J. Dairy Sci. 88:3435–3442

Ingestion of Milk Fermented by Genetically Modified *Lactococcus lactis* Improves the Riboflavin Status of Deficient Rats

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ABSTRACT

Riboflavin deficiency is common in many parts of the world, particularly in developing countries. The use of riboflavin-producing strains in the production of dairy products such as fermented milks, yogurts, and cheeses is feasible and economically attractive because it would decrease the costs involved during conventional vitamin fortification and satisfy consumer demands for healthier foods. The present study was conducted to assess in a rat bioassay the response of administration of milk fermented by modified *Lactococcus lactis* on the riboflavin status of deficient rats. Rats were fed a riboflavin-deficient diet during 21 d after which this same diet was supplemented with milk fermented by *Lactoccus lactis* pNZGBAH, a strain that overproduces riboflavin during fermentation. The novel fermented product, with increased levels of riboflavin, was able to eliminate most physiological manifestations of ariboflavinosis, such as stunted growth, elevated erythrocyte glutathione reductase activation coefficient values and hepatomegaly, that were observed using a riboflavin depletion-repletion model, whereas a product fermented with a nonriboflavin-producing strain did not show similar results. A safety assessment of this modified strain was performed by feeding rodents with the modified strain daily for 4 wk. This strain caused no detectable secondary effects. These results pave the way for analyzing the effect of similar riboflavin-overproducing lactic acid bacteria in human trials. The regular consumption of products with increased levels of riboflavin could help prevent deficiencies of this essential vitamin.
(**Key words:** riboflavin, lactic acid bacteria, fermented milk, genetically modified microorganism)

Abbreviation key: EGRAC = erythrocyte glutathione reductase activation coefficient, **FAD** = flavin adenine dinucleotide.

Received March 9, 2005.
Accepted June 7, 2005.
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INTRODUCTION

Riboflavin (vitamin B_2) is a water-soluble vitamin belonging to the B-complex group that is important for optimal body growth and red blood cell production and helps in releasing energy from carbohydrates and fatty acids. In the body, riboflavin is primarily found as an integral component of the coenzymes (**FAD**) and flavin mononucleotide. These flavin-containing coenzymes participate in redox reactions in numerous metabolic pathways such as the metabolism of carbohydrates, fats, and proteins. They are also involved in the metabolism of folate, cobalamin, vitamin B6, and other vitamins, explaining why plasma riboflavin is a determinant of plasma homocysteine levels, which influence the risk of cardiovascular disease, pregnancy complications, and cognitive impairment (Hustad et al., 2002).

Although riboflavin is found in a wide variety of foods (i.e., lean meats, poultry, fish, grains, broccoli, turnip greens, asparagus, spinach, and enriched products), vitamin B_2 deficiency is common in many parts of the world, not only in developing countries (Boisvert et al., 1993), but also in industrialized countries, in the elderly (Bailey et al., 1997; Madigan et al., 1998), and in young adults (Benton et al., 1997).

Vitamin B_2 status in humans has usually been assessed by measuring the erythrocyte glutathione reductase activation coefficient (**EGRAC**), which is the ratio between glutathione reductase activity determined with and without the addition of the cofactor, FAD (Glatzle et al., 1970). Glutathione reductase loses FAD at an early stage in vitamin B_2 deficiency, making EGRAC a useful method for the diagnosis of vitamin B_2 deficiency (Bates, 1993).

Riboflavin-deficient rat models have been used to study the biological effects of riboflavin. Using these models, it has been shown that riboflavin (i) is important in the early postnatal development of the brain (Ogunleye and Odutuga, 1989) and gastrointestinal tract (Williams et al., 1996; Yates et al., 2003), (ii) is able to modulate carcinogen-induced DNA damage (Pangrekar et al., 1993; Webster et al., 1996), (iii) plays a role in iron absorption and use (Butler and Topham,

1993; Powers et al., 1993), and (iv) can modulate inflammatory responses (Lakshmi et al., 1991). These models also allow the extrapolation of data to human clinical data (Greene et al., 1990).

