GENETIC DIVERSITY OF ***LACTOBACILLI*** ISOLATED FROM HUMAN INFANT FECES*

MAGALI SOARES DOS SANTOS POZZA**
LÚCIA HELENA DA SILVA MIGLIORANZA***
JOSÉ EDUARDO GARCIA****
PAULO CESAR POZZA*****
ROBERTA MERGUIZO******

Seventy-five strains isolated from fecal samples of six children were studied for their morphology and biochemical properties. Genetic diversity of 30 strains was analyzed by using Random Amplified Polymorphic DNA (RAPD). The intestinal isolates were grouped into four clusters. Some strains in group I were 100% similar (*Lactobacillus reuteri*) while high degree of genetic diversity was found in groups II (standard species), III and IV. Eight strains of group I and group III were identified through biochemical identification, as *Lactobacillus reuteri, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei* and *Lactobacillus salivarius,* and showed low genetic similarity with type strains. The findings in this study provide a strong basis for exploring the potential of these eight selected *lactobacilli* strains for use in fermented-milk based products.

KEY-WORDS: BACTERIA IDENTIFICATION; LACTIC ACID BACTERIA; Lactobacillus plantarum; PCR; PROBIOTICS; RAPD.

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** Doutora em Ciência de Alimentos, Professora, Centro de Ciências Agrárias, Universidade Estadual do Oeste do Paraná (UNIOESTE), Campus Marechal Cândido Rondon, Paraná (e-mail: magaspozza@hotmail.com).

*** Doutora em Ciência de Alimentos, Professora, Centro de Ciências Agrárias, Departamento de Tecnologia de Alimentos e Medicamentos, Universidade Estadual de Londrina, Perobal, PR (e-mail: luciah@uel.br).

**** Doutor em Biologia (Genética), Professor, Centro Acadêmico de Vitória, Universidade Federal de Pernambuco, Vitória de Santo Antão, PE (e-mail: jegarcia30@gmail.com).

***** Doutor em Nutrição de Monogástricos, Professor, Universidade Estadual de Maringá, Maringá, PR (e-mail: pcpozza@hotmail.com).

****** Química, graduada pela Universidade Estadual de Londrina, Londrina, PR (e-mail: romerguizo@yahoo.com.br).

1 INTRODUCTION

The increasing interest in healthy lifestyle has led to the use of probiotic in a variety of fermented milk products (FUJIMOTO *et al*., 2008). *Lactobacillus* strains are the most widely used probiotics, and there are many reports of their benefits to human health (WOLD, 2001).

Lactobacilli have been isolated from different sources, for several purposes, like preparation of naturally fermented products such Italian salami (MACIEL *et al*., 2003) or meat (OLIVEIRA, OLIVEIRA & GLÓRIA, 2008) and meat products, such as sausage (SAWITZKI *et al.*, 2007). Few studies have been published describing the isolation of *Lactobacilli* from the human (RYAN *et al.*, 2008) and other species gastrointestinal tract (McCOY & GILLILAND, 2007; RODRIGUEZ-PALACIOS *et al.*, 2009).

Lactobacilli are Gram positive rods belonging to the group of lactic acid bacteria (LAB) (BERNARDEAU, 2008). They are ubiquitous in the diet and are found in the gastrointestinal tract soon after birth. In healthy humans, *lactobacilli* are normally present in the oral cavity (10^3-10^4 cfu/g), in the ileum (10^3-10^7cfu/g) and in the colon (10^4-10^8 cfu/g), and are the dominant microorganism in the vagina (MERK *et al.,* 2005).

The subsequent development of gut microflora during infancy is a complex succession of bacterial species. Among intestinal bacterial species, lactic acid bacteria and bifidobacteria are considered particularly important for their health-promoting benefits such as the prevention of gut colonization by pathogens (McFARLAND, 2000).

