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Lactococcus lactis is capable of improving the riboflavin status in deficient rats

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Lactococcus lactis is a commonly used starter strain that can be converted from a vitamin B_2 consumer into a vitamin B_2 'factory' by over-expressing its riboflavin biosynthesis genes. The present study was conducted to assess in a rat bioassay the response of riboflavin produced by GM or native lactic acid bacteria (LAB). The riboflavin-producing strains were able to eliminate most physiological manifestations of ariboflavinosis such as stunted growth, elevated erythrocyte glutathione reductase activation coefficient values and hepatomegalia that were observed using a riboflavin depletion–repletion model. Riboflavin status and growth rates were greatly improved when the depleted rats were fed with cultures of *L. lactis* that overproduced this vitamin whereas the native strain did not show the same effect. The present study is the first animal trial with food containing living bacteria that were engineered to overproduce riboflavin. These results pave the way for analysing the effect of similar riboflavin-overproducing LAB in human trials.

Riboflavin: Lactic acid bacteria: Ariboflavinosis: Genetically modified micro-organisms

Riboflavin (vitamin B_2) is a water-soluble vitamin belonging to the B-complex group that is important for optimal body growth and erythrocyte production and helps in releasing energy from carbohydrates. In the body, riboflavin is primarily found as an integral component of the coenzymes FAD and FMN. 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, vitamin B_{12}, vitamin B_6, and other vitamins, which explains why plasma riboflavin is a determinant of plasma homocysteine, which is associated with CVD, pregnancy complications, and cognitive impairment (Hustad *et al.* 2000, 2002).

Although riboflavin is found in a wide variety of foods (dairy products, lean meats, poultry, fish, grains, broccoli, turnip greens, asparagus, spinach and enriched food products), vitamin B_2 deficiency is common in many parts of the world, particularly in developing countries (Boisvert *et al.* 1993). Several studies have indicated that vitamin B_2 deficiency may be widespread in industrialised countries as well, both in the elderly (Bailey *et al.* 1997; Madigan *et al.* 1998) and in young adults (Benton *et al.* 1997).

Vitamin B_2 status in human subjects has usually been assessed by measuring the erythrocyte glutathione reductase activation coefficient (EGRAC), which is the ratio between enzyme 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 the EGRAC a useful method for the diagnosis of vitamin B_2 deficiency (Bates, 1993).

Riboflavin-deficient rat models have been utilised for a number of years 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 & Odutuga, 1989) and gastrointestinal tract (Williams *et al.* 1995, 1996; Yates *et al.* 2001, 2003); (ii) is able to modulate carcinogen-induced DNA damage (Pangrekar *et al.* 1993; Webster *et al.* 1996); (iii) plays a role in Fe absorption and utilisation (Powers, 1987; Powers *et al.* 1988, 1991, 1993; Butler & Topham, 1993); (iv) can modulate inflammatory responses (Lakshmi *et al.* 1991). These models also allow the extrapolation of data obtained in an animal model 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 four biosynthetic genes (*ribG*, *ribH*, *ribB* and *ribA*) are over-expressed simultaneously (in *L. lactis* NZ9000 containing pNZGBAH). Also, spontaneous mutants (i.e. *L. lactis* strain CB010) capable of producing riboflavin in the growth medium, although at a lower level than the engineered

Abbreviations: EGRAC, erythrocyte glutathione reductase activation coefficient; LAB, lactic acid bacteria.

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strain, were identified. Such spontaneously riboflavin-overproducing strains have a considerable advantage over the genetically engineered strain as they can be promptly implemented in industrial fermentation.

The main objective of the present study was to evaluate the bioavailability of riboflavin from spontaneous and engineered riboflavin-overproducing *L. lactis* strains using a depletion–repletion rat bioassay. These strains could be used in the development of novel fermented foods containing increased levels of riboflavin, produced *in situ*, which eliminates the need for vitamin fortification.

