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ORIGINAL ARTICLE

Lactobacillus reuteri CRL 1098 prevents side effects produced by a nutritional vitamin B_{12} deficiency

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Abstract

Aims: To evaluate the efficiency of the vitamin B_{12}-producing *Lactobacillus reuteri* CRL1098 strain in preventing the symptoms caused by a nutritional cobalamin-deficient diet in pregnant female mice and their weaned offspring.
Methods and Results: Pregnant female mice were divided into three groups: animals fed with a B_{12}-deficient diet (DD), animals fed with DD plus *L. reuteri* CRL1098 and animals fed with a B_{12}-sufficient diet. The animals received the different feedings from the end of gestation up to weaning. At the end of the trials, they and their corresponding offspring were bled to determine haematological, immunological and histological parameters. The administration of the pseudovitamin B_{12}-producing strain prevented the symptoms observed in female and weaned young animals fed with a nutritional B_{12}-deficient diet.
Conclusions: Our data suggest that the pseudovitamin B_{12} produced by *L. reuteri* CRL1098 is biologically active and effective in preventing the pathologies caused by the nutritional deficiency of B_{12} both in pregnant mice and their offspring.
Significance and Impact of the Study: The ability of *L. reuteri* CRL1098 to prevent a nutritional vitamin deficiency was demonstrated for the first time. The addition of a GRAS micro-organism to complement the B_{12} content in deficient foods is an interesting biotechnological alternative.

Introduction

Lactobacillus reuteri (Reuter 2001) is a heterofermentative bacterium that resides in the gastrointestinal tract of humans and animals (Casas and Dobrogosz 2000) and is considered as one of the few truly autochthonous (indigenous) *Lactobacillus* species in humans (Dobrogosz *et al.* 1989; Mitsuoka 1992). The oral intake of *L. reuteri* revealed an effective gut colonization in healthy people that started rapidly within the first few days of ingestion but returned to low levels a few months after that the intake was discontinued (Wolf *et al.* 1995). *Lactobacillus reuteri* was also found in breast milk; the consumption of foods containing *L. reuteri* by nursing mothers could increase the concentration of these micro-organisms in breast milk and transfer these beneficial bacteria to the newborn (Peran *et al.* 2007).

Numerous studies have reported the probiotic properties of *L. reuteri,* such as prevention of human ulcerative colitis (Fabia *et al.* 1993), prevention of community-acquired diarrhoea in young children (Shornikova *et al.* 1997), reduction in bacterial translocation after acute liver failure (Wang *et al.* 1995), increase in intestinal resistance to *Cryptosporidium parvus* infection in immunodeficient mice (Alak *et al.* 1999) and regulation of serum cholesterol and triglycerides in both therapeutic and preventive treatments (Taranto *et al.* 1998, 2000).

Another important characteristic of *L. reuteri* is its ability to produce and excrete 3-hydroxypropionaldehyde (reuterin), a broad-spectrum antimicrobial agent, during anaerobic sugar-glycerol cofermentation (Talarico *et al.* 1988). The pathway for conversion of glycerol to 3-hydroxypropionaldehyde (3-HPA) is mediated through

a cobalamin-dependent glycerol dehydratase (Daniel *et al.* 1998).

In a previous paper, we reported the ability of *L. reuteri* CRL 1098 to produce a compound with vitamin B_{12} activity (Taranto *et al.* 2003). This finding constitutes the first evidence of vitamin B_{12} production in lactic acid bacteria (LAB). High-performance liquid chromatography coupled to an ultraviolet diode array detector, mass spectrometry and nuclear magnetic resonance spectroscopy enabled us to identify the compound as Coα-[α-(7-adenyl)]-Coβ-cyanocobamide or pseudovitamin B_{12} (Santos *et al.* 2007).

Vitamin B_{12} or cobalamin (deoxyadenosylcobalamin and methylcobalamin) is an important water-soluble vitamin belonging to the B complex group. Cobalamin, which is involved as a cofactor in a variety of enzymatic reactions and as a methyl donor in the synthesis of DNA and red blood cells, is essential for maintaining the integrity of the insulation sheath (myelin sheath) that surrounds the nerve cells. The clinical manifestations of cobalamin deficiency include haematological (megaloblastic anaemia, pancytopenia [leukopenia, thrombocytopenia]), neurological (peripheral neuropathy, demyelization of dorsal columns and corticospinal tract), psychiatric (irritability, personality change, mild memory impairment, dementia, depression, psychosis) and cardiovascular (increased risk of myocardial infarction and stroke) aspects. Vitamin B_{12} deficiency is frequent in strict vegetarians, in individuals with inadequate feeding and in those undergoing certain physiological states (pregnancy, old age), etc.