The traditional phenotypic *lactobacilli* identification mainly based on cell morphological characteristics and sugar fermentation profiles are still widely applied on a routine basis, but a reliable identification of members from these species is complex by strictly phenotypical testing, which is often highly unsatisfactory (VENTURA & ZINK, 2002).

Although useful for identification purposes, biochemical characterization has poor discriminating capacity and molecular techniques must be used for typing purposes. High-resolution genotypic techniques such as random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) are often used to generate species-specific eletrophoretic profiles when investigating the genetic diversity between *Lactobacillus* species. The identification of *Lactobacillus* species is an important step not only in the development of new and interesting cultures, but also in the identification at intra-species level (McLEOD *et al.*, 2008).

RAPD wide popularity can be attributed mainly to the fact that it is rapid, easy and does not require any prior knowledge of the target sequence; being also universally applicable to any genome. In principle, RAPD involves random amplification from the target bacterial genome with the aid of a single primer under less stringent conditions (SESENÃ, SÁNCHEZ & PALOP, 2004).

The combination of techniques, both biochemical and molecular ones can assure a better identification of isolated strains. Taking that into consideration, the goal of this research was to select potential probiotics amongst isolated strains from children feces using biochemical tests and RAPD technique.

2 MATERIAL AND METHODS

This research was carried out in the Science, Technology, Biochemistry and Biotechnology, both in the State University of Londrina (Universidade Estadual de Londrina), Brazil. The strains were obtained from children's feces (both genders). The children were one year old, previously diagnosed as healthy, not submitted to antibiotic therapy. The samples were collected during the months of November and December 2004, at the Day Care Center of the State University of Londrina.

Fecal samples from six children were collected using swab (KONEMAN, ALLENS &

DOWELL, 1997). The swab was placed in 10 mL of Rogosa broth BBL™ LBS broth (DIFCO, USA) and incubated at 37°C for 24 hours. *Lactobacillus* strains were grown on MRS agar (DE MAN, ROGOSA & SHARPE, 1960) (Himedia, Mumbai, India). Representative colonies were isolated from MRS plates of the highest sample dilutions. All colonies were picked out from plates containing less than 10 colonies and only some colonies were randomly selected from those plates containing more than 10 colonies. The purity degree of isolates was checked by streaking and sub culturing on fresh MRS broth as well as MRS agar, followed by microscopic examinations. Identified strains of *Lactobacillus* were preserved in MRS broth using 15% (v/v) glycerol at - 20ºC.

The primary classification was based on results obtained from Gram staining, cell morphology and catalase tests, being identified based on phenotypic properties such as carbon dioxide production from glucose (FRAZIER, MARTH & DEIBEL, 1968), growth at different temperatures and sugar fermentation patterns.

The ability of strains to grow at different temperatures was evaluated using tubes containing 10% of reconstituted skim milk and incubated at 15°C for 3 and 10 days, at 37°C for 2 days and at 45°C for 3 days (BERGEY *et al.*, 1986).

The ability of microorganisms to metabolize carbohydrates was verified using modified MRS broth and adding 2% of the carbohydrate being tested dextrose, fructose, galactose, lactose, maltose, mannose, rhamnose, saccharose, sorbitol and xylose, and bromocresol purple (0.004% weight/volume) as indicator, following incubation at 37ºC for 48h. The positive results were interpreted by the change in the indicator color from purple to yellow (DAVIS, 1995).

The *Lactobacillus* strains used for comparative purposes (reference strains) in the molecular analyses were obtained from the following Cultures Collection: *Lactobacillus acidophilus* (Korean Collection for Type Cultures - KCTC 3111)*, Lactobacillus casei* (KCTC 1121*), Lactobacillus rhamnosus (*KCTC 3237/ ATCC 7469*),* (ATCC- American Type Culture Collection) *Lactobacillus johnsoni* (KCTC 3138/ ATCC 332)*, Lactobacillus paracasei* (KCTC 3510), *Lactobacillus brevis* (00221/ATCC 367*), Lactobacillus fermentum* (00225/ATCC 9338)*, Lactobacillus plantarum* (00007/ ATCC 8014), *Lactobacillus acidophilus* (La-5 ™) *and Lactobacillus casei* (Lc-01 ™) and *Lactobacillus helveticus* ™ (Christian Hansen-Valinhos, Brazil)*.*