Materials and methods

Bacterial strains, media and culture conditions

L. lactis strains NZ9000 (*L. lactis* B_2-) and CB010 (*L. lactis* B_2+) were grown (12 h at 30 °C) in M17 medium (Biokar Diagnostics, Beauvais, France) supplemented with 0·5 % glucose (M17-Glu). *L. lactis* NZ9000 harbouring plasmid pNZGBAH (*L. lactis* B_2++) (Burgess *et al.* 2004) were grown at 30 °C in M17-Glu supplemented with chloramphenicol (5 μg/ml). Nisin was added (1 ng/ml) after 4 h growth when required.

Quantitative analysis 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 from a cell-free supernatant fraction were precipitated from a 1 ml sample by adding 10 % TCA. HPLC analysis (Isco model 2360; Teledyne Isco Inc., Lincoln, NE, USA) of the resulting liquid was performed using a C18 reverse-phase column (4 × 150 mm; Varian, Inc., Palo Alto, CA, USA) 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. Commercially obtained riboflavin, FMN and FAD were used as references and to obtain a standard curve (Sigma, Buenos Aires, Argentina).

Experimental design

The overall experimental protocol is summarised in Fig. 1. Ninety 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 (Argentina). Rats were individually housed in wire-based cages (to prevent coprophagy) and were allowed free access to a riboflavin-deficient diet (ICN Biomedicals Inc., Irvine, CA, USA) and water throughout the study.

The rats were weight matched into three main groups of animals. The first group was a depleted group where animals were fed the riboflavin-deficient diet during 42 d. The second group was a non-depleted group where animals received the riboflavin-deficient diet supplemented with commercial riboflavin (15 mg B_2/kg; Sigma, Buenos Aires, Argentina) during 42 d. The third group was a depleted–replete group where rats were fed the riboflavin-deficient diet for 21 d (depletion period) followed by a 21 d repletion period where animals were fed the same diet supplemented with (i) different levels of commercial riboflavin, or (ii) wild-type or engineered riboflavin-producing lactic acid bacteria (LAB; *L. lactis* B_2+ and *L. lactis* B_2++, respectively) or (iii) the control strain (*L. lactis* B_2-), which were grown in M17-Glu. 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 B_2/kg diet; Powers *et al.* 1991, 1993; Yates *et al.* 2001, 2003); (ii) the daily riboflavin requirement of laboratory rats (3·0 mg B_2/kg diet; Institute for Laboratory Animal Research, 1995). In the second depleted–replete group, rats were fed 20 ml LAB-containing M17-Glu broth twice daily during the repletion period in replacement of their drinking water, which contained 0·0 (SD 0·1), 0·5 (SD 0·2) or 15·0 (SD 5) mg/l for *L. lactis* B_2-, B_2+ and B_2++, respectively (determined by HPLC). Animal live weight and food intake (given *ad libitum*) were determined on a bi-daily basis. Growth rates were calculated during the repletion period (21 d) using the mean average bi-daily increase and were expressed as changes in live animal weight (g) per d.

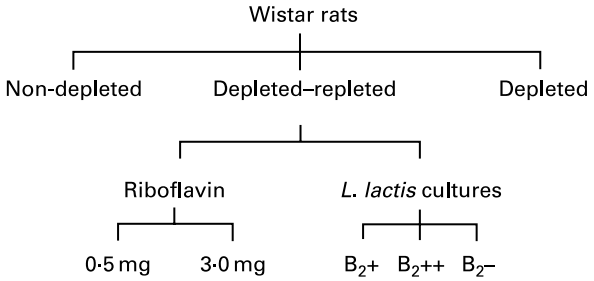

Fig. 1. Riboflavin depletion–repletion experimental protocol. The depleted group were fed a riboflavin-deficient diet (RDD) during 42 d; the non-depleted group received the RDD supplemented with commercial riboflavin during 42 d; the depleted–replete group were fed the RDD for 21 d (depletion period) followed by a 21 d repletion period where animals were fed the same diet supplemented with (i) different levels of commercial riboflavin, or (ii) wild-type or engineered riboflavin-producing lactic acid bacteria (*Lactococcus lactis* B_2+ and *L. lactis* B_2++, respectively) or the control strain (*L. lactis* B_2-).