The cobalamin is exclusively synthesized by certain bacteria and archaea. In cattle, sheep and other ruminants, micro-organisms present in the rumen can synthesize cobalamin. Humans, however, do not have such microflora in their small intestine and must absorb the coenzyme from natural sources such as animal organ meats (especially liver and kidney), fish, eggs and pharmaceutical products (Herbert 1996). Albert *et al.* (1980) reported that some apparently healthy southern Indian subjects harboured in their small intestine micro-organisms of the genera *Pseudomonas* and *Klebsiella* that were able to synthesize cobalamin. These authors also found that none out of the 12 lactobacilli tested produced detectable levels of coenzyme B_{12}. In this respect, information concerning the ability of LAB to produce vitamins is very scarce. *Lactobacillus reuteri* CRL 1098 is the only described LAB capabable of producing detectable amounts (50 $\mu g\ l^{-1}$) of a compound with vitamin B_{12} activity. The synthesis of this kind of compound by a LAB strain is a surprising finding considering the auxotrophic characteristics of this bacterial group to several vitamins and amino acids (Deguchi and Morishita 1992). Up to now, industrial vitamin B_{12} production has been carried out using *Pseudomonas* or *Salmonella* species (Blanche *et al.* 1992; Raux *et al.* 2000). Unlike these organisms, LAB has GRAS (generally regarded as safe) status; therefore, the finding of a LAB strain able to produce cobalamin would be of remarkable importance for both the food industry and the medical and veterinary fields.

The purpose of the present study is to evaluate the efficiency of the pseudovitamin B_{12} produced by *L. reuteri* CRL 1098 to prevent the symptoms produced by a cobalamin nutritional deficiency in pregnant females and their weaning offspring by using an experimental murine model previously standardized in our laboratory (Molina *et al.* 2007).

Materials and methods

Micro-organisms and growth conditions

Lactobacillus reuteri CRL 1098 (patent PA040103130) used in this study belongs to the culture collection (CRL) of the Centro de Referencia para Lactobacilos (CERELA, Tucumán, Argentina). Before experimental use, the cultures were grown in sterile De Man Rogosa Sharpe (MRS) broth (De Man *et al.* 1960) and incubated at 37°C for 16 h.

Animals and diet groups

Six-week-old pregnant female BALB/C mice (11 days pregnancy calculated from the first contact with the male) obtained from the closed colony of the breeding unit kept at the CERELA Institute (San Miguel de Tucumán, Argentina) were individually housed in plastic cages ($20 \times 30 \times 15$ cm) with a litter tray of $20 \times 30 \times 6$ cm; the animals were maintained at 20 ± 2°C with a 12-h light/dark cycle.

The animals were randomly allocated to three main groups (each of five mice) as follows: B_{12}-deficient females that received a B_{12}-deficient diet (DF group); B_{12}-deficient females that received a B_{12}-deficient diet plus *L. reuteri* CRL 1098 (RF group); B_{12}-sufficient females that receiving a B_{12}-sufficient diet (CF or the control group). The B_{12}-deficient diet used in this study was provided by Biomedical Inc/ICN (Irvine, CA, USA) and its composition is described in Table 1. The B_{12}-sufficient diet was identical to the deficient one except that 1·3 $\mu g\ kg^{-1}$ of commercial vitamin B_{12} (Parafarm, Bs. As., Argentina) was added.

