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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 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 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 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 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 B2 deficiency is common in many parts of the world, particularly in developing countries (Boisvert et al. 1993). Several studies have indicated that vitamin B2 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 B2 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 B2 deficiency, making the EGRAC a useful method for the diagnosis of vitamin B2 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% glucose (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 × 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 Bio-technicals 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 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 (Lactococcus lactis B2+, and L. lactis B2++, respectively) or the control strain (L. lactis 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

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**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 (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 (SD 0.1), 0.5 (SD 0.2) or 15 (SD 5) 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 (10%)–xylacin (2%) (3·0 ml/kg animal weight; 40:60, v/v; Alfa-san, Woerden, The Netherlands) and bled by cardiac puncture. Blood was transferred into tubes containing anticoagulant (heparin; Rivero, Buenos Aires, Argentina) and centrifuged (2000g 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 excited organs (liver, spleen and kidneys) were rinsed with 0·15 M-NaCl, weighed and stored at −70°C.
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 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
unit time 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 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 (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 bac-
terial strain, given here at the same concentration as the commer-
cially 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) μg/ml) was the highest used in the depleted–replete
animals. The food consumption during the depletion and reple-
tion 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-
vin 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
(B2—), CB010 (B2+), or NZ9000 (pNZGBAH) (B2++)) during 21 d (repletion period)
(Mean values and standard deviations)

<table>
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<tr>
<th>Group*</th>
<th>Growth rate (g/d)</th>
<th>Final weight (g)</th>
<th>Growth rate (g/d)</th>
<th>Final weight (g)</th>
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<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>B2—</td>
<td>5·81a</td>
<td>0·35</td>
<td>138·0a</td>
<td>8·3</td>
</tr>
<tr>
<td>L. lactis B2+</td>
<td>4·08b</td>
<td>0·24</td>
<td>102·2b</td>
<td>6·1</td>
</tr>
<tr>
<td>B2 (0·5 mg)</td>
<td>2·78c</td>
<td>0·17</td>
<td>188·9c</td>
<td>11·3</td>
</tr>
<tr>
<td>B2 (3·0 mg)</td>
<td>3·61d</td>
<td>0·22</td>
<td>204·0d</td>
<td>12·2</td>
</tr>
<tr>
<td>L. lactis (pNZGBAH) B2+</td>
<td>3·30e</td>
<td>0·20</td>
<td>200·0e</td>
<td>12·0</td>
</tr>
</tbody>
</table>

a,b,c,d,ef: 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.
Lactococcus lactis improves riboflavin status

**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). a,b,c,d Mean values with unlike letters are significantly different (P < 0.05).

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 arriboflavinosis 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

### Organ weight comparison

#### An increase in the weight of the liver in relation to body weight

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

#### 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.
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.

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

Acknowledgements

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