Blood and organ collection

Throughout the trial, rats from each group were placed into a home-made sampling chamber, and whole blood was collected from the tail and transferred into a tube containing anticoagulant for EGRAC evaluation (see p. 262). At the end of the trial, animals were anaesthetised with an intraperitoneal injection of ketamin (10 %)–xylacin (2 %) (3·0 ml/kg animal weight; 40:60, v/v; 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 cold 0·15 M-NaCl. Erythrocytes (0·5 ml) were haemolysed 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 the EGRAC using a modification of a previously described technique (Adelekan & Thurnham, 1986). Briefly, haemolysed blood was allowed to

thaw at room temperature under conditions of reduced light. Haemolysates (31·3 μl) were added to 1 ml potassium phosphate buffer (0·1 M; pH 7·4) containing 2·3 mM-ethylenediaminetetraacetic acid (dipotassium salt) and 0·89 mM-GSSG with or without 8 μM-FAD. The mixture was pre-incubated for 30 min at 37°C followed by the addition of 80 μM-NADPH to initiate the reaction. The absorbance at 340 nm was measured every 10 min during 1 h at 37°C. 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. EGRAC were measured in triplicate for each sample.

Statistics

Comparisons were performed using the software package SigmaStat (SPSS Inc., Chicago, IL, USA). Comparisons of multiple means were accomplished by one-way ANOVA followed by Tukey's *post hoc* test and $P<0·05$ was considered significant. Unless otherwise indicated, all values are the means of three independent trials and standard deviations (where n 30).

Results and discussion

In order to study the bioavailability of riboflavin from native and engineered *L. lactis* strains, a depletion–repletion rat bioassay 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 was compared with that of pure riboflavin given to rats at levels previously considered negligible (0·5 mg/B_2 kg diet) or at the daily recommended intake for such animals (3·0 mg B_2/kg diet; for details, see p. 262).

Animal growth during the depletion–repletion periods

It is well documented that rats, which are deprived of riboflavin, exhibit an impairment of 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 as compared with the non-depleted group (Table 1).

The animals supplemented with *L. lactis* B_2- showed significantly lower growth rates (2·78 (SD 0·17) g/d) as compared with those fed *L. lactis* B_2+ or 0·5 mg B_2/kg (3·30 (SD 0·20) and 3·19 (SD 0·16) g/d respectively), suggesting that the riboflavin produced by the latter strains can exert a biological function. The *L. lactis* B_2- rats showed higher growth rates than the depleted animals, which only received the riboflavin-deficient diet (0·68 (SD 0·04) g/d). This last difference could be due to other residual nutrients found in the M17 broth after bacterial growth besides riboflavin since this vitamin was not detected after growth of *L. lactis* B_2- (HPLC determination). Interestingly, the animals that received *L. lactis* B_2+ showed statistically similar growth rates as the group that received 0·5 mg B_2/kg, suggesting that the riboflavin produced by this bacterial strain, given here at the same concentration as the commercially available pure riboflavin, possesses similar bioavailability. The animals that received *L. lactis* B_2++ showed a significantly higher growth rate (4·41 (SD 0·26) g/d) and final weight (224·0 (SD 13·4) g) than all the other depleted–replete groups (Table 1), an expected result since the riboflavin concentration of this culture (15·0 (SD 5) 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 (erythrocyte glutathione reductase activation coefficient)

Activation assays such as the EGRAC are functional tests that show a decline in a specific enzyme activity as a result of riboflavin deficiency and a disproportionate increase in activity after the *in vitro* addition of this vitamin (Adelekan & Thurnham, 1986). 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. 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.

