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Risk Assessment of Genetically Modified Lactic Acid Bacteria Using the Concept of Substantial Equivalence

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Abstract The use of food-grade microorganisms such as lactic acid bacteria (LAB) is one of the most promising methods for delivering health promoting compounds. Since it is not always possible to obtain strains that have the ability to produce specific compounds naturally or that produce them in sufficient quantities to obtain physiological responses, genetic modifications can be performed to improve their output. The objective of this study was to evaluate if previously studied genetically modified LAB (GM-LAB), with proven in vivo beneficial effects, are just as safe as the progenitor strain from which they were derived. Mice received an elevated concentration of different GM-LAB or the native parental strain from which they were derived during a prolonged period of time, and different health parameters were evaluated. Similar growth rates, hematological values, and other physiological parameters were obtained in the animals that received the GM-LAB compared to those that were fed with the native strain. These results demonstrate that the GM-LAB used in this study are just as safe as the native strains from which they were derived and thus merit further studies to include them into the food chain.

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Introduction

Consumer interest in the relationship between diet and health has increased the demand and thus the production of foods containing ingredients that aid specific bodily functions in addition to being nutritious. Foods fitting this definition are commonly referred to as functional foods. The specific ingredients present in functional foods that can produce a beneficial effect on the host above and beyond its basic nutritional value are denominated nutraceuticals which can be defined as "a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease" [1].

Many methods can be used to produce functional foods, but one of the widely accepted and economically attractive options is the use of nutraceutical-producing microorganisms. Due to their long history of safe use in the elaboration of fermented foods throughout history, lactic acid bacteria (LAB) are ideal candidates to produce nutraceuticals (such as vitamins, enzymes, and metabolites). However, it is not always possible to obtain native strains that have the ability to produce specific compounds or that produce them in sufficient quantities to obtain physiological responses. Not only do LAB have great potential for in situ production in fermented foods, but they also have huge potential in metabolic engineering strategies [2], thus the use of genetically modified LAB (GM-LAB) specifically designed to produce nutraceuticals would be an ideal step to obtain novel functional foods. A review on the potential uses of GM-LAB has been published previously [3].

Although there is no scientific evidence that supports the notion that genetically modified foods or food ingredients are dangerous for human consumption, it is necessary to demonstrate that these are innocuous for consumers to alleviate the fears held by the general public associated

with the use of genetically modified organisms (GMO) in the food chain. In order to do this, it is necessary to demonstrate that engineered ingredients are just as safe as their conventional equivalents. Although GM-LAB designed for use in functional food production would probably be more acceptable than other GMO for the consumer because of their (health) benefits, a proper safety assessment must be made before introducing them in products intended for human consumption. The safety evaluation of GM-LAB has not been formally regulated neither in Europe nor in the US [3]; many researchers have proposed the using substantial equivalence as a concept guide for the development of new regulations for risk assessment of these promising microorganisms. The concept of substantial equivalence of GM-LAB involves demonstrating that these are just as safe as their non-modified progenitors with a long history of safe use, thereby eliminating the need for a time consuming and costly safety review.

Previously, we have described GM-LAB that have the ability to produce nutraceuticals (B-group vitamins and digestive enzymes) and their use in the promotion of specific health issues. *Lc. lactis* NZ9000 pNZ7010 was able to compensate for folate depletion using an animal model as manifested by increasing folate concentrations in organs and blood, improvement of hematological values and reversion of a partial megaloblastic anemia caused by folate deficiency [4]. *Lc. lactis* NZ9000 pNZGBAH was used to feed riboflavin deficient rodents and was able, in contrast to the control strain (*Lc. lactis* MG 1363), to eliminate most physiological manifestations of ariboflavinosis, such as stunted growth, elevated erythrocyte glutathione reductase activation coefficient values, and hepatomegaly [5]. Supplementation with α-gal-producing LAB such as *Lc. lactis* NZ9000 pVE5571 alleviated the undesired physiological effects associated to soy consumption in rats such as increased gas emissions (flatulence) [6, 7].

The objective of this study was to demonstrate that these GM-LAB, with proven beneficial effects in animal models, are just as safe as the progenitor strain from which they were derived.

Materials and Methods

Bacterial Strains

Lactococcus (*Lc.*) *lactis* strains used in this study are described in Table 1. Microorganisms were grown in M17-Glu during 16 h at 30°C, and gene expression was induced with nisin as described previously [8]. Plasmid maintenance was ensured by growing *Lc. lactis* strains in the presence of 5 μg/ml chloramphenicol. The cultures were centrifuged (4000×*g*), and the pellet was washed twice with 0.1% (w/v) peptone solution. The cell pellet was then suspended in 10% skim milk before being administered to mice.