The procedure for DNA extraction from *Lactobacillus* ssp. strains was according to methodology of Luchansky, Tennant & Klaenhammer (1991) with minor modifications. Briefly, cells from an overnight culture, previously grown at 37°C in 100 mL of MRS broth, were collected by centrifugation and resuspended in 200 mL of fresh MRS broth. The cells were harvested by centrifugation, washed twice in TES buffer (50 mmol NaCl - Vetec, 30 mmol Tris pH 8.0 and 5 mmol EDTA), and resuspended in 0.5 mL of lysis buffer (50 mmol Tris pH 8.0, 1.0 mmol EDTA; 20% sucrose - Vetec) containing mutanolysin (4.0 µg mL - Invitrogen) and RNase (44 µL/18 mL buffer-Invitrogen). This mixture was incubated at 37°C for 45 min. A 200 µL volume of sodium dodecyl sulphate 20% was added, and the mixture was incubated at 65°C until the solution cleared. After the addition of 20 µL of proteinase K and 15 min of incubation at 65°C, the lysate was extracted twice with phenol:chloroform (25:24) (700 µL sample) and once with chloroform (500 µL sample). Ethanol 95% was used to precipitate and ethanol 70% to wash the DNA. The samples were kept at 37ºC for 1h, followed by the addition of TE (10 mmol/L Tris pH 8.0 and 1.0 mmol/L EDTA) and DNA was incubated overnight at 37ºC.

RAPD analyses were carried out in a 10 µL final volume, containing 5 ng DNA template, 0.4 mmol/L primer, 200 µM of each dNTP's, 1.5 mmol/L $MgCl_2$ and 1 U *Taq* DNA polymerase (Invitrogen, Brazil) in 20 mmol/L Tris-HCl pH 8.4 containing 50 mmol/L KCl. The primers used were W5, X4, W2, W4, W20, W11, W19, X3, X5, X9, X13, X14, X12, W14, W6, X9 (Operon Technologies, Alameda, CA, USA) (Table 1). The thermal cycling was programmed for 40 cycles of 40 s at 92ºC, 1 min and 30 s at 40ºC and 2 min at 72ºC with a final extension of 5 min at 72ºC and 10 min at 10ºC. PCR products were resolved in 2% agarose gels in TBE (89 mmol/L Tris base, 89 mmol/L boric

acid, 0.2 mmol/L EDTA) and stained with ethidium bromide under UV light. All the reactions were performed twice and negative controls were used at all times.

TABLE 1 - PRIMER SEQUENCES USED IN THIS STUDY

Primers	Sequences 5' – 3'
W-02	ACCCCGCCAA
W-04	CAGAAGCGGA
W-05	GGCGGATAAG
W-06	AGGCCCGATG
W-11	CTGATGCGTG
W-14	CTGCTGAGCA
W-19	CAAAGCGCTC
W-20	TGTGGCAGCA
X-03	TGGCGCAGTG
X-04	CCGCTACCGA
X-05	CCTTTCCCTC
X-09	GGTCTGGTTG
X-12	TCGCCAGCCA
X-13	ACGGGAGCAA
X-14	ACAGGTGCTG

Only reproducible well-marked amplified fragments were scored. For each genotype, the presence and absence of fragments were recorded as 1 or 0. A pairwise comparison of banding patterns was evaluated by the Jaccard similarity coefficient (SNEATH & SOKAL, 1973) using the NTSYS-PC analysis software (ROHLF & NTSYS, 1987). The cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

3 RESULTS

The 75 isolated colonies were presented as Gram positive, mostly bacilli (cocci-bacilli, long and thin bacilli) and rarely cocci. The predominance of Gram positive rod-shaped bacilli was verified. Thirty isolates have been previously selected according to cell morphology (bacilli or rows), catalase reaction (negative) and spore production (non-spore formers). All lactobacilli were tested for their ability to ferment carbohydrates (Table 2), including D-xylose, which is typically fermented by *L. pentosus*.

