ACTA BIOCLINICA
Articulo Original
C. Vergel y col

Volumen 8, N° 15, Enero/Junio 2018
Depósito Legal: PPI201102ME3815
ISSN: 2244-8136

IDENTIFICATION AND QUANTIFICATION OF *Lactobacillus casei* subsp. *defensis* FROM FIRM YOGURT AND BUTTERMILK. NEED FOR PROPER LABELING OF THESE FOODS

Carlos Vergel[1,2], Carmen Mantilla[2], Solange Bravo[1], María Morales[1], Doris Medina[1], Jesús Ibarra[1], William Moreno[1], Luis Gutiérrez-Peña[3], Isbery Pérez[3], Roxy Chirinos[3], Juana Villarreal[4], José Vielma[3,5*].

1. **Escuela Técnica Robinsoniana Industrial Agropecuaria Comercial ``Tulio Febres Cordero´´ (ETRIAC), San Cristóbal, estado Táchira, Venezuela.**
2. **Instituto Universitario de Tecnología Agro-Industrial (IUTAI), Departamento de Transformación y Distribución de Materias Primas, San Cristóbal, estado Táchira, Venezuela.**
3. **Laboratorio de Análisis Químico (LAQUNESUR), Universidad Nacional Experimental Sur del Lago "Jesús María Semprum" (UNESUR), Santa Bárbara de Zulia, estado Zulia, Venezuela.**
4. **Escuela Técnica Agropecuaria Robinsoniana Nacional Mesa Cerrada, Timotes, estado Mérida, Venezuela.**
5. **Laboratorio de Neurobiología, Centro de Investigaciones Biomédicas (CIB), Instituto Venezolano de Investigaciones Científicas (IVIC), Hospital Universitario de Maracaibo, Maracaibo, estado Zulia, Venezuela.**

CORRESPONDENCE: José Ramón Vielma Guevara. Laboratorio de Neurobiología, Centro de Investigaciones Biomédicas (CIB), Instituto Venezolano de Investigaciones

Recibido: 30/08/2017
Aceptado: 11/10/2017

Científicas (IVIC), 9° Piso, Hospital Universitario de Maracaibo, Avenida Goajira/SN, Maracaibo, estado Zulia, Venezuela. Phone: +58261-3231795.

E-mail: joravig@yahoo.com

ABSTRACT

In order to identify and quantify *Lactobacillus casei* subsp. *defensis* from milk samples in the San Cristóbal city, Táchira State, a strategy of: biochemistry phenotyping, Gram staining, bile resistance, pH resistance, and viability of the strain in a food were realized. Both in Venezuela and the rest of the world the presence of lactic acid bacteria in yogurt (active and viable) in no fewer than 10^6 CFU/g of product it is accepted. With respect to liquid sweet whey and sweet whey powder the COVENIN standard (3495:1999) does not provide the required number of *Lactobacillus*. We verify the presence of *L. casei* subsp. *defensis* in a range of $11.0x10^8$-$1.0x10^{12}$ and $6.2x10^9$-$1.0x10^{12}$ CFU/mL from firm yogurt and buttermilk respectively in the selected period (0-15 days). Is necessary the inclusion of molecular tools such as the polymerase chain reaction (PCR) to characterize unequivocally of the subspecies identified by the large number of beneficial effects on human health of *L. casei* subsp. *defensis* included in these foods. In Venezuela, the labeling of these products only describes "probiotic mixture" in the case of buttermilk and fermented milk only *Lactobacillus bulgaricus* and *Streptococcus thermophylus* were used in yogurt.

Keywords: yogurt, buttermilk, immunity, COVENIN standard, functional food, probiotic, *Lactobacillus casei* subsp. *defensis*.

IDENTIFICACIÓN Y CUANTIFICACIÓN DE *Lactobacillus casei* subsp. *defensis* DE YOGUR FIRME Y SUERO DE LECHE. NECESIDAD DE CORRECTO ETIQUETADO DE ESTOS ALIMENTOS

Recibido: 30/08/2017
Aceptado: 11/10/2017

Volumen 8, N° 15, Enero/Junio 2018
Depósito Legal: PPI201102ME3815
ISSN: 2244-8136