Previously, we described the genetic analysis of the riboflavin biosynthetic (*rib*) operon in the lactic acid bacterium *Lactococcus lactis* ssp. *cremoris* strain NZ9000 (Burgess et al., 2004). This strain can be converted from a vitamin B_2 consumer into a vitamin B_2 "factory" by over-expressing its riboflavin biosynthesis genes. Substantial riboflavin overproduction is seen in the growth medium when all 4 biosynthetic genes (*ribG*, *ribH*, *rib*, and *ribA*) are overexpressed simultaneously (in *L. lactis* NZ9000 containing pNZGBAH). Spontaneous mutants (i.e., *L. lactis* strain CB010) capable of producing riboflavin in the growth medium, albeit at a lower level than the engineered strain, were also identified. Such spontaneously riboflavin-overproducing strains have a considerable advantage over the genetically engineered strain as they can be promptly implemented in industrial fermentation. We have previously shown that the bioavailability of the riboflavin produced by these strains is similar to pure commercial riboflavin (LeBlanc et al., accepted).

The main objective of this study was to demonstrate that milk fermented by a riboflavin-producing strain of *L. lactis* could be used to improve the riboflavin status of deficient rats, eliminating the need for costly fortification of this essential vitamin.

MATERIALS AND METHODS

Strains and Culture Conditions

Lactococcus lactis NZ9000 (*L. lactis* B_2–) was grown (12 h at 30°C) in M17 medium (Biokar Diagnostics, Beauvais, France) supplemented with 0.5% glucose (M17-Glu). *Lactococcus lactis* NZ9000 harboring plasmid pNZGBAH (*L. lactis* B_2++; Burgess et al., 2004), which expressed all 4 riboflavin biosynthesis genes using the nisin-induced expression system (de Ruyter et al., 1997), was grown at 30°C in M17-Glu supplemented with 5 μg/mL chloramphenicol. Nisin was added (1 ng/mL) after 4 h of growth when required. Before inoculation, strains were washed twice and resuspended in sterile peptone water (1:1, vol/vol).

Lactose utilization ability was introduced in *L. lactis* NZ9000 and NZ9000 (pNZGBAH) by means of transformation (de Vos et al., 1989) with the lactose miniplasmid pMG820 (Maeda and Gasson, 1986); selection was carried out on lactose indicator agar (McKay et al., 1972) supplemented with chloramphenicol when appropriate.

Fermented Milk Preparation

Commercially dried, low-fat, low-riboflavin milk (Bago, Buenos Aires, Argentina) was rehydrated as suggested by the manufacturer (13.5% wt/vol). The reconstituted milk was subjected to thermal treatment at 100°C for 5 min, cooled to 4°C in an iced bath, poured into sterile 500-mL Erlenmeyer flasks, and stored for 24 h before use. Before inoculation, the milk was supplemented with chloramphenicol (5 μg/mL). The flasks were then inoculated (2% vol/vol) with *L. lactis* NZ9000 pNZGBAH + pMG820 (*L. lactis* B_2++) and incubated for 16 h at 30°C (fermented milk B_2++). As a negative control, the same protocol was used and milk was fermented with *L. lactis* NZ9000 pMG820, a riboflavin-consuming strain (fermented milk B_2–). All inoculated milks were incubated statically at 30°C for 16 h. After 4 h incubation, nisin was added (1 ng/mL) to induce riboflavin production in *L. lactis* NZ9000 pNZGBAH.

Quantification of Riboflavin in Culture Medium

Extracellular riboflavin concentrations of *L. lactis* cultures were measured by reverse phase HPLC using a modification of a previously described technique (Capo-Chichi et al., 2000). Briefly, proteins were precipitated from a 1-mL sample by adding 10% TCA. The HPLC analysis (Isco model 2360, Lincoln, NE) of the resulting supernatant was performed using a C_{18} reverse-phase column (4 × 150 mm Microsorb MV; Varian, Inc., Palo Alto, CA) with a linear gradient of acetonitrile from 3.6 to 30% at pH 3.2 (HPLC-grade water containing 0.1% acetic acid). Fluorescent detection was used and the excitation and emission wavelengths were 445 and 530 nm, respectively, using a Gilson fluorescence detector (Middletone, WI). Commercially obtained riboflavin, flavin mononucleotide, and FAD were used as references and to obtain a standard curve (Sigma-Aldrich, St. Louis, MO).