Six carbohydrates were fermented by all strains within 24–48h. The thirty strains did not ferment D- xylose and some strains showed weak positive or negative reaction with rhamnose (29 strains), sorbitol (17 strains) and saccharose (6 strains) as the only carbohydrate source.

According to the identification tables of *Bergey's manual of systematic bacteriology* (BERGEY, 1986) it was observed that 11 isolates (36.6%) had metabolic behavior similar to *L. reuteri.* The sugar profile of the other strains suggested that these strains belong to *L. casei*, *L. plantarum*, *L. brevis, L. salivarius L. fermentum*, *L. rhamnosus* and *L. gasseri* species. Some researchers identified that *L. paracasei* ssp*. paracasei, L. rhamnosus, L. acidophilus, L. gasseri* and *L. reuteri* were isolated

from children's feces. The present study findings were similar to those of Xanthopoulos, Litopoulou-Tanetaki & Tzanetakis (2000).

TABLE 2 - ISOLATE PHENOTYPIC CHARACTERIZATION

Isolate	Phenotypic affiliation	Isolate	Phenotypic affiliation
1.2	*L. gasseri*	4.1	*L.reuteri / L. fermentum*
2.2	*L. fermentum*	4.6	*L.reuteri/ L. fermentum*
2.3	*L. fermentum*	4.3	*Nd*
2.5	*L. acidophilus*	5.2	*L.casei/ L. plantarum*
2.11	*L. acidophilus*	5.3	*L. casei/ L. plantarum*
2.15	*L.reuteri*	5.6	*L. plantarum*
2.16	*L.reuteri*	5.5	*L.. plantarum /L. paracasei*
2.17	*L.reuteri*	5.7*	*L. brevis*
2.19	*L.reuteri*	5.8	*L. salivarius /L. plantarum/ L. casei*
2.21	*L.reuteri*	5.9	*L. casei*
2.22	*L.reuteri*	6.7	*L. casei*
3.2	*L.casei*	6.8	*L. brevis*
3.3	*L.reuteri/ L. casei*	6.5	*L. acidophilus /L. salivarius*
3.7	*L.reuteri/ L. casei*	6.9	*L. rhamnosus /L. salivarius*
3.14	*L.reuteri/ L. casei*	6.10	*L. salivarius /L. acidophilus*

*undetermined; but sorbitol +.

The selected strains had different growth response and biochemical profile at different temperatures. According to the growth temperature, 2.5 and 2.11 could belong to the *L. acidophilus* group (BERGEY, 1986).

The RAPD results showed the existence of genetic differences between strains. Amongst the 16 primers used, six (OPW 14 and 16, OPX 9, 12, 13 and 14) were efficient in the amplification of the samples, generating a total of 110 polymorphic bands. The UPGMA dendrogram generated (Figure 1) and the band pattern relationships between some representative reference strains and isolates were analyzed.

The 17.5% similarity between strains from Chr. Hansen and ITAL for *L. acidophilus* was considered low, and a 34.8% similarity for *L. casei* types was verified. Comparing the standard strains from the same species, Nigatu (2006) and Sesenã, Sánchez & Palop (2004) also reported different clusters and did not find an explanation for the phenomenon but they verified higher variability.