The animals were allowed free access to the diets and water. The animals in the RF group received *L. reuteri* CRL 1098 resuspended in water at a concentration of 10^7 cells per day per mouse. The LAB strain was

Table 1 Composition of the B_{12}-deficient diet

Component	g kg^{-1}
Vitamin-free casein	220·0
DL-methionine	3·0
Iodinated casein	0·5
l-cystine	2·0
Sucrose	632·0
Alphacel, non-nutritive bulk	50·0
Cottonseed oil	50·0
Choline chloride	1·0
Calcium carbonate	6·250
Manganese sulfate·$5H_2O$	0·180
Zinc carbonate	0·05
Cupric sulfate·$5H_2O$	0·025
Chromium potassium sulfate	0·022
Sodium fluoride	0·005
Sodium selenite	0·001
Salt mixture no. 2, U.S.P.XXII	40·0
Vitamin A acetate (500 000 IU g^{-1})	1·8
Vitamin D_2 (855 000 IU g^{-1})	0·125
dl-α-tocopherol acetate	22·0
Ascorbic acid	45·0
Inositol	5·0
Choline chloride	75·0
Menadione	2·25
p-aminobenzoic acid	5·0
Niacin	4·25
Riboflavin	1·0
Pyridoxine hydrochloride	1·0
Thiamine hydrochloride	1·0
Calcium pantothenate	3·0
Biotin	0·02
Folic acid	0·09

administered by gavage in a single daily dose (0·5 ml per day per mice). No anaesthetic was used for intragastrical administration.

The pregnant females in groups DF and RF were fed the B_{12}-deficient diet for 30 days from the middle of gestation (11th day after mating) up to weaning (21st day after birth of offspring). The animals in the CF group were fed the B_{12}-sufficient diet for the same period. With this feeding schedule no spontaneous abortions were observed in the DF female group in contrast to feeding the DF diet prior to or during mating (data not shown).

The females belonging to the CF and RF groups gave birth to *c.* 10 young ones (CY and RY groups, respectively), while the females in the DF group gave birth to *c.* five B_{12}-deficient young ones (DY group). The young remained with their mothers until weaning and were selected at random for the studies irrespective of sex.

Females (CF, DF and RF groups) continued to receive the corresponding diets during the suckling period. The feed intake (5·2 ± 0·6 g of feed per day) was similar in all groups. The offspring only received maternal milk.

The body weight of the females was recorded from the beginning of the feeding up to the weaning period. The body weight of the young was determined at the end of weaning (21-day-old young). The results are expressed in grams (g). All determinations in females and offspring were performed in groups of five and ten mice, respectively, for statistical validation. Determinations in the offspring were carried out during the weaning period.

Blood and organ collection

At the end of the trials, the females in each group and their corresponding offspring were anaesthetized with an intraperitoneal injection of ketamin (5%)–xylacin (2%) (2·0 ml kg^{-1} animal weight; 20 : 1 v/v; Bayer S.A.) and bled by cardiac puncture. Blood was transferred (1) into tubes with EDTA solution (anticoagulant) to determine haematological parameters and (2) into plastic centrifuge tubes for immunoassay vitamin B_{12} determination. The freshly excised small intestine was removed and processed for paraffin inclusion using the Sainte-Marie's (1962) technique.

Haematological determinations

Haematocrit (Hto) values and number of leukocytes and red blood cells were determined by hematocytometric methods. Differential cell counts were performed by counting 100 cells in blood smears stained with May Grünwald–Giemsa. Haemoglobin concentration was determined by colorimetric assays.

For reticulocyte examination (% Ret), equal volumes (100 μl) of blood and 1% brilliant cresol blue (BCB) were mixed and incubated at 37°C for 15 min. Blood sample smears were prepared on glass slides with 5 μl of the cell suspension. Ret was counted under a microscope (1000 × magnification) in 10 areas of the stained smears, corresponding to approximately 1000 red blood cells. The results are expressed as percentage of total red blood cells.

Determination of B_{12} in serum

The concentration of vitamin B_{12} was measured in serum samples by electrochemiluminescence immunoassay (ECLIA) on a Roche Elecsys 2010 automatic analyzer (Roche Diagnostics, Basel, Switzerland) at the Laboratorio de Análisis Clínicos de Alta Complejidad – Quevedo S.R.L. (Tucumán, Argentina). Results are expressed as pg ml^{-1}.

Histological studies

The small intestine was removed at the end of each treatment and processed by a modified Saint-Marie's

technique (1962). Briefly, tissues were fixed in 10% formalin in PBS (phosphate buffer sodium 0·1 mol l^{-1}, pH 7) for 48 h at room temperature and then dehydrated in successive alcohols baths (40%, 50%, 70%, 96% and 100%) for 20 min in each alcohol. Finally, the samples were cleared by passage through three consecutive xylene baths for 45 min each. The tissue was embedded in paraffin at 56°C for 3 h. Sectioning was carried out as usual, and tissue sections (3–4 μm) were placed on glass slides.