Animals

Seventy-two BALB/c mice of 6 weeks of age, weighing 25–30 g, were obtained from the inbred closed colony maintained (12-h light cycle, 22 ± 2°C) at CERELA-CONICET (Tucumán, Argentina). The mice were separated into six experimental groups (each experimental group consisted of 12 mice with equal numbers of male and female mice). Each group of mice received one of the five lactococcal strains described in Table 1, and one group (control group) did not receive bacterial supplementation. All groups were fed water and balanced rodent diet (Cooperación, Argentina; containing 32% protein, 5% fat, 2% fiber, and 60% nitrogen-free extract) ad libitum. *Lc. lactis* strains were given to the mice at a concentration of 1.0×10^9 CFU LAB/day/animal by daily intraoesophagic

Table 1 Strains used in this study

Strain	Relevant properties	Reference
Lc. lactis MG1363	Plasmid-free derivative of *Lc. lactis* NCDO712	[14]
Lc. lactis NZ9000	*Lc. lactis* MG 1363 derivative, *pepN:nisRK*. Host strain for the nisin controlled gene expression (NICE) system	[8]
Lc. lactis NZ9000 pNZ7010	Plasmid pNZ7010 contains the *folKE* genes under the control of the *nisA* promoter (P_{nisA}) encoding the bifunctional protein 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyriphosphokinase and GTP-cyclohydrolase I. Increased intracellular folate is observed when induced with nisin	[15]
Lc. lactis NZ9000 pNZGBAH	Plasmid pNZGBAH contains the riboflavin biosynthesis genes *ribG*, *ribB*, *ribA*, and *ribH* under the control of P_{nisA}. Substantial riboflavin overproduction is seen when these four biosynthetic genes are overexpressed simultaneously	[16]
Lc. lactis NZ9000 pVE5571	Plasmid pVE5571 contains the *melA* gene from *Lactobacillus plantarum* ATCC8014 under the control of P_{nisA}. After nisin induction, the digestive enzyme α-galactosidase is produced	[17]

administration during 30 days. The control group consisted of mice that received 10% non-fat milk under the same conditions as the test groups.

Animal growth (live weight), food, and water intake were determined on a bi-daily basis.

All animal protocols were pre-approved by the Animal Protection Committee of CERELA and followed the latest recommendations of the Federation of European Laboratory Animal Science Associations and of the Asociación Argentina para la Ciencia y Tecnología de Animales de Laboratorio. All experiments comply with the current laws of Argentina.

Blood and Organ Samples Collection

At the end of the feeding period (30 days), mice were anaesthetized with an intraperitoneal injection of a solution containing ketamine (Holliday, Buenos Aires, Argentina) and xylacin (Rompum, Bayer S.A., Buenos Aires, Argentina) to obtain a final concentration of 100 μg and 5 μg/kg body weight, respectively. Animals were bled by cardiac puncture, and blood was transferred into tubes containing anticoagulant (Heparin, Rivero, Buenos Aires, Argentina).

Blood smears were prepared immediately and were stained with Giemsa (Biopur Quimica, Argentina). The remaining blood samples were used to determine the following hematological parameters: red (RBCS) and white (WBCS) blood cell counts, differential leukocyte counts, hematocrit, and hemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) according to the current guidelines of the Colegio Bioquímico de Tucumán (Tucumán, Argentina).

Microbial Translocation and Relative Organ Indexes

Microbial translocation to extra-gut organs and bacteremia in blood were determined following previously described protocols [9]. Briefly, the spleen and liver were aseptically removed, homogenized in sterile peptone solution, and serial dilutions were plated in triplicate in the following media: MRS for enumeration of lactobacilli, McConkey for analysis of enterobacteria, and blood-supplemented brain–heart infusion (BHI) for enumeration of anaerobic and aerobic bacteria. The relative weight of the organs was calculated dividing the organ weight by the live animal weight and is given as a percentile.

Histology

Tissues of visceral organs (spleen, liver, and kidney) and of the small and large intestines were prepared for histological evaluation as described previously [10]. Serial paraffin sections of 4 μm were made and stained with hematoxylin–eosin. The gross anatomy of the organs was examined under light microscopy at 100× magnification.

