-Rodriguez A, Tellez G, Hargis BM. 2008. Evaluation of a Lactobacillus-based probiotic culture for the reduction of Salmonella Enteritidis in neonatal broiler chicks. Poult Sci 87: 27–31. [Medline] [CrossRef]

9. Higgins SE, Vicente JL, Wolfenden AD, Henderson SN, Torres-Rodriguez A, Tellez G, Hargis BM. 2010. Effect of lactic acid bacteria probiotic culture treatment timing on Salmonella Enteritidis in neonatal broilers. Poult Sci 89: 243–247. [Medline] [CrossRef]

10. Vicente JL, Higgins SE, Bielke L, Tellez G, Donoghue DJ, Hargis BM. 2007. Effect of probiotic culture candidates on Salmonella prevalence in commercial turkey houses. J Appl Poult Res 6: 471–476.

11. Vicente JL, Aviña L, Torres-Rodriguez A, Hargis BM, Tellez G. 2007. Effect of a Lactobacillus spp-based probiotic culture product on broiler chicks performance under commercial conditions. Int J Poult Sci: 154–156.

12. Vicente J, Wolfenden A, Torres-Rodriguez A, Higgins SE, Tellez G, Hargis BM. 2007. Effect of a Lactobacillus-based probiotic and dietary lactose prebiotic on turkey poult performance with or without Salmonella Enteritidis challenge. J Appl Poult Res 16: 361–364.

13. Vicente JL, Torres-Rodriguez A, Higgins SE, Pixley C, Tellez G, Donoghue AM, Hargis BM. 2008. Effect of a selected Lactobacillus spp-based probiotic on Salmonella enteritidis-infected broiler chicks. Avian Dis 52: 143–146. [Medline] [CrossRef]

14. Wolfenden AD, Pixley CM, Higgins JP, Higgins SE, Torres A, Vicente JL, Hargis BM, Tellez G. 2007. Evaluation of spray application of a Lactobacillus-based probiotic on Salmonella enteritidis colonization in broiler chickens. Int J Poult Sci 6: 493–496. [CrossRef]

15. Wolfenden AD, Vicente JL, Brielke LR, Pixley CM, Higgins SE, Donoghue DJ, Donoghue AM, Hargis BM, Tellez G. 2007. Effect of a defined competitive exclusion culture for prophylaxis and reduction of horizontal transmission of Salmonella enteritidis in broiler chickens. Int J Poult Sci 6: 489–492. [CrossRef]

16. Gilliland SE, Staley TE, Bush LJ. 1984. Importance in bile tolerance of Lactobacillus acidophilus used as a dietary adjunct. J Dairy Sci 67: 3045–3051. [Medline] [CrossRef]

17. Wagner RD, Paine DD, Cerniglia CE. 2003. Phenotypic and genotypic characterization of competitive exclusion products for use in poultry. J Appl Microbiol 94: 1098–1107. [Medline] [CrossRef]

18. Hyronimus B, Le Marrec C, Hadj Sassi A, Deschamps A. 2000. Acid and bile tolerance of spore-forming lactic acid bacteria. Int J Food Microbiol 61: 193–197. [Medline] [CrossRef]

19. Amrane A, Prigent Y. 1999. Effect of the main culture parameters on the growth and production coupling of lactic acid bacteria. Appl Microbiol 2: 101–108. [CrossRef]

20. Dunne C, O’Mahony L, Murphy L, Thornton G, Morrissey D, O’Halloran S, Feeney M, Flynn S, Fitzgerald G, Daly C, Kiely B, O’Sullivan GC, Shanahan F, Collins JK. 2001. In vitro selection criteria of probiotic bacteria of human origin: correlation with in vivo findings. Am J Clin Nutr 73:(suppl): 3865–3925. [Medline]

21. Boonkumklaow P, Kongthong P, Assavaniag A. 2006. Acid and bile tolerance of Lactobacillus thermotolerans, a novel species isolated from chicken feces. Kasetsart J 40: 13–17 (Natural Science).

22. Wouters JA, Kamphuis HH, Hugenholzt J, Kuipers P, De Vos WM, Abe T. 2000. Changes in glycolytic activity of Lactococcus lactis induced by low temperature. Appl Environ Microbiol 66: 3686–3691. [Medline] [CrossRef]

23. Ibourahema C, Dauphin RD, Jacqueline D, Thonart P. 2008. Characterization of lactic acid bacteria isolated from poultry farms in Senegal. Afr J Biotechnol 7: 2006–2012.

24. Mohd Adnan AF, Tan IK. 2007. Isolation of lactic acid bacteria from Malaysian foods and assessment of the isolates for industrial potential. Biocentech Technol 98: 1380–1385. [Medline] [CrossRef]

25. Havenaar R, Brink BT, Huis Veld JHH. 1992. Selection of strains for probiotic use. In Probiotics, the scientific basis, Fuller, R. (ed), Chapman and Hall, London, UK, pp. 209–224.

26. Lee YK, Salimen S. 1995. The coming age of probiotics. Food Sci Technol 6: 241–245. [CrossRef]
27. Kristoffersen SM, Ravnum S, Tourasse NJ, Økstad OA, Kolstø AB, Davies W. 2007. Low concentrations of bile salts induce stress responses and reduce motility in *Bacillus cereus* ATCC 14570. *J Bacteriol* 189: 5302–5313. [Medline] [CrossRef]

28. Xanthopoulos V, Litopoulou-Tzanetaki E, Tzanetakis N. 1997. Lactic acid bacteria. Caen Presses Universitaires de Canada.

29. du Toit M, Franz C, Schillinger U, Warles B, Holzappfel W. 1998. Characterization and selection of probiotic lactobacilli for a preliminary mini pig-feeding trial and their effect on serum cholesterol level, faeces pH and faeces moisture contents. *Int J Food Microbiol* 40: 93–104. [Medline] [CrossRef]

30. Tanaka H, Doesburg K, Iwasaki T, Mireau I. 1999. Screening of lactic acid bacteria for bile salt hydrolase activity. *J Dairy Sci* 82: 2530–2535. [Medline] [CrossRef]

31. Rattanachaikunsopon P, Phumkhachorn P. 2010. Lactic acid bacteria: their antimicrobial compounds and their uses in food production. *Ann Biol Res* 1: 218–228.

32. Tannock GW. 2004. A special fondness for lactobacilli. *Appl Environ Microbiol* 70: 3189–3194. [Medline] [CrossRef]

33. Higgins SE, Wolfenden AD, Tellez G, Hargis BM, Porter T. 2011. Transcriptional profiling of cecal gene expression in probiotic and *Salmonella*-challenged neonatal chicks. *Poult Sci* 90: 901–913. [Medline] [CrossRef]