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

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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.

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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 10^7 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 10^6 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 10^6 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.

Table 1. Morphological characteristics of lactic acid bacteria isolates 18 and 48 present in FloraMax®-B11

LAB- ID	Anatomic region isolated	Gram stain	Observation	Catalase	Oxidase
18	Ceca	+	Cocci (clusters)	–	–
48	Ceca	+	Rods	–	–

Table 2. MIDI System Identification, Biolog Identification System, and 16S rRNA sequence analyses identification of isolates 18 and 48 present in FloraMax®-B11

LAB- ID	16S RNA sequencing (first 500 bp) Microbial Identification Inc.	MIDI system Identification MicroTest Laboratories Inc.	MIDI system Identification Microbial Identification Inc.	Biolog Identification Dept. of Poultry Science U. of Arkansas
18	*Pediococcus parvulus*	*Enterococcus cecorum*	*Lactobacillus gasseri*	Unable to identify
48	*Lactobacillus salivarius*	*Lactobacillus helveticus*	*Lactobacillus gasseri*	*Lactobacillus salivarius*

Table 3. Tolerance of lactic acid bacteria isolates 18 and 48 present in FloraMax®-B11 to pH, temperature and NaCl

LAB ID	pH2		pH3		15°C		45°C		3.5% NaCl		6.5% NaCl	
	2hr	4hr	2hr	4hr	2hr	4hr	2hr	4hr	2hr	4hr	2hr	4hr
18	–	–	+	+	+	+	+	+	+	+	+	+
48	–	–	+	+	+	+	+	+	+	+	+	+

Symbols: +, tolerant; –, nontolerant

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

Table 4. Evaluation of bile salt tolerance of FloraMax®-B11 isolates 18 and 48

LAB ID	2 hours				4 hours				24 hours			
	0%	0.4%	0.5%	0.6%	0%	0.4%	0.5%	0.6%	0%	0.4%	0.5%	0.6%
18	+	+	+	+	+	+	+	+	+	+	+	+
48	+	+	+	+	+	+	+	+	+	+	+	+

Symbols: +, tolerant; –, nontolerant

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].

Table 5. *In vitro* assessment of antimicrobial activity of lactic acid bacteria isolates 18 and 48 present in FloraMax®-B11 against enteropathogenic bacteria

LAB- ID	*Salmonella* Enteritidis	*Escherichia coli* (O157:H7)	*Campylobacter jejuni*
18	+	+	+
48	+	+	+

Symbols: +, inhibition

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.

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