Experimental Design

The overall experimental protocol is summarized in Figure 1. Eighty weanling specific-pathogen-free conventional Wistar rats (weighing 60 ± 3 g) were obtained from the inbred colony maintained (12-h light cycle, 22 ± 2°C) in the Nutrition Department of the Universidad Nacional de Tucumán (San Miguel de Tucumán, Argentina). Rats were individually housed in wire-based cages (to prevent coprophagy) and were allowed free access to a riboflavin-deficient diet (Riboflavin Deficient Diet, MP Biomedicals Inc. (ICN), Irvine, CA) and water throughout the study.

The rats were weight-matched into 3 main groups of animals: i) depleted group where animals were fed a

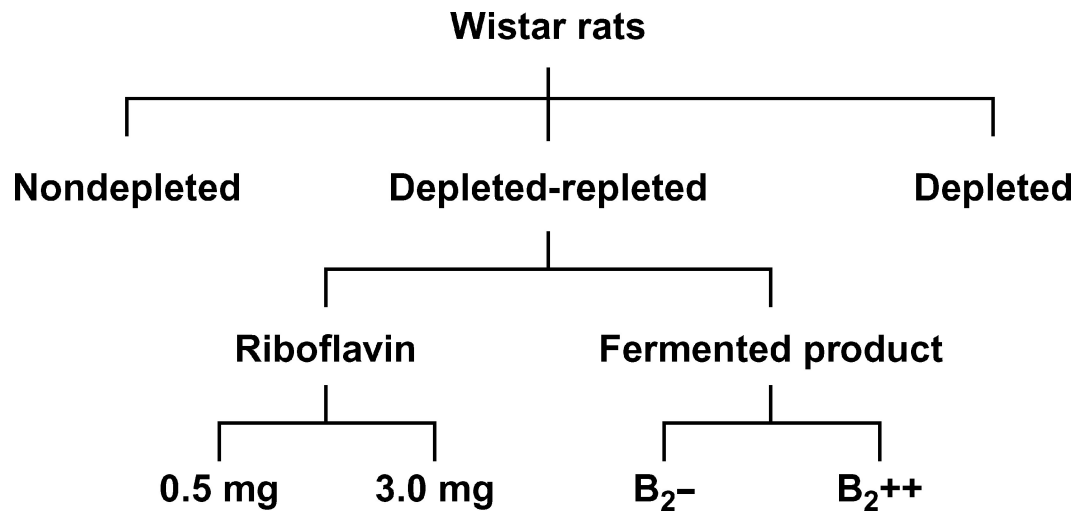

Figure 1. Riboflavin depletion-repletion experimental protocol. The depleted group was fed a riboflavin-deficient diet (RDD) for 42 d; the nondepleted group received RDD supplemented with commercial riboflavin for 42 d; and the depleted-repleted group was fed RDD for 21 d (depletion period) followed by a 21-d repletion period, during which animals were fed the same diet supplemented with different levels of commercial riboflavin, or one of the fermented milk products (*L. lactis* B_2– and *L. lactis* B_2++, respectively).

riboflavin-deficient diet for 42 d; ii) nondepleted group where animals received riboflavin-deficient diet supplemented with commercial riboflavin (15 mg of B_2/kg, Sigma-Aldrich) for 42 d; and iii) depleted-repleted group where rats were fed riboflavin-deficient diet for 21 d (depletion period) followed by a 21-d repletion period where animals were fed the same diet supplemented with either different levels of commercial riboflavin, or one of the fermented products (fermented milk B_2- or fermented milk B_2++). Each subgroup contained a minimum of 10 animals. Commercial riboflavin was added at concentrations equivalent to: i) the residual riboflavin found in B_2-free diets used in previous deficiency studies (0.5 mg of B_2/kg of diet; Powers et al., 1993; Yates et al., 2003), and ii) the daily riboflavin requirement of laboratory rats (3.0 mg of B_2/kg of diet; ILAR, 1995). In the second depleted-repleted group, rats were fed 20 mL of either fermented product twice daily during the repletion period in place of their drinking water which contained 0.0 ± 0.1 or 13.0 ± 4.0 mg of riboflavin/L for *L. lactis* B_2– and B_2++, respectively (determined by HPLC). Animal live weight and food intake (given ad libitum) were determined twice daily. Growth rates were calculated during the repletion period (21 d) using the mean average twice daily increase and were expressed as changes in live animal weight (g) per day.