Statistical Analysis

Statistical analyses were performed with the software package Minitab 14 (Minitab) using ANOVA GLM followed by a Tukey's post hoc test, and $P \leq 0.05$ was considered significant. Unless otherwise indicated, all values were expressed as the mean ± standard deviation.

Results and Discussion

Lactococcus (*Lc.*) *lactis* subsp. *cremoris* MG1363 is the lactococcal strain most intensively studied throughout the world, and the genome of this plasmid-free strain has recently been completely sequenced [11]. This strain has become the prototype among LAB in applied and fundamental research due to its GRAS (generally regarded as safe) status and the development of elegant systems for the expression of proteins and peptides in this strain. *Lc. lactis* NZ9000 is a MG1363 derivative in which *nisRK* was integrated into the *pepN* gene [8] and is the most commonly used host of the *Ni*sin-*C*ontrolled gene *E*xpression system, also referred to as NICE. This system has become one of the most successful and widely used tools for regulated gene expression in Gram-positive bacteria and has been introduced into *Leuconostoc lactis*, *Lactobacillus* (*L.*) *brevis*, *L. helveticus*, *L. plantarum*, *Streptococcus* (*St.*) *pyogenes*, *St. agalactiae*, *St. pneumoniae St. zooepidemicus*, *Enterococcus faecalis*, and *Bacillus subtilis* [12].

Although *Lc. lactis* is not a natural inhabitant of the gastrointestinal tract, it does survive gut passage and has been successfully used to pioneer the gut delivery of bioactive molecules, such as vaccine antigens and immune modulators [13]. In this study, three genetically modified *Lc. lactis* strains that produce nutraceuticals (folates, riboflavin, and α-galactosidase) using the NICE system were used as examples to evaluate the risk of genetically modified LAB (GM-LAB) compared to the native strain (*Lc. lactis* MG1363) from which they were derived.

The strains used in this study were previously shown to exert beneficial effects in mice after very short feeding periods (some observed after a few hours of their administration). Prolonged feeding of mice (during 30 days, a significant period of time in the lifetime of mice) with 1.0×10^9 CFU/day of either of the three above mentioned genetically engineered strains (equivalent to 5×10^{10} CFU/kg of animal live weight) did not significantly affect animal growth or final animal weight compared to the animals fed the native strain or to those that did not receive any bacterial supplementation (Fig. 1). No