In order to show that EGRAC values correlate with the riboflavin status of rats, a validation test was performed where the EGRAC was followed at a weekly basis in animals fed with the

Table 1. Growth rate and live weight of animals fed a riboflavin-deficient diet during 21 d (depletion period), after which they received the same diet supplemented with commercial riboflavin or *Lactococcus lactis* strains (NZ9000 (B_2-), CB010 (B_2+), or NZ9000 (pNZGBAH) (B_2++)) during 21 d (repletion period)

(Mean values and standard deviations)

	Depletion period				Repletion period			
	Growth rate (g/d)		Final weight (g)		Growth rate (g/d)		Final weight (g)	
Group*	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Non-depleted	5·81[a]	0·35	138·0[a]	8·3	5·34[a]	0·32	299·2[a]	18·0
Depleted	4·08[b]	0·24	102·2[b]	6·1	0·68[b]	0·04	141·1[b]	8·5
L. lactis (B_2-)					2·78[f]	0·17	188·9[c]	11·3
B_2 (0·5 mg)					3·19[c]	0·16	190·0[c]	9·5
B_2 (3·0 mg)					3·61[d]	0·22	204·2[c,d]	12·5
L. lactis (B_2++)					4·41[e]	0·26	224·0[e]	13·4
L. lactis (B_2+)					3·30[c]	0·20	200·0[c,d]	12·0

[a,b,c,d,e,f] Mean values within a column with unlike superscript letters are significantly different ($P<0·05$).

*Sixty rats in the depleted group during the depletion period and ten rats in all groups during the repletion period and for the non-depleted group during the depletion period.

riboflavin-deficient diet (depleted group). A significant increase in EGRAC values can be observed in the depleted group as compared with the non-depleted groups (Fig. 2). After only 7 d, a significant increase in the EGRAC is seen in the depleted group (1·62 (SD 0·09)) and this value continues to increase in function of time (2·02 (SD 0·07) and 2·41 (SD 0·06) after 21 and 42 d, respectively). In the non-depleted group, EGRAC values did not vary significantly (1·18–1·29) showing that their riboflavin status remained normal throughout the study.

In order to determine if native and engineered LAB could improve the riboflavin status of deficient rats, cultures grown in M17-Glu were used to supplement the riboflavin-deficient diet for 21 d (repletion period) of previously depleted animals. HPLC analysis showed significant levels of riboflavin in the M17-Glu medium following growth of *L. lactis* B_2+ or B_2++ (0·5 (SD 0·2) and 15 (SD 5) mg/l respectively). This vitamin was below the detection level in the medium after growth of the non-producing strain (*L. lactis* B_2-).

As was the case in the validation test, the depleted rats showed increased EGRAC values (2·41 (SD 0·06)) compared with the non-depleted animals (1·18 (SD 0·04)) after the repletion period (Fig. 3). The rats whose diet was supplemented with the non-producing strain (*L. lactis* B_2-) showed statistically similar EGRAC values as those found in the depleted animals (2·35 (SD 0·06)). This result confirms that the increase in growth observed in the animals supplemented with the non-producing strains is not caused by riboflavin but by other residual nutrients found in the cultures broth. The rats whose diet was supplemented with either of the two riboflavin producing strains (*L. lactis* B_2+ and B_2++) exhibited significantly lower EGRAC values (1·65 (SD 0·09) and 1·31 (SD 0·05), respectively) as compared with rats of the depleted group (2·41 (SD 0·06)) or rats whose diet was supplemented with the non-producing strain (*L. lactis* B_2-) (2·10 (SD 0·06)) (Fig. 3). Interestingly, the animals that received *L. lactis* B_2+ showed statistically similar EGRAC values as the group that received 0·5 mg B_2/kg, suggesting that the riboflavin produced by this bacterial strain, given here at the same concentration as the commercially available pure riboflavin, possesses similar bioavailability, confirming the results seen in growth (Table 1).