Blood and Organ Collection

Throughout the trial, rats from each group were placed into a homemade sampling chamber; whole blood was collected from the tail, and transferred into a tube containing anticoagulant for EGRAC evaluation (see below). At the end of the trial, animals were anesthetized with an i.p. injection of (3.0 mL/kg of BW) ketamin (10%)-xylacin (2%) (40:60 vol/vol; Alfasan, Woerden, The Netherlands) and bled by cardiac puncture. Blood was transferred into tubes containing heparin (Rivero, Buenos Aires, Argentina) and centrifuged ($2000 \times g$ for 15 min at 4°C). Plasma was removed and stored at –70°C until analysis. The sedimented cells were washed 3 times with cold 0.15 *M* NaCl. Erythrocytes (0.5 mL) were hemolysed by adding distilled water (9.5 mL), and stored at –70°C for EGRAC determinations. Freshly excised organs (liver, spleen, and kidneys) were rinsed with 0.15 *M* NaCl, weighed, and stored at –70°C.

Riboflavin Status

Riboflavin status was assessed by measuring EGRAC using a modification of a previously described technique (Adelekan and Thurnham, 1986). Briefly, frozen hemolysed blood was allowed to thaw at room temperature under conditions of reduced light. Hemolysates (31.3 μL) were added to 1 mL of potassium phosphate buffer (0.1 *M*, pH 7.4) containing 2.3 m*M* EDTA (dipotassium salt) and 0.89 m*M* oxidized glutathione with or without 8 μ*M* FAD. The mixture was preincubated for 30 min at 37°C followed by the addition of 80 μ*M* NADPH to initiate the reaction. The absorbance at 340 nm was measured every 10 min for 1 h at 37°C (Cecil CE 2021 spectrophotometer). Riboflavin status was calculated as the ratio (activity coefficient) of the rate of change

of absorbance per time unit in the presence or absence of FAD. The EGRAC ratio was measured in triplicate for each sample.

Safety Assessment of *L. lactis* NZ9000 pNZGBAH

The general safety of *L. lactis* pNZGBAH was investigated in feeding trials where animals received 5×10^{10} cfu/kg of BW per d for 4 wk (concentrated in peptone water; sterile peptone water in the control group) as described previously (Zhou et al., 2000). Throughout this time, feed intake, water intake, and live BW were monitored. At the end of the 4-wk observation period, samples of blood, liver, and spleen were collected to determine: i) hematological parameters (red and white blood cell counts, differential leukocyte counts, hematocrit, and hemoglobin concentration, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration), ii) microbial translocation to extragut tissues (liver and spleen) as previously described (LeBlanc et al., 2004), and iii) relative organ weight (liver and spleen).

Statistical Analyses

Comparisons were performed using the software package SigmaStat (SPSS, Chicago, IL). Comparisons of multiple means were accomplished by 1-way ANOVA followed by a Tukey's posthoc test, and $P < 0.05$ was considered significant. Unless otherwise indicated, all values were the means of 3 independent trials ± standard deviation (SD) where n = 30 (each assay was performed in triplicate on a minimum of 10 animals).

All animal protocols were approved by the Animal Protection Committee of CERELA and followed the latest recommendations of Federation of European Laboratory Animal Science Associations (FELASA). All experiments comply with the current laws of Argentina.

RESULTS AND DISCUSSION

To study the effect of milk fermented with *L. lactis* on the riboflavin status of rats, a depletion-repletion rat bioassay described previously (LeBlanc et al., accepted) was used. Conventional Wistar rats were fed a riboflavin-deficient diet and their riboflavin status was followed using growth rate and EGRAC as indicators. The bioavailability of the riboflavin produced by the bacterial strains during the fermentation process was compared with that of pure riboflavin given to rats at levels previously considered negligible (0.5 mg of B_2/kg of diet) or at the recommended daily intake for such animals (3.0 mg of B_2/kg of diet).