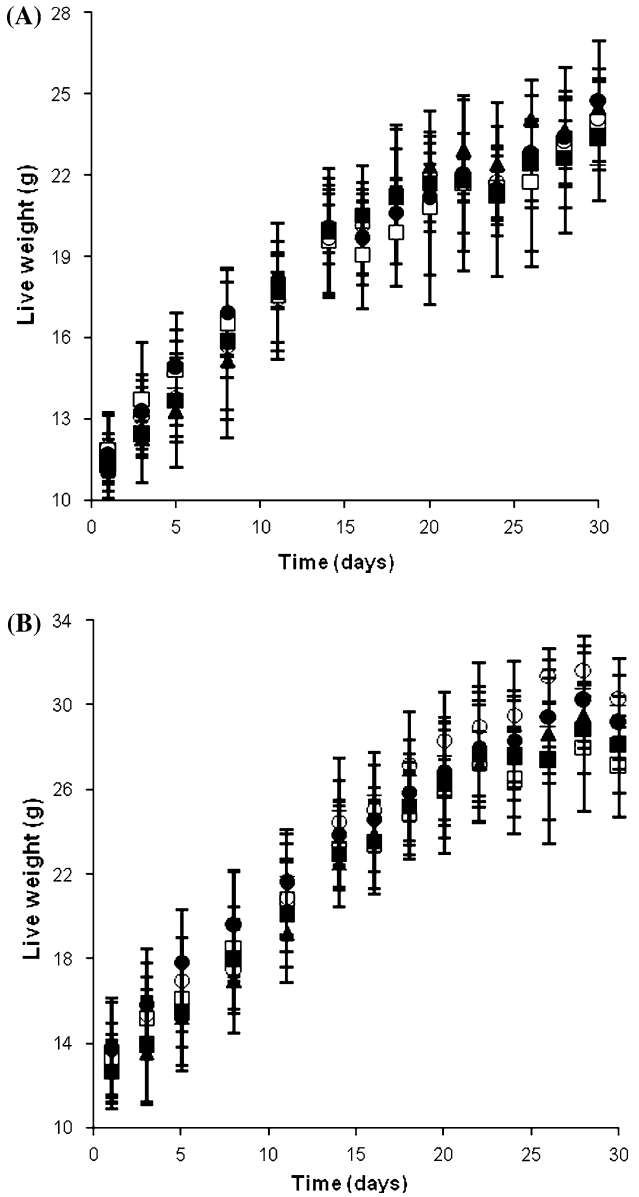

Fig. 1 Animal growth of female (**a**) and male (**b**) mice during the feeding period (30 days). Rodents received 1.0×10^9 CFU/day of *Lc. lactis* MG1363 (*open squares*), *Lc. lactis* NZ9000 (*open circles*), *Lc. lactis* NZ9000 pNZ7011 (*filled squares*), *Lc. lactis* NZ9000 pNZGBAH (*filled circles*), or *Lc. lactis* NZ9000 pVE5571 (*filled triangles*). The animals from the control group (*crosses*) did not receive microbial supplementation

significant differences were observed in food or water intake of all animals regardless of the experimental group nor were any changes in animal behavior or in the general aspects (hair color or thickness, eye coloration, etc.) of the animals observed (data not shown). The feeding period in this trial is quite long considering that 30 days in the life of a mouse (with an average life expectancy of 2 years) is equivalent to 3 years of consumption of a human being (using an average life expectancy of 75 years).

No significant differences were observed in the relative weights of liver or spleens of the animals fed the plasmid carrying strains compared to those receiving the native strains or to those of the control group (Fig. 2).

Neither microbial translocation to extra-gut organs nor changes in the morphology of the small and large intestines were observed in any of the animal groups, showing that the genetic modifications or the liberation of the products of gene expression did not cause a microbial imbalance in the gut (data not shown).

When blood smears and blood samples were analyzed (Table 2), no significant differences were observed in the animals in the groups that received the GM-LAB compared to those of the control groups or those that received *Lc. lactis* MG 1363 or its derivative NZ9000.

No adverse effects were observed on the general health, growth rates, hematological parameters, or other physiological parameters of the animals that received the GM-LAB. These strains did not cause any type of immediate pathology or infection and did not translate (or cause microbial translocation) to visceral organs from their initial contact site with the host (gastrointestinal tract). No significant differences were observed between the sexes of the assayed animals except for growth rates.

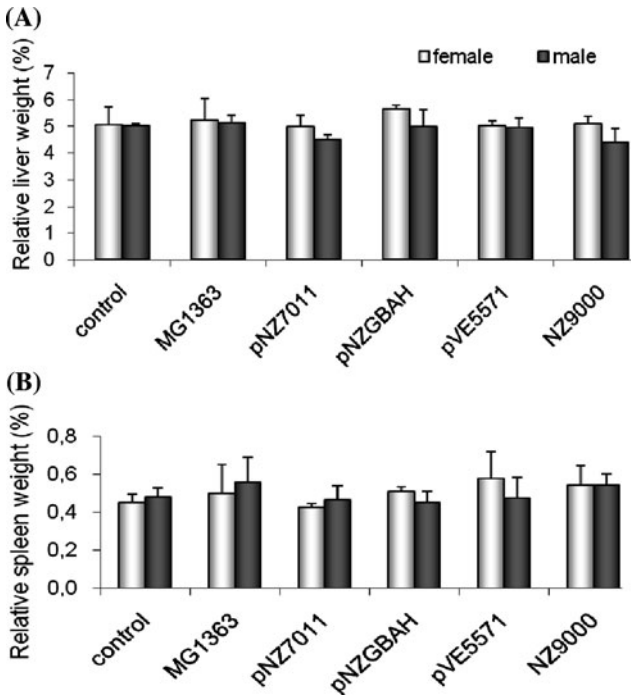

Fig. 2 Relative weight of liver (**a**) and spleen (**b**) of animals that received 1.0×10^9 CFU/day of *Lc. lactis* MG1363 (MG1363), *Lc. lactis* NZ9000 (NZ9000) *Lc. lactis* NZ9000 pNZ7011 (pNZ7011), *Lc. lactis* NZ9000 pNZGBAH (pNZGBAH) or *Lc. lactis* NZ9000 pVE5571 (pVE5571). The animals from the control group (Control) did not receive microbial supplementation

Table 2 Hematology values of female and male mice that received 1.0×10^9 CFU/day of *Lc. lactis* MG1363 (MG1363), *Lc. lactis* NZ9000 (NZ9000) *Lc. lactis* NZ9000 pNZ7011 (pNZ7011), *Lc. lactis* NZ9000 pNZGBAH (pNZGBAH) or *Lc. lactis* NZ9000 pVE5571 (pVE5571)

