ELSEVIER

Nutrition 22 (2006) 645–651

NUTRITION

www.elsevier.com/locate/nut

Basic nutritional investigation

A novel dairy product fermented with *Propionibacterium freudenreichii* improves the riboflavin status of deficient rats

Jean Guy LeBlanc, M.Sc.,[a,*] Ger Rutten[b], Paul Bruinenberg, Ph.D.,[c] Fernando Sesma, Ph.D.,[a] Graciela Savoy de Giori, Ph.D.,[a,d] and Eddy J. Smid, Ph.D.[b]

[a] *Centro de Referencia para Lactobacilos, Consejo Nacional de Investigaciones Científicas y Técnicas (CERELA-CONICET), Tucumán, Argentina*
[b] *NIZO Food Research, Ede, The Netherlands*
[c] *Campina Innovation, Wageningen, The Netherlands*
[d] *Cátedra de Microbiología Superior, Universidad Nacional de Tucumán, Tucumán, Argentina*

Manuscript received September 27, 2005; accepted January 4, 2006.

Abstract

Objective: 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 milk, yogurt, and cheese 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 in a rat bioassay assessed the response of administration of yogurt containing a riboflavin-producing strain of *Propionibacterium freudenreichii* on the riboflavin status of deficient rats.

Methods: *Propionibacterium freudenreichii* NIZO B2336 is a spontaneous roseoflavin-resistant mutant derived from *P. freudenreichii* B374 that produces larger amounts of riboflavin than the parental stain. Rats were fed a riboflavin-deficient diet for 21 d (depletion period), after which this same diet was supplemented with conventional yogurt, yogurt containing the riboflavin-producing strain (B2336), or the parental non-producing strain (B374) and fed to animals for 28 d (repletion period). As controls, rats were fed the same diet with different concentrations of commercial riboflavin.

Results: The novel fermented product containing *P. freudenreichii* B2336, with increased levels of riboflavin, eliminated most physiologic manifestations of ariboflavinosis such as stunted growth, high erythrocyte glutathione reductase activation coefficient values, and hepatomegaly that were observed when using a riboflavin depletion-repletion model, whereas the product fermented with the non–riboflavin-producing strain did not show this beneficial effect.

Conclusions: Consumption of such products with increased levels of riboflavin on a regular basis may help prevent deficiencies of this essential vitamin.

Keywords: Riboflavin; *Propionibacterium freudenreichii*; Yogurt; Lactic acid bacteria; Fermented milk

Introduction

Riboflavin (vitamin B2) is a water-soluble vitamin belonging to the B-complex group that is important for optimal body growth, red blood cell production, and release of energy from carbohydrates and fatty acids. In the body, riboflavin is found primarily as an integral component of the coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide. These flavin-containing coenzymes participate in redox reactions in numerous metabolic pathways such as metabolism of carbohydrates, fats, and proteins. In addition, flavin-containing coenzymes are associated with the metabolism of folate, cobalamin, vitamin B6, and other vitamins, which is the reason plasma riboflavin is one of the determinants of plasma homocysteine levels, a factor known to influence

All listed authors contributed to the conception and design of the study, analysis/interpretation of data, drafting of the manuscript, and approved the final version of the manuscript.

* Corresponding author. Tel.: +54-381-431-0465; fax: +54-381-400-5600.

E-mail address: leblanc@cerela.org.ar (J.G. LeBlanc).

0899-9007/06/$ – see front matter
doi:10.1016/j.nut.2006.01.002

the risk of cardiovascular disease, pregnancy complications, and cognitive impairment [1].

Although riboflavin is found in a wide variety of foods (i.e., lean meats, poultry, dairy products, fish, grains, broccoli, turnip greens, asparagus, spinach, and enriched products), vitamin B2 deficiency is common in many parts of the world, particularly in developing countries [2], but can also be found in industrialized countries in the elderly [3,4] and young adults [5,6].

Vitamin B2 status in humans has usually been assessed by measuring the erythrocyte glutathione reductase activation coefficient (EGRAC), which is the ratio between glutathione reductase activity as determined with and without the addition of the cofactor FAD [7]. Glutathione reductase loses FAD at an early stage in vitamin B2 deficiency, making EGRAC a useful method for the diagnosis of vitamin B2 deficiency [8].