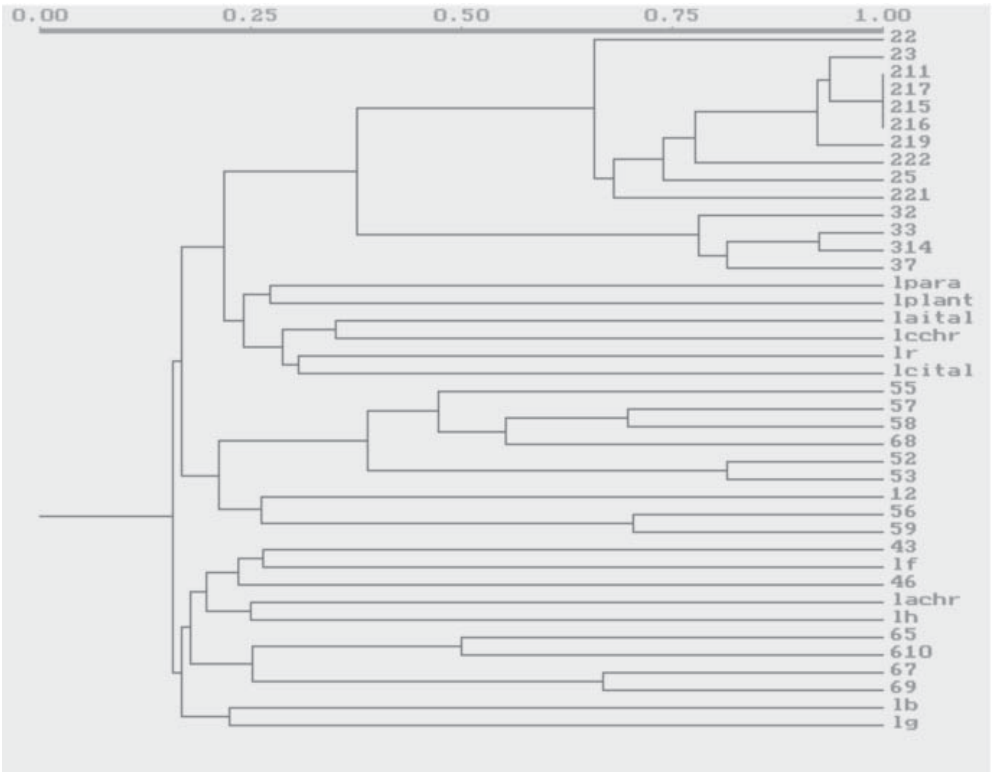

FIGURE 1 - DENDOGRAM OF GENETIC SIMILARITY USING THE JACCARD COEFFICIENT AND UPGMA METHOD FOR THE ISOLATED INTESTINAL BACTERIA, APPLYING RAPD TECHNIQUE

Note: Sequence: 2.2, 2.3, 2.11, 2.17, 2.15, 2.16, 2.19, 2.22, 2.5, 2.21, 3.2, 3.3, 3.14, 3.7, *L. paracasei*, *L. plantarum*, *L. acidophilus* Ital, *L. casei* (Chr. Hansen- Lc 01™), *L. reuteri, L. casei*- ITAL, 5.5, 5.7, 5.8, 6.8, 5.2, 5.3,1.2, 5.6, 5.9, 4.3, *L. fermentum,* 4.6, *L. acidophilus* (Chr. Hansen- La -5™), L. *helveticus,* 6.5, 6.10, 6.7, 6.9, *L. brevis* and *L. johnsoni.*

The RAPD methodology for characterization of *Lactobacillus* strains, isolated from the human intestinal tract, is considered a complementary alternative to traditional systematic methods. There was concordance between both methodologies used in this experiment, and the dendrogram obtained (Figure 1) demonstrated a clear presence of four distinct groups.

Group I had low variability and was formed by 14 strains, from 2.2 to 3.7. These results agree with those obtained by biochemical tests, because some of the strains could belong to the same specie (*L. reuteri*). Strains 2.11, 2.15, 2.16 and 2.17 were 100% similar, although 2.11 had been previously classified as *L. acidophilus*.