Determination of immunoglobulin A (IgA)-producing cells in the small intestine

The number of IgA-producing cells was determined in the histological slices of samples from the ileal region near the Peyer's patches by direct immunofluorescence test (DIFT; Vintiñi *et al.* 2000). The DIFT test was performed by using α-chain-specific FITC-conjugated anti-mouse IgA (Sigma-Aldrich, USA). Deparaffinized histological samples were incubated with the antibody dilution (1/100) in PBS solution for 30 min at 37°C. The samples were then washed thrice with PBS solution and examined by using a fluorescent light microscope. The results are expressed as the number of IgA-producing cells (positive: fluorescent cell) per 10 fields (magnification 100 ×). Results are the means of three histological slices for each animal.

Statistical analysis

A Student's test was used to compare the data form the vitamin B_{12}-deficient groups with the control group (females and young). Significant differences were considered at $P < 0·05$. Experimental data were expressed as mean ± SD.

The Ethical Committee for Animal Care at CERELA approved all animal protocols. All assays complied with the current laws of Argentina and followed the most recent recommendations of the Federation of European Laboratory Animal Science Associations.

Results

Animal weight

The B_{12}-deficient females (DF group) showed 25% less body weight than the females in the control group. In contrast, the deficient females that received *L. reuteri* CRL 1098 (RF group) showed a weight gain similar ($P > 0·05$) to the control females fed a B_{12}-sufficient diet (Table 2).

Likewise, the weaned young (DY group) from the DF group displayed 38% lower body weight compared with the weaned young (CY group) from the control females. Consumption of the probiotic strain by the deficient females (RF group) resulted in offspring (RY group) of sizes similar to those from the normal females (Table 2). A significant delay in growth (size = 55 mm) was also observed in the offspring of the DY group compared with the young from females in the CF (size = 65 mm) and RF (size = 66 mm) groups fed a B_{12}-sufficient and -deficient diet plus *L. reuteri* CRL 1098, respectively.

Table 2 Effect of the maternal vitamin B_{12} deficiency on the body weight of mouse females (A) and their corresponding offspring (B)

Experimental group*		Body weight (g)
A	CF	36 ± 1·5
	DF	27 ± 1·2†
	RF	35·0 ± 1·9
B	CY	13 ± 1·4
	DY	8 ± 0·9†
	RY	13·4 ± 1·6

*CF, B_{12}-sufficient females (control group); DF, B_{12}-defficient females; RF, B_{12}-defficient females treated with *Lactobacillus reuteri* CRL 1098; CY, weaned young coming from B_{12}-sufficient females; DY, weaned young from B_{12}-deficient females; RY, weaned young from B_{12}-deficient females, which were treated with *L. reuteri* CRL 1098.
†$P < 0·05$ *vs* control group.
Values are means ± SD. Female mouse group: $n = 5$ (each one); young groups: $n = 30$ (each one).

Haematological determinations

The haematological values of females and their offspring are shown in Table 3. The B_{12}-deficient females (DF group) showed a significant decrease ($P < 0·05$) in Hto (23%), Ret percentage (37%) and haemoglobin (34%)

Table 3 Haematological parameters of females (A) and their corresponding offspring (B)

Experimental group*		Haematocrit (%)	Reticulocytes (%)	Haemoglobin (g dl^{-1})
A	CF	50·2 ± 4·0	4·3 ± 0·3	15·7 ± 0·8
	DF	38·3 ± 1·1†	2·7 ± 0·3†	10·3 ± 1·2†
	RF	53·0 ± 4·9	3·9 ± 0·6	14·5 ± 1·1
B	CY	43·7 ± 3·1	4·1 ± 0·4	12·0 ± 1·2
	DY	33·5 ± 2·1†	2·2 ± 0·2†	8·2 ± 2·0†
	RY	43·0 ± 2·6	3·8 ± 0·5	11·4 ± 2·0

*CF, B_{12}-sufficient females (control group); DF, B_{12}-defficient females; RF, B_{12}-defficient females treated with *Lactobacillus reuteri* CRL 1098; CY, weaned young coming from B_{12}-sufficient females; DY, weaned young from B_{12}-deficient females; RY, weaned young from B_{12}-deficient females, which were treated with *L. reuteri* CRL 1098.
†$P < 0·05$ *vs* control group.
Values are means ± SD. Female mouse group: $n = 5$ (each one); young groups: $n = 30$ (each one).

values compared with the CF group. In contrast, the deficient females that received *L. reuteri* CRL 1098 (RF group) showed a normalization in the haematological parameters assayed with values similar ($P > 0{\cdot}05$) to those in the normal animals (control group). A similar tendency was observed in the corresponding weaned young.