Riboflavin-deficient rat models have been used to study a wide range of biological effects exerted by riboflavin. Using these models, it has been shown that riboflavin (1) is important in the early postnatal development of the brain [9] and gastrointestinal tract [10,11], (2) is able to modulate carcinogen-induced DNA damage [12,13], (3) plays a role in iron absorption and utilization [14,15], and (4) can modulate inflammatory responses [16]. Data obtained using these models can also be extrapolated to human clinical data [17].

It has been reported that *Lactococcus lactis* subspecies *cremoris* strain NZ9000 can be converted from a vitamin B2 consumer into a vitamin B2 "factory" by overexpressing its riboflavin biosynthesis genes [18]. This genetically modified strain was capable of producing riboflavin with a bioavailability similar to that of commercially available riboflavin [19]. This strain was also used in the elaboration of a novel fermented milk-based food that was able to improve the riboflavin status of depleted animals [20]. However, the use of such genetically modified bacteria in the manufacture of novel fermented foods is not feasible in most countries due to the lack of confidence in the safety of genetically modified organisms by consumers. Moreover, current legislation in most countries does not permit the addition of live genetically modified strains in food products for human consumption, thus strongly limiting the use of the overproducing strain used in these previous studies.

Kukanova et al. [21] described a method to select roseoflavin-resistant mutants of *Bacillus subtilis*, which were found to produce high levels of riboflavin. We used this method to select a spontaneous riboflavin overproducing strain of *Propionibacterium freudenreichii*. Roseoflavin-resistant mutants of *L. lactis* have recently been characterized by Burgess et al. [18]. They demonstrated the presence of nucleotide replacements and deletions in the regulatory region of the *rib* operon. Such spontaneously riboflavin-overproducing strains have a considerable advantage over the genetically engineered strain because they can be promptly implemented in industrial fermentation for human consumption (European Council Directive 90/220/EEC) and because these strains are not considered genetically modified organisms.

Table 1
Riboflavin content of freeze-dried yogurt samples and final concentration in the repletion diet

Yogurt sample	Riboflavin	
	Yogurt (μg/g)	Diet (mg/kg)
WT*	12.9 ± 0.12	0.32
B374†	10.5 ± 0.15	0.26
B2336‡	19.7 ± 0.20	0.49

* Control yogurt prepared with the starter culture *Campina MUH306.*
† Yogurt produced with *Proprionibacterium freudenreichii* B374 as an adjunct culture.
‡ Yogurt produced with *P. freudenreichii* B2336 as an adjunct culture.

The main objective of this study was to demonstrate that the addition of a spontaneous riboflavin overproducing strain of *P. freudenreichii* in a fermented milk product could be used to improve the riboflavin status of deficient rats, thereby eliminating the need of costly fortification of this essential vitamin.

Materials and methods

Yogurt preparation and riboflavin quantification

Riboflavin-enriched yogurt was prepared on pilot plant scale at the facilities of Campina Innovation (Wageningen, The Netherlands). Batches of pasteurized yogurt milk (20 L) were preincubated with the strains *P. freudenreichii* NIZO B2336 (a spontaneous roseoflavin-resistant mutant derived from *P. freudenreichii* B374 with increased riboflavin production) and *P. freudenreichii* NIZO B374 (parental wild-type strain with low riboflavin production, control) for 24 h at 31°C to allow full growth of these cultures. Subsequently, the prefermented yogurt milk was inoculated with the yogurt starter culture *Campina MUH306*. Yogurt fermentation was performed for 18 h at 31°C until the pH was 4.4. As an additional control, an experimental yogurt was made without addition of the adjunct culture of *Propionibacterium*. Plate counting in the final yogurt products showed that the *Propionibacterium* strains reached cell numbers of about 5×10^8 colony-forming units/mL.

Subsequently, the three yogurt batches were freeze dried and ground. Each batch yielded approximately 2 kg of yogurt powder. The vitamin B2 content of the powder was determined as described by Burgess et al. [18]. For the feeding trials, yogurt powder was mixed with a riboflavin-deficient diet at a ratio of 1:40 (wt/wt). The final amounts of riboflavin in the repletion diets are presented in Table 1.