Group II was formed by standard *L. paracasei*, *L. plantarum*, *L. acidophilus* (ITAL), *L. casei* (Chr. Hansen Lc 01™), *L. rhamnosus* and *L. casei* (ITAL) species, since they had common characteristics, possibly because some strains have been selected for presenting an active metabolism in the gastrointestinal tract. These results demonstrated that the RAPD technique allows the differentiation between several species of *Lactobacillus*, since they presented low similarity, although the two standards of *L. casei* were in the same cluster.

In group III, strains 5.2, 5.3, 5.5, 5.7, 5.8 and 6.8 were in the same cluster and the similarity was around 27%. Some of these isolates were classified as belonging to the same group, according to the *Bergey's manual of systematic bacteriology* (1986). They had high similarity in the carbohydrate metabolism, besides the fact that they are facultative heterofermentative bacteria. It was also observed that the isolates had only 16.8% of similarity and 16.2% with the standard strains (*L. plantarum* and *L. casei*).

Group IV, which contained standard *L. fermentum*, *L. acidophilus* (Chr. Hansen - La-5 ™) and *L. helveticus,* also showed high polymorphism.

4 DISCUSSION

In spite of this study results demonstrated a concordance between the biochemical and molecular species identification or grouping, the RAPD clusters did not correspond to the API 50 CH grouping pattern of isolates showed in the experiment carried out by Nigatu (2006), who used 42 *lactobacilli* isolated from *tef* and *koncho,* when compared with 30 types of strains. This inconsistency between the results observed at the two methodologies should be explained by the great plasmidial mobility in this group of bacteria, once the most carbohydrate fermentation ability is plasmid-encoded.

Nishitani *et al.* (2004) evaluated 45 *Lactobacillus* strains isolated from human feces and fermented products by RAPD and verified conflicting results when compared to the PCR identification (16S 23S rDNA spacer regions) because they found species related to *L. plantarum* such as *L. paraplantarum* and *L. pentosus*. The authors grouped these strains into 4 clusters, A, B, C and D, and verified low similarity between them, around 40%, and that these clusters were not species-specific. However, 10 out of the 14 strains from humans were included in the same cluster and 8 strains presented low similarity with any of the other clusters, indicating that RAPD is a useful tool to distinguish related *Lactobacillus* species.

Using RAPD PCR, Wall *et. al* (2007) evaluated 292 isolates obtained from adult and infant stool samples and verified prevalence of *L. gassei* and *L. salivarius* among the *Lactobacillus* species found in samples collected from infants.

A study carried out by Ahrné *et al.* (2006) used RAPD to identify *Lactobacillus* species from children's feces with 1, 2, 4 and 8 weeks of age and with 6, 12 and 18 months. The authors considered that, when the strains from the same child showed the same band profile in the RAPD analysis, they were considered as belonging to the same species. The following *Lactobacillus* species were identified: *L. rhamnosus*, *L. paracasei*, *L. gasseri*, *L. fermentum*, *L. reuteri*, *L. plantarum*, *L. delbrueckii* and *L. salivarius*. Using the same parameter to this study data, it is possible to consider the isolates 2.11, 2.15, 2.16 and 2.17 as the same species in spite of the isolate 2.11 was classified as *L. acidophilus* by the biochemical tests. A more sensitive method, like ARDRA for example, have to be used aiming to perform the correct identification of this isolate.

The species diversity observed can be compared to those obtained by Marzotto *et al.* (2006) that used PCR-DGGE to characterize more than 150 *lactobacilli* isolates at different sampling times from feces of infants who received *L. paracasei* A. Those analyses revealed the complex (16 species) and varied composition of the *Lactobacillus* community, which contained typical intestinal species (*L. ruminis, L. reuteri, L. rhamnosus and L. gasseri*), as well as food–associated species (*L. paracasei, L. plantarum, L. fermentum*). The variability was observed among the species isolated throughout the experiment, though *L. casei/L. paracasei* was found as the prevalent species (43% to the isolates). Finding food-associated species in the analyzed samples indicates a promising future for testing these isolates to be used as probiotics lineages.