RESUMEN

Con el objetivo de identificar y cuantificar *Lactobacillus casei* subsp. *defensis* a partir de muestras de leche en la ciudad de San Cristóbal, estado Táchira, se realizó una estrategia de fenotipificación bioquímica, tinción de Gram, resistencia a la bilis, resistencia al pH, y la viabilidad de la cepa en alimentos. Tanto en Venezuela y el resto del mundo la presencia de bacterias de ácido láctico en el yogur (activo y viable) en no menos de 10^6 UFC/g de producto se acepta. Con respecto al suero dulce líquido y de suero dulce en polvo, la norma COVENIN (3495: 1999) no proporciona el número requerido de *Lactobacillus*. Nosotros verificamos la presencia de *L. casei* subsp. *defensis* en un rango de $11.0x10^8$-$1.0x10^{12}$ y $6.2x10^9$-$1.0x10^{12}$ CFU/mL de yogur firme y suero de leche respectivamente, en el período seleccionado (0-15 días). Es necesario la inclusión de herramientas moleculares tales como la reacción en cadena de la polimerasa (PCR, acrónimo en inglés) para caracterizar de forma inequívoca la subespecie identificada, por el gran número de efectos benéficos sobre la salud humana de *L. casei* subsp. *defensis* incluido en alimentos. En Venezuela, el etiquetado de éstos productos solo describe "mezcla de probióticos" en el caso de suero de leche y para leche fermentada solamente *Lactobacillus bulgaricus* y *Streptococcus thermophylus* son utilizados en el yogur.

Palabras claves: yogur, suero de leche, normas COVENIN, inmunidad, alimentos funcionales, probióticos, *Lactobacillus casei* subsp. *defensis*.

INTRODUCTION

The rich biodiversity of species within the intestinal ecosystem facilitates the life and development of the whole, which includes not only the microbiota, but also the human host. For a number of bacterial species, the whole is essential for life: unicellular organisms

Recibido: 30/08/2017
Aceptado: 11/10/2017

require community and biodiversity to development normally. Various bacterial genera and species use metabolic products generated by other microorganisms for their proliferation. The human intestine is the natural habitat of these bacteria that have evolved and adapted to living with humans for thousands of years, so many of them do not grow spontaneously out of that habitat. Amongst the metabolic benefits it brings, formation of adaptive immune system and maintenance of its homeostasis are functions that play an important role. The United Nations for Food and Agriculture (FAO) designated "the microflora or microbiota as the set of microbial communities that inhabit the mucosal surfaces of a host individual". Every human individual harbors about 100 trillion bacteria of 400 different species. For example, in the gastric juice, the bacteria content is relatively low, about 1000 bacteria per mL, and this is due to the acidity of the environment (1-4). Up to day, the concept of nutrition has greatly evolved through constant research and the growth of the information available. The prevention of chronic non-communicable diseases has become the focus of interest both from the public health from research and biotechnology. Fuller in 1989 (5) in this frame presents as "functional food", specially designed components that may affect specific body functions and positively promoting a physiological or psychological beyond its conventional nutritional value effect. This effect may contribute to the maintenance of health and welfare, reducing the risk of illness, or both. In this sense, the studies on functional foods include topics as: fiber,

Recibido: 30/08/2017
Aceptado: 11/10/2017

Volumen 8, N° 15, Enero/Junio 2018
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ISSN: 2244-8136

prebiotics, probiotics and symbiotics, analyzing the technical for each available scientific information and legal frameworks. Moreover, information about the different products available on the market in those categories to identify functional foods available, highlighting the characteristics necessary for its proper use and the recommendation was collected, emphasizing the importance of a full and healthy eating for a good immune system (5-6).

Lactobacillus casei subsp. *defensis* is a lactic acid bacteria, Gram-positive bacilli or cocci. They is anaerobic and/or aerotolerant microorganisms. They may be homo or hetero fermentative, according to the characteristics of its metabolism and mesophilic or thermophilic, due to optimum temperatures for development. Another feature is its ability to adhere to the mucous membranes and produce bacteriostatic substances and/or bactericidal (bacteriocins). *Lactobacillus casei* is probiotic bacteria used in food. Probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (1).

This microorganism should be able to survive passage through the digestive tract and proliferate in the intestine (7). Two genera are mainly used: *Lactobacillus* and *Bifidobacterium*. These are known as acid lactic bacteria (LAB), by its ability to convert carbohydrates to lactic acid. The three species most used and studied are: *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium* spp.