Animals

The overall experimental protocol is summarized in Fig. 1. Weanling, specifically pathogen-free, conventional

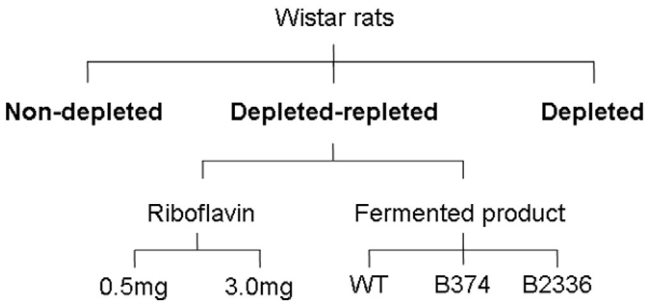

Fig. 1. Riboflavin depletion/repletion experimental protocol. The depleted group was fed a riboflavin-deficient diet for 49 d; the non-depleted group received a riboflavin-deficient diet supplemented with commercial riboflavin (15 mg/kg of vitamin B2) for 49 d; and the depletion/repletion groups were fed a riboflavin-deficient diet for 21 d (depletion period) followed by a 28-d repletion period when animals were fed the same diet supplemented with 1) different levels of commercial riboflavin (0.5 or 3.0 mg/kg of vitamin B2) or 2) one of the fermented milk products (WT for the control yogurt prepared with the starter culture *Campina MUH306* and B374 or B2336 for yogurt produced with *Proprionibacterium freudenreichii* B374 or B2336 as an adjunct culture, respectively).

Wistar rats (weighing 60 ± 3 g) were obtained from an inbred colony and 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-bottom cages (to prevent coprophagy) and were allowed free access to a riboflavin-deficient diet (MP Biomedicals Inc./ICN, Irvine, CA, USA) and water throughout the study.

Rats were matched by weight and assigned to one of three main groups: (1) a depleted group in which animals were fed the riboflavin-deficient diet for 21 d (D21) or 49 d (D49); (2) a non-depleted group in which animals received the riboflavin-deficient diet supplemented with commercial riboflavin (Sigma-Aldrich, St. Louis, MO, USA; 15 mg of vitamin B2 per kilogram of diet, the amount of riboflavin present in the balanced rodent diet used in our laboratory; balanced/autoclaved Rodent Diet, Batistela, Buenos Aires, Argentina) for 21 d (ND21) or 42 d (ND49); and (3) a depletion/repletion group in which rats were fed the riboflavin-deficient diet for 21 d (depletion period) followed by a 28-d repletion period when animals were fed the same diet supplemented with different levels of commercial riboflavin or with one of the fermented products (DR-B374, DR-B2336, or DR-WT yogurt). Commercial riboflavin was added at concentrations equivalent to 1) the residual riboflavin found in B2-free diets used in previous deficiency studies (0.5 mg of vitamin B2 per kilogram of diet [11,15]) and 2) the daily riboflavin requirement of laboratory rats (3.0 mg of vitamin B2 per kilogram of diet [22]). Live weight and food intake (given ad libitum) were determined twice daily. Growth rates were calculated during the depletion (first 21 d) and repletion (final 28 d) periods by using the mean average twice-daily increase and were expressed as daily changes in live animal weight (grams).

Blood and organ collection

Throughout the trial, rats from each group were placed into a homemade sampling chamber, and 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 intraperitoneal injection of 3.0 mL/kg of ketamine (10%, wt/vol) plus xylazine (2%, wt/vol; 40:60 vol/vol; Alfasan, Woerden, The Netherlands) and bled by cardiac puncture. Blood was transferred into tubes containing anticoagulant heparin (Rivero, Buenos Aires, Argentina) and centrifuged (2000*g* for 15 min at 4°C). Plasma was removed and stored at −70°C until analysis. The sedimented cells were washed three times with 0.15 mol/L of cold NaCl. Erythrocytes (0.5 mL) were hemolyzed 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 mol/L of NaCl, weighed, and stored at −70°C.