Albesharat *et al*. (2011) isolated LAB from 70 different samples (15 infants, 15 adults, 15 breast milk and 25 fermented foods) and verified diversity of RAPD types found in food versus human samples. Such findings suggest the relevance of host factors in colonization and individual host specificity and support the hypothesis that there is a vertical transfer of intestinal LAB to the mother's gut to her milk, and subsequently through the milk to the infants gut.

Fujimoto *et al.* (2008) evaluated the ability of *L. casei* (LcS) to proliferate in human intestines after being ingested and developed a PCR-based method to identify and quantify LcS using specific primer set (pLcS) derived from a randomly amplified polymorphic DNA (RAPD) analysis. They tested a total of 27 RAPD primers and the primer p1252 generated a 0.7kb LcS- specific band.

In infant feces, the presence of *L. salivarius* CECT 5713, strain originally isolated from feces of a breast-fed infant, has been observed by Martin *et al*. (2006) using RADP analysis Kit. Later, species-specific PCR showed that *L. salivarius* were present in the milk from the respective mother. The found of four suspect *L. salivarius* lineages in the present study sample should be explained by the same way, but it was also observed a high degree of genetic dissimilarity between those lineages. Three of them grouped at the cluster IV (6.5, 6.9 and 6.10) with low genetic similarity and one (5.8) clustered out of this group demonstrating a high genetic diversity at this species. A more sensitive method for species identification will be used at these lineages at the continuing of this research with the aim to confirm these observations.

As previously reported in literature, RAPD-PCR has proven to be a successful technique for recognizing unique sequences for a selected strain, especially due to its ability to detect differences between bacteria at genomic level (VENTURA & ZINK, 2002). Although a simple and rapid method, RAPD is prone to poor reproducibility in the band pattern due to the present small changes in the reaction conditions. The time saved by the direct application of RAPD is often lost in achieving consistency and in confirming the reproducibility of the results. This standardization of the best resolving conditions and maintenance of very high consistency in all parameters are considered the most important aspects in applying RAPD (SINGH *et al.*, 2009).

The eight lineages identified as *L. reuteri, L. gasseri, L. casei, L. plantarum, L. crispatus, L. salivarius, L. rhamnosus* and *L. acidophilus* in the analyzed samples are considered strong candidates to the continuance of this research, in which is intended to submit them to a molecular characterization with 16S rRNA ARDRA method using it to develop a fermented milk beverage with probiotic characteristics.

5 CONCLUSION

The strains were grouped into four clusters and one of these clusters had good concordance with the biochemical data. Results obtained by this study provide bases to explore the potential of those eight selected *lactobacilli* strains regarding its usage in fermented products.

RESUMO

DIVERSIDADE GENÉTICA DE LACTOBACILOS ISOLADOS DE FEZES DE CRIANÇAS

Setenta e cinco cepas isoladas de fezes de seis crianças foram estudadas quanto a sua morfologia e propriedades bioquímicas. Analisou-se a diversidade genética de 30 cepas pelo método Random Amplified Polymorphic DNA (RAPD). Os isolados intestinais foram agrupados em quatro clusters. Algumas estirpes do grupo I mostraram-se 100% similares (*Lactobacillus reuteri*). Encontrou-se alto grau de diversidade genética nos grupos II (cepas de referência), III e IV. Oito cepas dos grupos I e III foram identificadas bioquimicamente, como *Lactobacillus reuteri, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei* e *Lactobacillus salivarius,* as quais mostraram baixa similaridade genética com as estirpes padrões. Os resultados obtidos neste estudo proporcionam bases para explorar o potencial dessas oito estirpes de lactobacilos selecionadas para uso em produtos fermentados.

PALAVRAS-CHAVE: IDENTIFICAÇÃO DE BACTÉRIAS; BACTÉRIAS ÁCIDO LÁTICAS; Lactobacillus plantarum; PCR, PROBIÓTICOS; RAPD.

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