	Control	NZ9000	MG1363	pNZ7011	pNZGBAH	pVE5571
Female						
Hematology						
RBCS ($\times 10^6$/mm^3)	6.5 ± 0.2	6.7 ± 0.3	6.5 ± 0.1	6.7 ± 0.1	6.7 ± 0.3	6.7 ± 0.3
Hemoglobin (g/dl)	20.3 ± 0.5	20.0 ± 1.0	19.9 ± 0.1	20.9 ± 0.1	20.7 ± 0.5	21.0 ± 1.0
Hematocrit (%)	60 ± 2	61 ± 3	59 ± 1	61 ± 1	61 ± 2	61 ± 3
WBCS ($\times 10^3$/mm^3)	4.6 ± 0.5	4.9 ± 0.5	5.0 ± 1.0	5.0 ± 2.0	4.4 ± 0.5	5.0 ± 2.0
Differential (%)						
Neutrophils	13 ± 4	14 ± 5	12 ± 8	15 ± 6	15 ± 4	17 ± 7
Lymphocytes	86 ± 4	85 ± 6	87 ± 8	84 ± 6	84 ± 4	82 ± 7
Monocytes	0.3 ± 0.6	0.4 ± 0.6	0.7 ± 0.6	0.7 ± 0.6	1 ± 1	0.7 ± 0.6
Eosinophils	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Basophils	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
MCV (fl)	91.4 ± 0.8	90.9 ± 0.1	90.9 ± 0.0	90.9 ± 0.0	90.7 ± 0.3	90.9 ± 0.0
MCHC (%)	34.0 ± 0.1	34.0 ± 0.2	33.7 ± 0.5	34.4 ± 0.5	34.1 ± 0.1	34.1 ± 0.1
MCH (pg)	31.1 ± 0.4	30.9 ± 0.4	30.7 ± 0.5	31.2 ± 0.5	30.9 ± 0.1	31.0 ± 0.1
Male						
Hematology						
RBCS ($\times 10^6$/mm^3)	6.0 ± 0.1	6.0 ± 0.2	5.3 ± 0.5	6.2 ± 0.2	6.0 ± 0.6	5.8 ± 0.7
Hemoglobin (g/dl)	17.6 ± 0.8	18.6 ± 0.7	16.0 ± 2.0	18.8 ± 0.7	18.0 ± 2.0	18.0 ± 2.0
Hematocrit (%)	53 ± 3	55 ± 2	48 ± 4	57 ± 2	55 ± 6	53 ± 6
WBCS ($\times 10^3$/mm^3)	6.0 ± 1.0	5.0 ± 1.0	5.7 ± 0.5	5.7 ± 0.7	5.5 ± 0.1	5.8 ± 0.4
Differential (%)						
Neutrophils	20 ± 8	16 ± 5	12 ± 3	13 ± 3	19 ± 6	11 ± 2
Lymphocytes	75 ± 8	83 ± 5	87 ± 3	86 ± 3	79 ± 2	89 ± 2
Monocytes	1.3 ± 0.6	0.7 ± 0.6	0.3 ± 0.6	0.7 ± 0.6	1.0 ± 1.0	0.3 ± 0.6
Eosinophils	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Basophils	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
MCV (fl)	88.0 ± 5.0	91.4 ± 0.9	90.9 ± 0.1	91.9 ± 0.8	90.9 ± 0.1	90.9 ± 0.1
MCHC (%)	33.4 ± 0.3	34.0 ± 0.1	33.5 ± 0.7	33.2 ± 0.4	33.6 ± 0.1	34.0 ± 0.1
MCH (pg)	29.4 ± 1.4	31.1 ± 0.3	30.4 ± 0.7	30.5 ± 0.4	30.6 ± 0.1	30.9 ± 0.1

The animals from the control group (Control) did not receive microbial supplementation

Statistics are not shown since no significant differences were observed between the means of a similar row

These results provide evidence that the nutraceutical-producing GM-LAB used in this study are just as safe as the native strains from which they were derived and thus merit further studies to include them into the food chain. It should be stated here that the folate, riboflavin, and α-galactosidase-overproducing strains were developed as proofs of principle, and in the current state contain plasmids with chloramphenicol (an antibiotic) resistant markers. These strains could therefore only be applied in food fermentations after the substitution of the antibiotic resistance marker with a food-grade marker. The presence of antibiotic resistance determinants would not exclude their safe use for production purposes provided that only the fermentation products are retained in the final product.

This study therefore advances our knowledge on the safety of GM-LAB that are intended for consumption as functional foods.

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