Riboflavin status

Riboflavin status was assessed by measuring the EGRAC according to a modification of a previously described technique [23]. Briefly, frozen hemolyzed blood was allowed to thaw at room temperature under conditions of low light. Hemolysates (31.3 μL) were added to 1 mL of potassium phosphate buffer (0.1 mol/L, pH 7.4) containing 2.3 mmol/L of ethylene-diaminetetra-acetic acid (dipotassium salt) and 0.89 mmol/L of oxidized glutathione with or without 8 μmol/L of FAD. The mixture was preincubated for 30 min at 37°C followed by the addition of 80 μmol/L of nicotinamide adenine dinucleotide phosphate to initiate the reaction. Absorbance at 340 nm was measured every 10 min for 1 h at 37°C (Cecil CE 2021 spectrophotometer, Cecil Instruments Ltd., Cambridge, UK). Riboflavin status was calculated as an activity coefficient, which was defined as the ratio between the rate of change of absorbance per time unit in the presence and absence of FAD. EGRACs were measured in triplicate for each sample.

Statistics

Comparisons were performed with SigmaStat (SPSS Inc., Chicago, IL, USA). Comparisons of multiple means were accomplished by one-way analysis of variance followed by Tukey's post hoc test, and $P < 0.05$ was considered statistically significant. Unless otherwise indicated, all values are the means of three independent trials ± standard deviation ($n = 30$).

All animal protocols were approved by the animal protection committee of the Centro de Referencia para Lactobacilos and followed the most recent recommendations of the Federation of European Laboratory Animal Science Associations. All experiments complied with the current laws of Argentina.

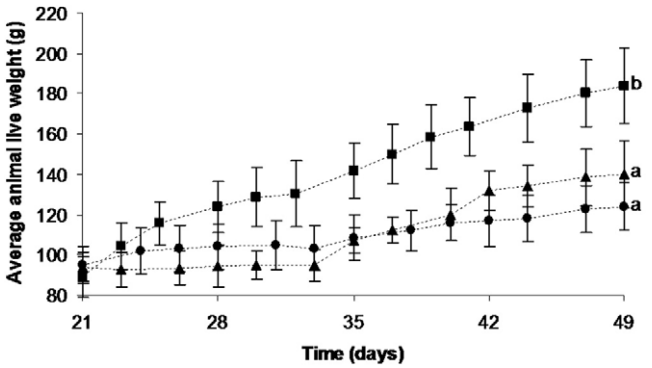

Fig. 2. Live weight during depletion/repletion in Wistar rats during the depletion period. Animals were fed a riboflavin-deficient diet for 21 d (depletion period), after which they received the same diet supplemented with different yogurt samples (B374, circles; B2336, squares; WT, triangles) for 28 d (repletion period). Results are expressed as means ± standard deviation ($n = 10$), and points without a common letter differ significantly ($P < 0.05$).

All materials were supplied by Sigma-Aldrich Corporation unless otherwise stated.

Results

Animal growth

Animals supplemented with B2336 yogurt showed a statistically significant increase in growth compared with those that received the B374 or WT yogurt (Fig. 2 and Table 2). Animals that received WT yogurt showed increased live weight at day 49 (139.9 ± 8.9 g) compared with those fed B374 yogurt (124.0 ± 7.0) after comparing mean values of live animal weight, although no statistically significant difference was observed between these latter two groups of animals.

The B374-fed rats showed statistically similar growth rates (1.14 ± 0.15 g/d) to the depleted animals, which received only the riboflavin-deficient diet (1.04 ± 0.04), and these rates were slightly lower than that of animals fed the control WT yogurt (1.71 ± 0.20; Table 2). The animals that received B2336 yogurt showed statistically similar growth rates (3.28 ± 0.22) as the group that received 0.5 mg/kg of vitamin B2 (3.50 ± 0.16), suggesting that the riboflavin produced by this bacterial strains, which were administered at the same concentration as the commercially available pure riboflavin, possess the same general growth-stimulating capacity.

Riboflavin status

The activation assay EGRAC is a functional test that shows a decrease in enzyme activity when riboflavin deficiency is present and a disproportionate increase in activity after the in vitro addition of this vitamin. The rate of change of the assay is proportional to the amount of enzyme present. EGRAC values of 1.30 to 1.40 or higher are indicative of biochemical riboflavin deficiency.