Animal Growth During Depletion-Repletion Periods

It is well documented that rats that are deprived of riboflavin exhibit impaired growth (Glatzle et al., 1968). Animal growth was followed throughout the trials. At the end of the depletion and repletion periods, a significant decrease was observed in the growth rate and final weight of the riboflavin depleted rats compared with the nondepleted group (Table 1).

Fermented milk B_2++ greatly improved the growth rate and final animal weight of the riboflavin-depleted animals (Table 1). The animals supplemented with fermented milk B_2– showed significantly lower growth rates (2.78 ± 0.17) compared with those fed fermented milk B_2++, 3.0 mg of B_2/kg, or 0.5 mg of B_2/kg (4.31 ± 0.25, 3.61 ± 0.22, and 3.19 ± 0.16, respectively), suggesting that the riboflavin-enriched product can exert a positive biological function. The rats fed fermented milk B_2– showed higher growth rates than the depleted animals that only received the riboflavin-deficient diet (0.68 ± 0.04). This difference could be due to other nutrients found in the milk after bacterial growth besides riboflavin because this vitamin was not detected after fermentation with *L. lactis* B_2– (HPLC determination). Interestingly, the animals that received fermented milk B_2++ showed a significantly higher growth rate (4.31 ± 0.25) and final weight (214.0 ± 10.2) than all the other depleted-replete groups (Table 1), an expected result because the riboflavin concentration in this fermented product (13.0 ± 4.0 mg/L) was the highest used in the depleted-replete animals. The food consumption during the depletion and repletion periods did not differ significantly between the different experimental groups (data not shown).

Riboflavin Status (EGRAC)

Activation assays such as EGRAC are functional tests that show a decline in a specific enzyme activity as a result of a co-factor deficiency (riboflavin in the case of EGRAC), and a disproportionate increase in activity after the in vitro addition of this co-factor (Adelekan and Thurnham, 1986). The rate of change of the assay is proportional to the amount of enzyme present—EGRAC values of 1.30 or higher are indicative of biochemical riboflavin deficiency. Riboflavin status, expressed in terms of the activation coefficient for the FAD-dependent enzyme, erythrocyte glutathione reductase (EC 1.6.4.2), was determined throughout the study.

To determine if the novel fermented product inoculated with riboflavin-producing *L. lactis* could improve the riboflavin status of deficient rats, 2 different fermented milks were used to supplement the riboflavin-deficient diet for 21 d (repletion period) of previously

Table 1. Growth rate and live weight of animals fed a riboflavin-deficient diet (RDD) for 21 d (depletion period) after which they received the same diet supplemented with commercial riboflavin or the fermented products (fermented milk B_2++ or B_2–) for 21 d (repletion period). The depleted group received only RDD for 42 d.[1]

	Depletion period		Repletion period	
Group	Growth rate, g/d	Final weight, g	Growth rate, g/d	Final weight, g
Nondepleted	5.81 ± 0.35[a]	138.0 ± 8.3[a]	5.34 ± 0.32[a]	299.2 ± 18.0[a]
Depleted	4.08 ± 0.24[b]	102.2 ± 6.1[b]	0.68 ± 0.04[b]	141.1 ± 8.5[b]
0.5 mg B_2			3.19 ± 0.16[c]	190.0 ± 9.5[c]
3.0 mg B_2			3.61 ± 0.22[d]	204.2 ± 12.5[cd]
Fermented milk B_2–			2.78 ± 0.17[e]	188.9 ± 11.3[c]
Fermented milk B_2++			4.31 ± 0.25[f]	214.0 ± 10.2[cd]

[a,b,c,d,e,f]Means in a column without a common letter differ, $P < 0.05$.

[1]Values are means ± SD; n = 60 for the depleted group during the depletion period and n = 10 for all groups during the repletion period and for the nondepleted group during the depletion period.

depleted animals. Analysis using HPLC showed significant levels of riboflavin in fermented milk B_2++ following growth of *L. lactis* B_2++ (13 ± 4 mg/L), whereas this vitamin was below the detection level in fermented milk B_2– after growth of the nonproducing strain (*L. lactis* B_2–).