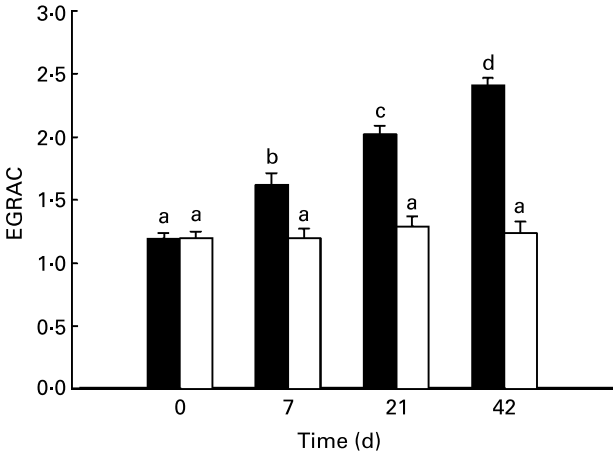

Fig. 2. Erythrocyte glutathione reductase activation coefficient (EGRAC) values of rats fed a riboflavin-deficient diet (■) or the same diet supplemented with commercial riboflavin (15 mg/kg; □). Values are means, with standard deviations represented by vertical bars (*n* 10). [a,b,c,d] Mean values with unlike letters are significantly different ($P<0·05$).

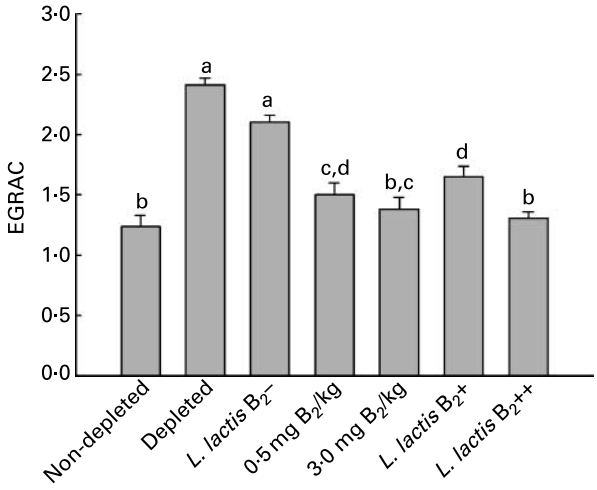

Fig. 3. Erythrocyte glutathione reductase activation coefficient (EGRAC) values of rats fed a riboflavin-deficient diet during 21 d followed by a 21 d repletion period where the diet was supplemented with different amounts of riboflavin (0, 0·5 or 3·0 mg/kg diet) or with cultures of *Lactococcus lactis* (NZ9000 (B_2-), CB010 (B_2+), or NZ9000 (pNZGBAH) (B_2++)). Values are means, with standard deviations represented by vertical bars (*n* 10). [a,b,c,d] Mean values with unlike letters are significantly different ($P<0·05$).

The animals that received *L. lactis* B_2++ showed the lowest EGRAC values and, as was the case with the growth rates, this result was not surprising since the riboflavin concentration of this culture was the highest used in the depleted–replete animals. Surprisingly, no statistically significant differences in EGRAC values were observed between the animals that received 0·5 mg B_2/kg and those receiving 3·0 mg B_2/kg (however, absolute values were lower in the 3·0 mg B_2/kg group compared with the 0·5 mg B_2/kg group); a longer repletion period in future studies could improve the sensibility of the experiment.

Organ weight comparison

Another physiological effect of ariboflavinosis is hepatomegaly, which is the enlargement of the liver beyond its normal size. This problem is normally found in rats deficient in riboflavin (Glatzle *et al.* 1968).

An increase in the weight of the liver in relation to body weight was observed in the depletion groups where riboflavin deficiency was observed (Fig. 4).

The groups supplemented with *L. lactis* B_2- showed a significant increase in relative liver weight (5·4 (SD 0·5) g) as compared with the non-depleted group (4·4 (SD 0·2) g) and were statistically similar to the depletion groups (5·2 (SD 0·4) g) and the group that received 0·5 mg B_2/kg (5·3 (SD 0·5) g). The groups supplemented with *L. lactis* B_2+ or *L. lactis* B_2++ showed no significant differences in relative liver weight compared with the non-depleted group or the group that received 3·0 mg B_2/kg. These results suggest that the riboflavin-producing strains are able to decrease the relative liver weight increases observed in the depleted animals. However, it is not possible to assess bioavailability of the riboflavin produced by the bacterial strains with these results since no significant differences were observed in the animals fed with either the producing strain (B_2+) or the overproducing strain (B_2++), which have very important