Animals supplemented with B2336 yogurt showed improved riboflavin status in erythrocytes (lower EGRAC levels, 1.68 ± 0.12) compared with the D21 or D49 animals (2.03 ± 0.14 and 2.43 ± 0.15) and those that received B374 or WT yogurt (2.26 ± 0.09 and 2.30 ± 0.10; Fig. 3). Interestingly, the B2336-supplemented animals showed statistically similar EGRAC values as the group of animals that received 0.5 mg/kg of vitamin B2 (1.56 ± 0.10), suggesting that the riboflavin found in this fermented product, which was administered at the same concentration as the commercially available pure riboflavin, possesses similar bioavailability, thus confirming the results seen in growth patterns (Table 2). DR-B374 and DR-WT animals showed statistically similar EGRAC values; however, absolute EGRAC values of DR-B374 animals were lower than those observed in DR-WT animals. DR-B374 animals had statistically similar EGRAC values as D49 animals, demonstrating that B374 and the WT control yogurts did not improve the riboflavin status of depleted animals; only the fermented product containing *P. freudenreichii* B2336 improved the riboflavin status of depleted animals. The non-depleted an-

Table 2
Growth rate and live weight of animals during depletion and repletion of riboflavin*

Group	Depletion period		Repletion period	
	Growth rate (g/d)	Final weight (g)	Growth rate (g/d)	Final weight (g)
Non-depleted	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]	92.3 ± 9.1[b]	1.04 ± 0.04[b]	111.3 ± 8.5[b]
0.5 mg/kg of vitamin B2			3.50 ± 0.16[c]	190.0 ± 9.5[c]
3.0 mg/kg of vitamin B2			4.00 ± 0.22[d]	204.2 ± 12.5[c]
WT			1.71 ± 0.20[e]	139.9 ± 8.9[e]
B374			1.14 ± 0.15[b]	124.0 ± 7.0[b]
B2336			3.28 ± 0.22[c]	183.9 ± 9.3[c]

* Animals were fed a riboflavin-deficient diet for 21 d (depletion period) after which they received the same diet supplemented with commercial riboflavin or fermented products for 28 d (repletion period). The depleted group received only the riboflavin-deficient diet for 49 d. Values are mean ± standard deviation ($n = 80$ for depleted group during depletion period; $n = 10$ for all groups during repletion period and for non-depleted group during depletion period). Means in a column without a common superscript letter differ ($P < 0.05$).

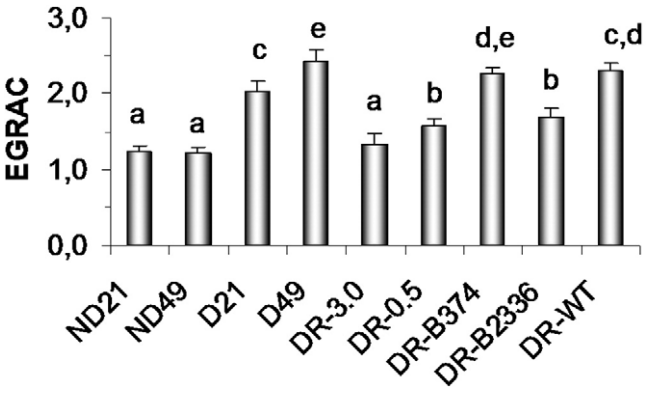

Fig. 3. EGRAC values of rats fed a riboflavin-deficient diet for 21 d followed by a 28-d repletion period when the diet was supplemented with different amounts of riboflavin (DR-0.5 and DR-3.0 groups) or with different yogurt samples (DR-B374, DR-B2336, and DR-WT groups).. Results are expressed as means ± standard deviation ($n = 10$), and bars without a common letter differ significantly ($P < 0.05$). D21, depleted animals fed a riboflavin-deficient diet for 21 d; D49, depleted animals fed a riboflavin-deficient diet for 49 d; DR, depletion/repletion; DR-0.5, 0.5 mg of vitamin B2 per kilogram of diet; DR-3.0, 3.0 mg of vitamin B2 per kilogram of diet; EGRAC, erythrocyte glutathione reductase activation coefficient; ND21, non-depleted animals fed a riboflavin-deficient diet containing high levels of riboflavin for 21 d; ND49, non-depleted animals fed a riboflavin-deficient diet containing high levels of riboflavin for 42 d.

imals (ND21 and ND49) and the animals that received 3.0 mg/kg of vitamin B2 showed normal EGRAC values (1.23 ± 0.08, 1.21 ± 0.08, and 1.34 ± 0.13, respectively).