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Aceptado: 11/10/2017

Volumen 8, N° 15, Enero/Junio 2018
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The goal of the study was to identify and quantify *Lactobacillus casei* subsp. *defensis* from firm yogurt and buttermilk from commercial located in the city of San Cristóbal, Táchira State. Similarly the need to include the proper labeling of probiotic cultures contained in these foods is highlighted.

MATERIALS AND METHODS

The research was carried out in the laboratories of Microbiology of ETRIAC "Tulio Febres Cordero" Colón, Táchira State. The milk samples were obtained directly from farms in the area.

a) **Isolation of lactic acid bacteria from fermented milk**. The milk was fermented about 25 days before starting the isolation of lactic acid bacteria. These were isolated in selective MRS agar (Man Rogosa and Sharpe, MERCK, Ref.1.106614) and incubated anaerobically for 48 hours at 37°C. Isolated colonies in the MRS medium were morphologically characterized by Gram staining and biochemical tests as conventional reducing sugars, catalase, oxidase, gelatinase, growth in 4% NaCl broth, among others (8). Colonies that did not conform to these parameters were discarded.

b) **Evaluation of probiotic lactic acid bacteria to native.**

b.1) **Stability in the passage through the stomach**: as the pH of the human stomach is 2.5 and the average time from a food entering and leaving the stomach are 90 minutes, to assess the ability of the microorganism to survive in MRS was adjusted to pH 2.5 with commercial acetic acid, subjecting the isolate to this acidity for four hours.

b.2) **Resistance to bile salts**: in the same manner as microorganism

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Aceptado: 11/10/2017

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resistance to the action of the pH was assessed resistance to bile salts in a concentration of 0.3% for four hours.

b.3) **Viability of the strain in a food**: 1x10^{12} CFU/mL were inoculated into 250 mL of freshly yogurt firm prepared, making CFU count every 5 days until day 15 (0-15 days). To compare the results was used as control 250 mL sterilized whey obtained from farms in the area, which was subjected to 121°C and 15 pounds of pressure/15 minutes, and inoculated with the same amount of *L. casei* subsp. *defensis*.

c) **Statistical analysis of data** from accounts CFU/Ml of *L. casei* subsp. *defensis* was performed for the two foods made from milk (yogurt and buttermilk) with three replicates respectively. The "t test" was applied to the differences between them, using the statistical program "Statistical 4.0". It was assumed as null hypothesis that there were no significant differences in registration count CFU/mL of *L. casei* subsp. *defensis* firm yogurt and buttermilk as a witness.

RESULTS AND DISCUSSION

About 10 different microorganisms in fermented milk were isolated colonies; the colonies that did not meet the identification parameters were discarded. The microorganism endured testing pH and bile salts, thus probiotic was considered. In this sense, colonies of lactic acid bacteria isolated, only met all microscopic identification parameters was considered for viability assays in foods: short bacillus Gram positive (+), non-spore forming, catalase negative (-), oxidase negative (-) (see table 1). In the biochemical characterization, their behavior in

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reducing sugars was very similar to *Lactobacillus casei* I reported by the Bergey's Manual.

Table 1. Biochemical behavior and staining characteristics of *Lactobacillus casei* subsp. *defensis* in the firm yogurt and buttermilk.

Parameter	**Microorganism:** *Lactobacillus casei* subsp. *defensis*
	Result
Gram	+
Temperature	
15 ^{o}C	+
45 ^{o}C	V
Reducing sugars	
Lactose	+/-
Sucrose	+/-
Mannitol	+
Melibiose	+
Sorbitol	+
Xylose	-
Nh_2 Arginine	-
Growth in 4% NaCl broth	+
Catalase	-
Oxidase	-
Gelatinase	-
Indole	-

(+) Positive, (-) Negative, (V) Variable.

Lactobacillus casei were recovered using a validated method in European Economic Community. Polymerase chain reaction (PCR)-based methods