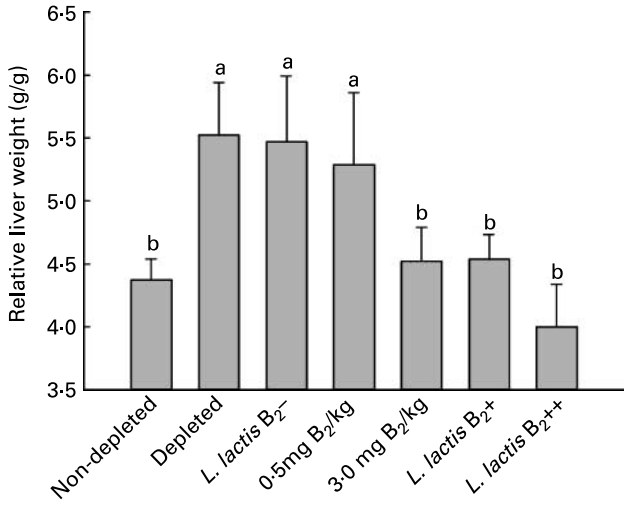

Fig. 4. Relative weight of liver of animals fed a riboflavin-deficient diet during 21 d followed by a 21 d repletion period where the diet was supplemented with different amounts of riboflavin (0, 0·5 or 3·0 mg/kg diet) or with cultures of *Lactococcus lactis* (NZ9000 (B_2-), CB010 (B_2+), or NZ9000 (pNZGBAH) (B_2++)). Values are means, and standard deviations represented by vertical bars (n 10). [a,b]Mean values with unlike letters are significantly different ($P<0·05$).

differences in bioavailable riboflavin as determined by HPLC, growth rates and EGRAC values.

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

Conclusions

The objective of the present study was to evaluate the bioavailability of riboflavin from spontaneous and engineered riboflavin-overproducing *L. lactis* strains using a depletion–repletion rat bioassay. The bioavailability of the riboflavin produced by these strains is similar to that of pure riboflavin, taking into account growth rates and EGRAC values as indicators of the biological function of this vitamin. The addition of riboflavin-producing strains was shown to clearly improve the growth (Table 1) and riboflavin status of the depleted animals as shown by significant decreases to EGRAC values in rats supplemented with the engineered or native riboflavin-producing strains, where values reach similar levels as those seen in the non-depleted group (Fig. 3). Also, the riboflavin-producing strains were capable of curing hepatomegaly resulting from ariboflavinosis (Fig. 4).

The safety of use of novel strains must be addressed when they are to be proposed to be inserted into the food chain. In the present study no secondary effects were observed in animals fed the GM strains and haematological values, morphology of blood cells, and relative weight of organs of these animals were all similar to those obtained in the non-depleted groups. Only positive results were observed with the use of these strains, such as improved animal growth, EGRAC values and relative organ weight. The GM riboflavin-producing strain (*L. lactis* B_2++) has been the object of a complete biosafety assessment in our laboratory and has been shown to be innocuous to the host (LeBlanc *et al.* 2005). Current legislation in most countries does not allow the addition of live GM strains in food products for human consumption, strongly limiting the use of the overproducing strain used in the present study. However, the use of spontaneous mutants, such as the riboflavin-producing strain, is generally accepted, greatly improving the possibilities that this strain could be included in novel products in a relatively short timeframe.

The present study has provided the first animal trial with food containing living bacteria that were selected or engineered to produce extracellular riboflavin in the fermented product. These results pave the way for analysing the effect of similar riboflavin-overproducing LAB in human trials. The development of fermented foods containing increased levels of riboflavin, produced *in situ*, which eliminates the need for vitamin fortification is currently underway. Since 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, yoghurt, and cheeses in order to increase riboflavin concentrations is feasible and economically attractive since it would decrease the costs involved in current practices of vitamin fortification. The consumption of such products with increased levels of riboflavin on a regular basis could help prevent deficiencies of this important vitamin. Such products could decrease the costs incurred when mandatory fortification programmes are elaborated, such as those now in place in many industrialised countries.

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

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