Organ weight

Animals depleted of riboflavin for 49 d showed statistically significant increases in relative liver weight compared to non-depleted animals and those given 3.0 mg/kg of vitamin B2 (Fig. 4). Animals supplemented with B2336 or WT yogurt showed no increases in relative liver weight compared with non-depleted animals, whereas animals supplemented with B374 yogurt showed statistically similar relative liver weights as D49 animals (Fig. 4). Rats fed the B2336 and WT yogurts showed statistically similar relative liver weights as non-depleted animals, demonstrating that these two latter products were able to prevent hepatomegaly (an abnormal enlargement of the liver typical of ariboflavinosis) in previously depleted animals.

No significant differences were observed in relative organ weights of spleens and kidneys in all experimental groups (data not shown).

Physical observations

The depleted animals showed important loss of hair (thinner coat, yellowish coloring) at the end of the depletion period (21 d of depletion). Animals supplemented with B2336 yogurt (DR-B2336) regained a normal appearance of hair (full white coat) during the repletion period (at the end of the repletion period, animals were similar to those in the non-depleted group), whereas those supplemented with WT (DR-WT) or B374 (DR-B374) yogurt showed no significant improvement in hair appearance.

The D49 animals also showed signs of psychological disorders (dizziness when walking in their cages, trembling, and delayed behavioral responses). These symptoms were not observed in the other experimental groups whose diets were supplemented with a yogurt sample or with commercial riboflavin or in the D21 animals.

No lactose-intolerance symptoms were observed in the rats that received the yogurt samples (data not shown). This observation is in accordance with previous studies that have shown that fermented milks such as yogurt do not cause lactose-associated disorders in conventional rats [24].

Discussion

The objective of this study was to demonstrate that the use of a riboflavin-producing strain of *P. freudenreichii*, supplied as an adjunct culture in the yogurt fermentation process, could improve the nutritional value of yogurt.

Inoculation of milk with *P. freudenreichii* B2336 (a spontaneous roseoflavin-resistant mutant derived from *P. freudenreichii* B374 that shows increased riboflavin production) before inoculation with commercial yogurt cultures significantly increased the riboflavin content (almost doubling it) compared with a similar protocol using *P. freudenreichii* B374 (a low riboflavin-producing strain) or without

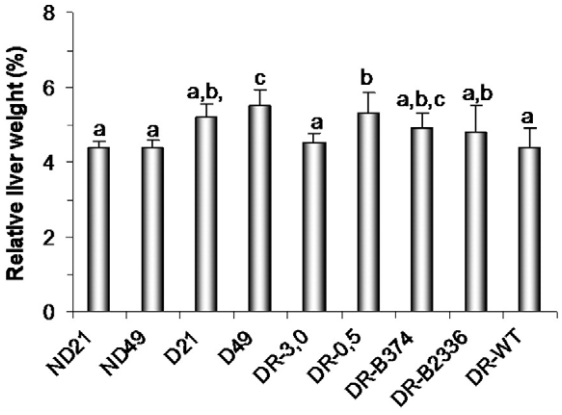

Fig. 4. Relative liver weight of rats fed a riboflavin-deficient diet for 21 d followed by a 28-d repletion period when the diet was supplemented with different amounts of riboflavin (DR-0.5 and DR-3.0 groups) or with different yogurt samples (DR-B374, DR-B2336, and DR-WT groups). Results are expressed as mean ± standard deviation ($n = 10$), and columns without a common letter differ significantly ($P < 0.05$). D21, depleted animals fed a riboflavin-deficient diet for 21 d; D49, depleted animals fed a riboflavin-deficient diet for 49 d; DR, depletion/repletion; DR-0.5, 0.5 mg of vitamin B2 per kilogram of diet; DR-3.0, 3.0 mg of vitamin B2 per kilogram of diet; ND21, non-depleted animals fed a riboflavin-deficient diet containing high levels of riboflavin for 21 d; ND49, non-depleted animals fed a riboflavin-deficient diet containing high levels of riboflavin for 42 d.

the addition of adjunct cultures. This novel fermented milk product was evaluated in a riboflavin depletion/repletion animal model similar to the one used previously to evaluate the bioavailability of riboflavin produced by genetically modified *L. lactis* strains [19,20].