The depleted rats showed increased EGRAC values (2.41 ± 0.06) compared with the nondepleted animals (1.18 ± 0.04) after the study period (Figure 2). The rats whose diet was supplemented with milk fermented by the nonproducing strain (*L. lactis* B_2–) showed statistically similar EGRAC values as those found in the depleted animals (2.29 ± 0.08). This result confirms that the increase in growth observed in the animals supplemented with fermented milk B_2– was not caused by riboflavin but by other residual nutrients found in the fermented product. The rats whose diet was supplemented with fermented milk B_2++ exhibited significantly lower EGRAC values (1.59 ± 0.07) compared with rats of the depleted group (2.41 ± 0.06) or rats whose diet was supplemented with fermented milk B_2– (Figure 2). Interestingly, the animals that received fermented milk B_2++ showed statistically similar EGRAC values to the group that received 3.0 mg of B_2/kg, suggesting that this fermented product is capable of conferring all the necessary riboflavin needed to meet the daily requirements for the rodents in this study, as was observed for animal growth (Table 1). Surprisingly, no statistically significant differences in EGRAC values were observed between the animals that received 0.5 mg of B_2/kg and those receiving 3.0 mg of B_2/kg; however, mean values were lower in the group receiving the higher dose. A longer repletion period in future studies could improve the sensitivity of the differences.

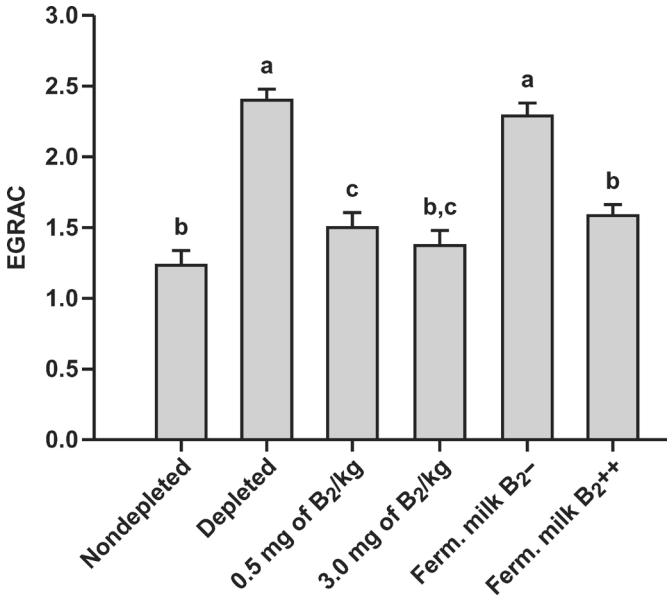

Figure 2. Erythrocyte glutathione reductase activation coefficient values of rats fed a riboflavin-deficient diet for 21 d followed by a 21-d repletion period, in which the diet was supplemented with different amounts of riboflavin (0, 0.5, or 3.0 mg/kg of diet) or one of the fermented products (fermented milk B_2++ or B_2–). Results are expressed as means ± standard deviation (n = 10). [a,b,c]Bars without a common letter differ significantly ($P < 0.05$).

Organ Weight Comparison

Another physiological effect of ariboflavinosis in rats is hepatomegaly, which is the enlargement of the liver beyond its normal size due to excessive lipid accumulation (steatosis) caused by decreased fatty acid oxidation (Glatzle et al., 1968).

An increase in the weight of the liver in relation to BW was observed in the depletion groups in which riboflavin deficiency was observed (Figure 3).

The group supplemented with the fermented milk B_2– showed a significant increase in relative liver weight (5.2 ± 0.4) compared with the nondepleted group (4.4 ± 0.2), and were statistically similar to the depletion

