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<th>Lactococcus lactis is capable of improving the riboflavin status in deficient rats</th>
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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 hepatomegaly 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 \textit{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 \textit{in situ}, which eliminates the need for vitamin fortification.

**Materials and methods**

**Bacterial strains, media and culture conditions**

\textit{L. lactis} strains NZ9000 (\textit{L. lactis} \textit{B2} –) and CB010 (\textit{L. lactis} \textit{B2} +) were grown (12 h at 30°C) in M17 medium (Biokar Diagnostics, Beauvais, France) supplemented with 0.5% glucose (M17-Glu). \textit{L. lactis} NZ9000 harbouring plasmid pNZGBAH (\textit{L. lactis} \textit{B2}++) (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 \textit{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 of \textit{L. lactis} were precipitated from 1 ml sample by adding 10% TCA. HPLC analysis (Isco model 2360; Teledyne Isco Inc., Lincoln, NE, USA) was performed using a C18 reverse-phase column (4 x 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 weaning 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 \textit{B2}/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 (\textit{Lactococcus lactis} \textit{B2} + and \textit{L. lactis} \textit{B2}+++, respectively) or the control strain (\textit{L. lactis} \textit{B2} –).

**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. Hae-
molysates (31.3 μl) were added to 1 ml potassium phosphate buffer (0.1 M; pH 7.4) containing 2.3 mM-ethylenediaminetetra-
acetic 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 Sigma-
Stat (SPSS Inc., Chicago, IL, USA). Comparisons of multiple 
comparisons were performed using the software package Sigma-
Statistics measured in triplicate for each sample.

**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 riboflavins pro-
duced by the bacterial strains was compared with that of pure 
riboflavin given to rats at levels previously considered negligible 
(0.5 mg/B2 kg diet) or at the daily recommended intake for such 
animals (3.0 mg B2/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* B2− showed significa-
cantly lower growth rates (2.78 (SD 0.17)) g/d) as compared with 
those fed *L. lactis* B2+ or 0.5 mg B2/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* B2− 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* B2− (HPLC determination). Interest-
ingly, the animals that received *L. lactis* B2+ showed 
statistically similar growth rates as the group that received 
0.5 mg B2/kg, suggesting that the riboflavin produced by this 
bacterial strain, given here at the same concentration as the com-
mercially available pure riboflavin, possesses similar bioavailability. 
The animals that received *L. lactis* B2++ 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 exper-
imental 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 ribofla-
vin 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 ribofla-
vlin status of rats, a validation test was performed where the 
EGRAC was followed at a weekly basis in animals fed with the

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| 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 (B2−), CB010 (B2−), or NZ9000 (pNZGBAH) (B2++) during 21 d (repletion period) (Mean values and standard deviations) |
|---------------------------------|------------------|------------------|------------------|------------------|
| **Depletion period**           | **Repletion period** |
| **Group**                      | **Growth rate (g/d)** | **Final weight (g)** | **Growth rate (g/d)** | **Final weight (g)** |
|                                | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| Non-depleted                   | 5.81a   | 0.35   | 138.0a  | 8.3    | 5.34a   | 0.32   | 299.2a  | 18.0   |
| Depleted                       | 4.08b   | 0.24   | 102.2b  | 6.1    | 0.68b   | 0.04   | 141.4b  | 8.5    |
| *L. lactis* (B2−)              |          |        |         |        | 2.78e   | 0.17   | 188.6e  | 11.3   |
| B2− (0.5 mg)                   |          |        |         |        | 3.19f   | 0.16   | 190.0f  | 9.5    |
| B2+ (3.0 mg)                   | 3.61a   | 0.22   | 204.9rd | 12.5   | 4.41a   | 0.26   | 224.0e  | 13.4   |
| *L. lactis* (B2++)             |          |        |         |        | 3.30c   | 0.20   | 200.0g  | 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 riboflavimin-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 B2++ or B2++ (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 B2−).

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 B2−) 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 B2+ and B2++) 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 B2−) (2·10 (SD 0·06)) (Fig. 3). Interestingly, the animals that received L. lactis B2+ showed statistically similar EGRAC values as the group that received 0·5 mg B2/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).

The animals that received L. lactis B2++ 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 B2/kg and those receiving 3·0 mg B2/kg (however, absolute values were lower in the 3·0 mg B2/kg group compared with the 0·5 mg B2/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 B2− 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 B2/kg (5·3 (SD 0·5) g). The groups supplemented with L. lactis B2+ or L. lactis B2++ showed no significant differences in relative liver weight compared with the non-depleted group or the group that received 3·0 mg B2/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 (B2+) or the overproducing strain (B2++), which have very important
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 B2⁺++) 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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