Because riboflavin is essential in various biochemical reactions that are related to obtaining metabolic energy from carbohydrates and fatty acids, the deficiency of this vitamin can cause severe symptoms in animals such as stunted growth [7]. The present experiments indicate that administration of the fermented milk that was produced with a spontaneous riboflavin overproducing strain of *P. freudenreichii* (strain B2336) was able to improve animal growth compared with conventional yogurt (WT) and with the same product fermented with the parental wild-type strain, which produces low levels of this essential vitamin. The growth rates of depleted rats that received B2336 yogurt was similar to those of rats that received commercial riboflavin at a concentration similar to that produced by *P. freudenreichii* B2336.

This novel fermented product was also able to improve riboflavin status (decreased EGRAC values) of previously depleted animals, with values similar to those of animals that received 0.5 mg/kg of commercial riboflavin. This was not the case for the fermented product that contained only the yogurt starter cultures or *P. freudenreichii* B374, where riboflavin status and growth of depleted rats were similar to those obtained in animals that did not receive any dietary riboflavin (depleted animals). The fermented product containing only the yogurt starter culture (WT yogurt) showed only a slight improvement in growth and riboflavin status compared with rats that were depleted of this essential vitamin.

Yogurt containing *P. freudenreichii* B2336 was able to prevent hepatomegaly, an abnormal enlargement of the liver due to riboflavin deficiency. This product was also able to revert morphologic changes observed in riboflavin-deficient animals (hair loss).

These results clearly show that the use of *P. freudenreichii* B2336 as an adjunct culture in yogurt fermentation increased the riboflavin content, with similar bioavailability as the commercially available vitamin, thereby improving the nutritional value of yogurt and eliminating the costly need to fortify this fermented milk product with vitamin B2.

Propionibacterium freudenreichii B2336 could be used for the production of yogurt or fermented milk to increase levels of riboflavin, thus increasing their commercial and nutritional value and eliminating the need for subsequent fortification with this essential vitamin. Such novel products could be used in the goal of decreasing the number of persons with clinical and subclinical riboflavin deficiency, which is common in many parts of the world, not only in developing countries but also in many industrialized countries.

In conclusion, the use of riboflavin overproducing *P. freudenreichii* B2336 in yogurt preparation increased its riboflavin content. This novel fermented milk product was able to improve growth and riboflavin status (lower EGRAC values) and prevent hepatomegaly of riboflavin-depleted animals.

Acknowledgments

This work has been funded by the European Union through contract 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 are endebted to Dr. Jeroen Hugenholtz for coordination of the EU-project. Finally, the authors would like to thank Analia Rossi and Silvia Burke for their help with the care of the animals and sampling.

References

[1] Hustad S, McKinley MC, McNulty H, Schneede J, Strain JJ, Scott JM, Ueland PM. Riboflavin, flavin mononucleotide, and flavin adenine dinucleotide in human plasma and erythrocytes at baseline and after low-dose riboflavin supplementation. Clin Chem 2002;48:1571–7.

[2] Boisvert WA, Castaneda C, Mendoza I, Langeloh G, Solomons NW, Gershoff SN, Russell RM. Prevalence of riboflavin deficiency among Guatemalan elderly people and its relationship to milk intake. Am J Clin Nutr 1993;58:85–90.

[3] Bailey AL, Maisey S, Southon S, Wright AJ, Finglas PM, Fulcher RA. Relationships between micronutrient intake and biochemical indicators of nutrient adequacy in a 'free-living' elderly UK population. Brit J Nutr 1997;77:225–42.

[4] Madigan SM, Tracey F, McNulty H, Eaton-Evans J, Coulter J, McCartney H, Strain JJ. Riboflavin and vitamin B-6 intakes and status and biochemical response to riboflavin supplementation in free-living elderly people. Am J Clin Nutr 1998;68:389–95.