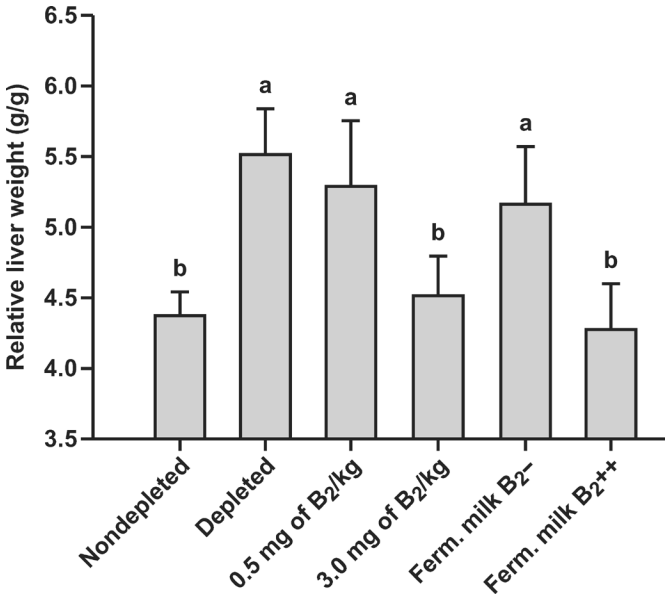

Figure 3. Relative weight of liver of animals fed a riboflavin-deficient diet for 21 d followed by a 21-d repletion period, in which the diet was supplemented with different amounts of riboflavin (0, 0.5, or 3.0 mg/kg of diet) or one of the fermented products (fermented milk B_2++ or B_2–). Results are expressed as means ± standard deviation (n = 10). [a,b]Bars without a common letter differ significantly ($P < 0.05$).

groups (5.2 ± 0.4) and the group that received 0.5 mg of B_2/kg (5.3 ± 0.5). The groups supplemented with milk fermented by *L. lactis* B_2++ showed no significant differences in relative liver weight compared with the nondepleted group or the group that received 3.0 mg of B_2/kg. These results suggest that the fermented product manufactured using the riboflavin-producing strain is able to decrease the relative liver weight increases observed in the depleted animals.

No changes in hematological values or morphology of blood cells were observed in these trials (data not shown). This was an expected finding because it was previously shown that riboflavin deficiency alone is not sufficient to change the hematological status of rats (Adelekan and Thurnham, 1986). There were no differences in relative spleen and kidney weights in all experimental groups (data not shown).

Safety Assessment of *L. lactis* NZ9000 pNZGBAH

Because a genetically modified strain of *L. lactis* was used in the preparation of the fermented milk product used as a source for riboflavin intake, a complete safety assessment was performed to prove that the product was innocuous to the host/consumer. Feeding rodents with *L. lactis* NZ9000 pNZGBAH at a dose of 5×10^{10} cfu/kg BW per day for 4 wk had no adverse effects on general health status, growth, hematology, and other physiological parameters examined in this study (Table 2). The strain did not cause infection and did not translocate (or cause microbial translocation) from the original colonization site (gut) after feeding for 4 wk (Table 2). Therefore, the oral LD_{50} (the dose predicted to cause 50% mortality) for *L. lactis* NZ9000 pNZGBAH would be greater than 20 g/kg per d; that is, 1.4 kg of dry bacteria/d for a 70-kg person assuming that 1 g of dry bacterial preparation contains 10^{11} bacterial cells (Zhou et al., 2000). The acceptable daily intake for this individual would be 14 g of dry bacteria/d (100 times the LD_{50}), which is several hundred times the amount of lactic acid bacteria normally recommended for human consumption (Donohue et al., 1998). From this it can be inferred that *L. lactis* NZ9000 pNZGBAH is nonpathogenic and safe for human consumption.

CONCLUSIONS

The objective of this study was to demonstrate that milk fermented by a riboflavin-producing strain of *L. lactis* could be used to improve the riboflavin status of deficient rats, eliminating the need of costly fortification of this essential vitamin.

The addition of a novel fermented product inoculated with a riboflavin-producing strain of *L. lactis* (*L. lactis* B_2++) was shown to clearly improve growth (Table 1) and riboflavin status of the depleted animals as shown by statistically significant decreases to EGRAC values (which reached levels similar to those seen in the nondepleted group; Figure 2). Moreover, this fermented product was capable of reverting hepatomegaly resulting from ariboflavinosis (Figure 3).

The safety of novel strains must be addressed when they are proposed for introduction into the food chain. In this study, no secondary effects were observed in animals fed the genetically modified *L. lactis* strain; hematological values, morphology of blood cells, and relative weight of organs of these animals were all similar to those of animals in the nondepleted groups. Only positive results were observed with the use of this strain, including improved animal growth, EGRAC values, and relative organ weight. Current legislations in most countries do not allow the addition of live genetically modified strains to food products for human consumption, strongly limiting the use of the strain used in this study. However, the use of spontaneous mutants, such as the one described in previous studies could be included in novel products in a relatively short timeframe (European Council Directive 90/220/EEC, 1990). However, because the latter strain is not able to use lactose as a carbon source, another carbohydrate (such as glucose) would have to be added. A solution to this

Table 2. Safety assessment in which animals were fed 5×10^{10} cfu *Lactococcus lactis* NZ9000 pNZGBAH/kg of BW per d for 4 wk (*L. lactis*) compared with animals that did not receive this bacterial supplementation (control). Results are expressed as means ± standard deviation (n = 10).