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were used to assess the accuracy of labelling with regard to genus and species, and pulsed-field gel electrophoresis (PFGE) was used to identify strains. Five products were mislabeled with respect to the numbers and three with respect to species of lactobacilli. In four cases, the specified strains were not detected. Four fermented milks sold fewer than three trademarks contained the same strain, which was named differently on each label. As safety and functionality of probiotics are strain dependent, these results demonstrated the need to control lactobacilli present in commercially probiotic human food products, not only at the species but also at the strain level, to ensure their quality and protect the consumer (9). *Lactobacillus casei* subsp. *defensis* is a microaerophilic bacillus, Gram positive, and catalase negative. This organism is a lactic acid as the main product of the fermentation of sugars therefore considered homofermenters microorganisms. In evaluating the capability of reducing sugars in the investigation, the inability to utilize xylose as a source of energy was confirmed (table 1), as homofermenters not use the pentose pathway for metabolism as do heterofermenters. According to nutritional parameters viability of probiotics in food, *Lactobacillus casei* fulfilled the requirement of viability, which should be over 10^6 (10). In this study the counts obtained after 15 days of storage were of $11x10^8$ CFU/mL and $6.2x10^9$ CFU/mL firm yogurt and buttermilk respectively (table 2). Although no adverse effect on the viability firm yogurt probiotic was found, he appeared in both samples

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decreased counts made in time; cooling still likely to have had an impact on the decline. Similar results were reported per Jaskari, *et al*., in 1998 (11), who found that the counts made of *Lactobacillus plantarum* decreased cooled cheese as in the control, but still remained a workable amount.

Lactobacillus casei and *Escherichia coli* show stability in terms of viability and purity preserved by three techniques food: freezing, cryoprotectants and glass beads without change in morphology, Gram staining and results of biochemical tests for identification (12). An added bonus was the identification *L. casei* through the API system in the González, Martínez and Miro design (12), which was not available in our studio.

The biochemical identification of microorganisms using biochemical tests and depending on their morphology may have limited by variability in metabolic pathways, other sources of error are associated with the origin and preservation of crops, resulting in atypical characteristics associated with phenotypic characterization (12-13). Techniques based on DNA studies are subject to less variability and based on the analysis of the unique composition of the nucleic acids are encoded by the same nucleic acid (13).

The probiotic *Lactobacillus casei* subsp. *defensis* isolated has activity from firm yogurt and buttermilk, remained viable during the two-week study (Table 2). Assessments count CFU/ mL indicate that these foods are a suitable vehicle for the administration of probiotic microorganisms, allowing the survival of these in the amount needed to produce beneficial effects on the health

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of consumers giving nutritional support and its turn strengthens the human immune system (table 3). It is recommended for future work with not only probiotic dairy products but also fruit drinks and oligosaccharides.

Table 2. *Lactobacillus casei* subsp. *defensis* counts obtained firm yogurt and buttermilk.

Time (days)	Yogurt firm *	Buttermilk *
0	$1.0x10^{12}$ CFU/mL	$1.0x10^{12}$ CFU/mL
5	$3.0x10^{10}$ CFU/mL	$4.5x10^{10}$ CFU/mL
10	$2.0x10^{9}$ CFU/mL	$4.0x10^{9}$ CFU/mL
15	$11.0x10^{8}$ CFU/mL	$6.2x10^{9}$ CFU/mL

*** The value of the count of colony forming units/mL (CFU/mL) for each food reflects the average of three determinations.**

Table 3 summarizes the main effects in vivo and in vitro obtained with different strains of *Lactobacillus casei* on the immune system, infectious diseases, vaccines (adjuvant), among many other properties (14-35). In our country the information on the food label only include limited information about the use of probiotics in fermented milk according to COVENIN standards: *Lactobacillus bulgaricus* and *Streptococcus thermophylus* in the case of yogurt. In buttermilk only the use of "mixtures of probiotics" in the food label is designated and genera and species were unspecified. This situation is different from countries like Mexico where a large amount of information is

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disseminated *Lactobacillus casei* strain Shirota and *Lactobacillus casei* subsp. *defensis* through mass media, including audiovisual.

Table 3. Effects *in vivo* and/or *in vitro* of different strains of *Lactobacillus casei*.