[5] Benton D, Haller J, Fordy J. The vitamin status of young British adults. Int J Vitam Nutr Res 1997;67:34–40.

[6] O'Brien M, Kiely M, Harrington K, Robson P, Strain J, Flynn A. The North/South Ireland Food Consumption Survey: vitamin intakes in 18–64-year-old adults. Public Health Nutr 2001;4:1069–79.

[7] Glatzle D, Körner WF, Christeller S, Wiss O. Method for the detection of a biochemical riboflavin deficiency. Stimulation of NADPH2-dependent glutathione reductase from human erythrocytes by FAD in vitro. Investigations on the vitamin B2 status in healthy people and geriatric patients. Int Z Vitaminforsch 1970;40:166–83.

[8] Bates C. Riboflavin. Int J Vitam Nutr Res 1993;63:274–7.

[9] Ogunleye AJ, Odutuga AA. The effect of riboflavin deficiency on cerebrum and cerebellum of developing rat brain. J Nutr Sci Vitaminol (Tokyo) 1989;35:193–7.

[10] Williams EA, Rumsey RD, Powers HJ. Cytokinetic and structural responses of the rat small intestine to riboflavin depletion. Br J Nutr 1996;75:315–24.

[11] Yates CA, Evans GS, Pearson T, Powers HJ. Absence of luminal riboflavin disturbs early postnatal development of the gastrointestinal tract. Dig Dis Sci 2003;48:1159–64.

[12] Pangrekar J, Krishnaswamy K, Jagadeesan V. Effects of riboflavin deficiency and riboflavin administration on carcinogen-DNA binding. Food Chem Toxicol 1993;31:745–50.

[13] Webster RP, Gawde MD, Bhattacharya RK. Modulation of carcinogen-induced DNA damage and repair enzyme activity by dietary riboflavin. Cancer Lett 1996;98:129–35.

[14] Butler BF, Topham RW. Comparison of changes in the uptake and mucosal processing of iron in riboflavin-deficient rats. Biochem Mol Biol Int 1993;30:53–61.
[15] Powers HJ, Weaver LT, Austin S, Beresford JK. A proposed intestinal mechanism for the effect of riboflavin deficiency on iron loss in the rat. Br J Nutr 1993;69:553–61.
[16] Lakshmi R, Lakshmi AV, Divan PV, Bamji MS. Effect of riboflavin or pyridoxine deficiency on inflammatory response. Indian J Biochem Biophys 1991;28:481–4.
[17] Greene HL, Specker BL, Smith R, Murrell J, Swift L. Plasma riboflavin concentrations in infants fed human milk versus formula: comparison with values in rats made riboflavin deficient and human cord blood. J Pediatr 1990;117:916–20.
[18] Burgess C, O'Connell-Motherway M, Sybesma W, Hugenholtz J, van Sinderen D. Riboflavin production in Lactococcus lactis: potential for in situ production of vitamin-enriched foods. Appl Environ Microbiol 2004;70:5769–77.
[19] LeBlanc JG, Burgess C, Sesma F, Savoy de Giori G, van Sinderen D. Lactococcus lactis is capable of improving the riboflavin status in deficient rats. Br J Nutr 2005;94:262–7.
[20] LeBlanc JG, Burgess C, Sesma F, Savoy de Giori G, van Sinderen D. Ingestion of milk fermented by genetically modified Lactococcus lactis improves the riboflavin status of deficient rats. J Dairy Sci 2005;88:3435–42.
[21] Kukanova AI, Zhdanov VG, Stepanov AI. Bacillus subtilis mutants resistant to roseoflavin. Genetika 1982;18:319–21.
[22] Institute for Laboratory Animal Research. Nutrient requirements of laboratory animals. 4th revised ed. Washington, DC: National Academy of Sciences; 1995.
[23] Adelekan DA, Thurnham DI. The influence of riboflavin deficiency on absorption and liver storage of iron in the growing rat. Br J Nutr 1986;56:171–9.
[24] Shah N, Jelen P. Lactose absorption by postweaning quarg, and quarg whey rats from yogurt. J Dairy Sci 1991;74:1512–20.