	Control	*L. lactis*
Weekly weight gain (g)	4.13 ± 0.38	4.10 ± 0.43
Relative organ weight		
Liver (g/g × 100)	5.02 ± 0.09	5.15 ± 0.28
Spleen (g/g × 100)	0.48 ± 0.05	0.52 ± 0.09
Hematology		
Red blood cell count ($\times 10^6$/mm^3)	6.53 ± 0.22	6.67 ± 0.34
White blood cell count ($\times 10^3$/mm3)	4.57 ± 0.49	4.90 ± 0.51
Neutrophils, %	13.67 ± 4.04	14.33 ± 5.03
Eosinophils, %	0.00 ± 0.00	0.00 ± 0.00
Basophils, %	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes, %	86.00 ± 4.36	85.33 ± 5.51
Monocytes, %	0.33 ± 0.58	0.35 ± 0.55
Hematocrit, %	59.67 ± 1.53	60.67 ± 3.06
Hemoglobin, g/dL	20.30 ± 0.46	20.63 ± 1.04
Mean corpuscular volume, fL	91.39 ± 0.84	90.91 ± 0.09
Mean corpuscular hemoglobin, pg	31.10 ± 0.38	30.92 ± 0.14
Mean corpuscular hemoglobin concentration, g/100 mL	34.02 ± 0.10	34.01 ± 0.16
Translocation[1]		
Liver	0/20	0/20
Spleen	0/20	0/20

[1]Animals with any tissue(s) where viable bacterial cell(s) were recovered were defined as microbial translocation positive animals (MT+). The numbers included in this table are MT+ animals/total examined animals.

problem would be the insertion of plasmid pMG820 into the strain, allowing it to use lactose, as was done in this study, or the isolation of spontaneous mutants from strains that can grow in milk.

This study has provided the first animal trial with milk fermented by *L. lactis* engineered to produce extracellular riboflavin in a novel product. These results prepare the way for examining the effect of similar riboflavin-overproducing lactic acid bacteria in human trials. Because fermentation with *L. lactis* is a common practice in the dairy industry, the addition of the riboflavin-producing strain into products such as fermented milks, yogurt, and cheeses to increase riboflavin concentrations is feasible and economically attractive. The regular consumption of such products with increased levels of riboflavin could help prevent deficiencies of this important vitamin. Such products could decrease the costs incurred when mandatory fortification programs are elaborated, such as those now in place in many industrialized countries and could be used to satisfy consumer demands for healthier functional foods.

The present study is one of many currently being addressed by the European NutraCells consortium (www.nutracells.com; Hugenholtz et al., 2002). The achievements of this multinational project should open the door to many applications in the development of new food products with enhanced nutritional value and probiotic preparations with well-demonstrated in vivo activities.

The present study clearly showed that milk fermented by a genetically modified riboflavin-producing *Lactococcus lactis* strain was as effective as addition of commercial riboflavin using an animal model. The manufacture of a product of this nature would decrease the costs compared with current vitamin fortification programs and could be used as a tool against malnutrition in developing countries. The final use of such genetically modified bacteria may rely on the acceptability of genetically modified organisms in nutrition and nutraceuticals preparations. Undoubtedly, consumers will play a major part in this decision and their position should be greatly influenced by the scientifically proven health benefits that can be gained by consumption of genetically modified organisms.

ACKNOWLEDGMENTS

This work has been funded by the European Union project QLK1-CT-2000-01376 (www.nutracells.com), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia de Promoción Científica y Tecnológica and CIUNT (Argentina). The authors would like to thank Analia Rossi and Silvia Burke for their help with the care of the animals and sampling. The authors would also like to thank Oscar Peinado Reviglione for his technical assistance in the HPLC analyses.

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