Microorganism	*in vivo* effect	*in vitro* effect	Associations with others microorganisms	Effects	Reference
Lactobacillus casei	+	-	*Lactobacillus acidophilus*	Use of Dixentil is an easy, safe, and feasible approach to protect patients against the risk of radiation-induced diarrhea.	14
Lactobacillus casei DN 114-001	-	+	*Lactobacillus mucosae* 0988, *Bifidobacterium animalis* subsp. *lactis* Bb-12.	*L. casei* DN 114-001 exhibited the strongest antigenotoxic activity against fecal water, with fermented inulin reducing genotoxicity by 75%.	15
Lactobacillus casei P4	-	+	None.	*L. rhamnosus* P1, P2, P3 and *L. casei* P4 had the highest abilities to adhere to Caco-2 cells.	16
Lactobacillus casei LOCK0919	+	-	*Lactobacillus rhamnosus* LOCK0900, *Lactobacillus rhamnosus* LOCK0908.	The three *Lactobacillus* strains shows potential for use in the prevention of increased gut permeability and the onset of allergies in humans.	17
Lactobacillus casei NWP08	+	-	None.	*L. rhamnosus* NWS29 and NWP13 but not *L. casei* NWP08 might be good candidates for the prevention of allergic airway inflammation.	18
Lactobacillus casei LBC80R	+	+	None or *Lactobacillus acidophilus* CL1285, *Lactobacillus rhamnosus* CLR2.	Individual strains and the finished products have shown antimicrobial activity against *Clostridium difficile* and toxin A/B neutralization capacity *in vitro*.	19
Lactobacillus casei strain Shirota	+	-	None.	The consumption of probiotic beverages containing *L. casei* strain Shirota can modulate the composition of the intestinal microbiota on a long-term basis, resulting in decreased concentrations of short chain fatty acids in the gut.	20
Lactobacillus casei strain Shirota	+	+	None.	Reduction of IgE anti-ovalbumine, increase of Th1-cell associated cytokines, and reduction of Th2-cell associated cytokines.	21
Lactobacillus casei strain Shirota	-	+	None.	Increase the levels of interleukin-12 (IL-12) and gamma interferon (INF-γ).	22
Lactobacillus casei	+	+	*Lactobacillus acidophilus*	Increase the phagocytosis and lymphocyte activity.	23
Lactobacillus casei	+	-	None	Increase the tumoral necrosis factor alpha (TNF-α) in serum, liver, lung and gut in mice.	24

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Table 3.

Microorganism	*in vivo* effect	*in vitro* effect	Associations with others microorganisms	Effects	Reference
Lactobacillus casei strain Shirota	+	-	None.	Regular intake of a *L. casei* strain Shirota-containing probiotic product may modify the gut microbiota composition and intestinal environment in pre-school and school-age Japanese children.	26
Lactobacillus casei DN-114 001	-	+	None.	*L. casei* DN-114 001 inhibits the increase in paracellular permeability of enteropathogenic *E. coli*-infected T84 cells.	27
Lactobacillus casei	+	-	None.	Protection against *Salmonella typhimurium* and increase of the level of specific intestinal IgA.	28
Lactobacillus casei LC71	-	+	None.	Two *L. reuteri* strains but not *L. casei* were deemed as the most appropriate candidates to be used as potential probiotics against microbial gastric disorders; with strong anti- *Helicobacter* and antioxidative activities.	29
Lactobacillus casei	+	-	None.	Synbiotics containing *L. casei* (4 × 10^8 CFU) with prebiotic Inulin (400 mg) may have a positive influence on human plasma antioxidant capacity and the activity of selected antioxidant enzymes .	30
Lactobacillus casei DG	-	+	None.	The probiotic *L. casei* DG has a bactericidal effect on all analyzed species isolated from dental plaque and that the mix culture of probiotic *L. acidophilus* LA-5 and *Bifidobacterium* BB-12 has only a bacteriostatic effect.	31
Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus acidophilus	-	+	None.	*Lactobacillus* species may antagonize *C. albicans* host colonization	32
Lactobacillus casei strain Shirota	+	-	None	*L. casei strain Shirota* could reduce the incidence of antibiotic-associated diarrhoea in hospitalized spinal cord injury patients.	33

***Meta-analysis was designed to evaluate the efficacy of *Lactobacillus* spp. in the prevention of *Clostridium difficile*-associated diarrhea**.

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CONCLUSION

From an inoculum of 1.0×10^{12} CFU/mL of *Lactobacillus casei* subsp. *defensis* placed in milk subjected to fermentation, we obtained viable colonies during the fifteen days of observation in yogurt and buttermilk, with higher counts than those required by the COVENIN standards. To date we have no information about *Lactobacillus casei* subsp. *defensis* included in processed foods elaborated in Venezuela and the inclusion of this probiotic, and proper food labeling is necessary.

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Aceptado: 11/10/2017

ACTA BIOCLINICA
Articulo Original
C. Vergel y col

Volumen 8, N° 15, Enero/Junio 2018
Depósito Legal: PPI201102ME3815
ISSN: 2244-8136

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Recibido: 30/08/2017
Aceptado: 11/10/2017