**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 B₂ consumer into a vitamin B₂ ‘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 B₂) 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 B₁₂, vitamin B₆, and other vitamins, which explains why plasma riboflavin is a determinant of folate, vitamin B₁₂, vitamin B₆, and other vitamind 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 B₂ consumer into a vitamin B₂ ‘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
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 B2−) and CB010 (L. lactis B2+) were grown (12 h at 30 °C) in M17 medium (Biokar Diagnostics, Beauvais, France) supplemented with 0.5 % glucone (M17-Glu). L. lactis NZ9000 harbouring plasmid pNZGBAH (L. lactis 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 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 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 B2/kg; Sigma, Buenos Aires, Argentina) during 42 d. The third group was a depressed–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 (Lactococcus lactis B2+ and L. lactis B2++, respectively) or the control strain (L. lactis B2−).

riboflavin, or (ii) wild-type or engineered riboflavin-producing lactic acid bacteria (LAB; L. lactis B2+ and L. lactis B2++, respectively) or (iii) the control strain (L. lactis B2−), which were grown in M17-Glu. Commercial riboflavin was added at concentrations equivalent to: (i) the residual riboflavin found in B2-free diets used in previous deficiency studies (0·5 mg B2/kg diet; Powers et al. 1991, 1993; Yates et al. 2001, 2003); (ii) the daily riboflavin requirement of laboratory rats (3·0 mg B2/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 5·0) mg/l for L. lactis B2−, B2+ and B2++, 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.

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 p. 262). At the end of the trial, animals were anaesthetised with an intraperitoneal injection of ketamin (50 mg/kg; Sigma, Buenos Aires, Argentina) and xylazine (5 mg/kg; 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
than 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 reac-
tion. 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.

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 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. The food consumption during the depletion and repletion
periods, a validation test was performed where the
bioavailability of riboflavin from native and
engineered *L. lactis* strains, a depletion–repletion rat bioassay
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periods, a validation test was performed where the
bioavailability of riboflavin from native and
cellular riboflavin, possesses similar bioavailability.
The animals that received *L. lactis* B2þ+ showed statistically similar growth rates as the group that received
0·5 mg B2/kg, suggesting that the riboflavin produced by this bact-
erial strain, given here at the same concentration as the commer-
cially available pure riboflavin, possesses similar bioavailability.
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-
vin status of rats, a validation test was performed where the
EGRAC was followed at a weekly basis in animals fed with the

Statistics
Comparisons were performed using the software package Sigma-
Stat (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 inde-
pendent trials and standard deviations (where *n* 30).

Animal growth during the depletion–repletion periods
It is well documented that rats, which are deprived of riboflavin,
**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)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Depletion period</th>
<th>Repletion period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth rate (g/d)</td>
<td>Final weight (g)</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Non-depleted</td>
<td>5.81 ± 0.35</td>
<td>138.0 ± 8.3</td>
</tr>
<tr>
<td>Depleted</td>
<td>4.08 ± 0.24</td>
<td>102.2 ± 6.1</td>
</tr>
<tr>
<td><em>L. lactis (B2 − )</em></td>
<td>2.78 ± 0.17</td>
<td>188.9 ± 11.3</td>
</tr>
<tr>
<td>B2 (0·5 mg)</td>
<td>3.61 ± 0.22</td>
<td>204.0 ± 12.5</td>
</tr>
<tr>
<td><em>L. lactis (B2++)</em></td>
<td>3.30 ± 0.20</td>
<td>200.0 ± 12.0</td>
</tr>
</tbody>
</table>

*a,b,c,d* Mean values within a column with unlike superscript letters are significantly different (*P<0.05*).
*a,b,c,d* 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.
Lactococcus lactis improves riboflavin status

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 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 0-044) 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

![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). ab,c,d Mean values with unlike letters are significantly different (P<0-05).](image)

![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 (B2 – ), CB010 (B2 +), or NZ9000 (pNZGBAH) (B2++)). Values are means, with standard deviations represented by vertical bars (n 10). ab,c,d Mean values with unlike letters are significantly different (P<0-05).](image)
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 riboflavinosis (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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