No statistically significant differences ($P > 0{\cdot}05$) in the total number of leukocytes between the B_{12}-deficient and the -sufficient groups (females and young) were observed (data not shown).

Serum vitamin B_{12} levels

The concentration of vitamin B_{12} in serum samples from the DY group ($275{\cdot}2 \pm 38$ pg ml^{-1}) was significantly lower ($P < 0{\cdot}001$) than in the ones from the control group ($735{\cdot}3 \pm 47$ pg ml^{-1}; Table 4). The weaned young from the deficient females treated with the probiotic strain (RY group) showed values similar ($P > 0{\cdot}05$) to those of the young from the B_{12}-sufficient females (Table 4). A similar behaviour was found in the corresponding female groups (data not shown).

Determination of IgA^+ cells in the small intestine

The number of IgA^+ cells in the small intestine of females and their offspring are shown in Table 5. A significant decrease ($P < 0{\cdot}05$; 27% and 47%) in the number of the IgA-producing cells in the B_{12}-deficient animals (DF and DY groups, respectively) compared with the control groups (CF and CY) was observed. The number of IgA-producing cells in the deficient females fed *L. reuteri* CRL 1098 and their weaned young showed enhanced values similar ($P > 0{\cdot}05$) to those in the control groups.

Discussion

Diseases caused by serious vitamin deficiencies frequently occur in cases of inadequate feeding leading to undernourishment. In particular, vitamin B_{12} deficiency can cause severe pathologies, some of them being irreversible. This deficiency is often found among strict vegetarians (Koebnick *et al.* 2004). It is well documented that animals deprived of vitamin B_{12} exhibit growth retardation (Ebara *et al.* 2001) besides immune system malfunctions and severe haematological and neurological abnormalities (Stabler 2000). During pregnancy and lactation, micronutrient deficiencies are one of the major complications promoting infectious processes owing to the high nutritional requirements to support foetal and infant growth as well as maternal metabolism. Casella *et al.* (2005) reported insidious developmental regression in 6-month-old infants from strictly vegetarian mothers. The infants were exclusively breastfed. The clinical symptoms were normalized after commercial vitamin B_{12} administration.

In the present study, the efficiency of *L. reuteri* CRL supplementation to correct the nutritional vitamin B_{12} deficiency in pregnant females and thus to prevent irreversible damage to the offspring was demonstrated using an in-house animal model based on a vitamin B_{12}-deficient diet.

In the experimental animal model, the nutritional vitamin B_{12} deficiency caused a significant reduction in the haematological parameters (haemoglobin, haematocrit and reticulocyte values) and anthropometric alterations in pregnant females compared with the control animals that were fed a B_{12}-sufficient diet. Moreover, the deficient females gave birth to a smaller number of young ones, which showed growth retardation (smaller size) and a diminution in haematological values. These results constitute a strong evidence of the way in which the nutritional status of the mother affects the normal development of her offspring. Severe growth retardation in weaned young

Table 4 Serum vitamin B_{12} concentration of the weaned young

Experimental group*	Vitamin B_{12} (pg ml^{-1})
CY	735·3 ± 47
DY	275·2 ± 38†
RY	673·6 ± 34

*CY, weaned young coming from B_{12}-sufficient females; DY, weaned young coming from B_{12}-deficient females; RY, weaned young from B_{12}-deficient females, which were treated with *Lactobacillus reuteri* CRL 1098.

†$P < 0{\cdot}05$ *vs* control group.

Values are means ± SD. Young groups: $n = 30$ (each one).

Table 5 Effect of the maternal vitamin B_{12} deficiency on the number of immunoglobulin A (IgA)-producing cells of mouse females (A) and their corresponding offspring (B)

Experimental group*		Number of IgA^+ cells per 10 field
A	CF	92 ± 4·5
	DF	67 ± 4·2†
	PD	90 ± 3·9
B	CY	72 ± 4·4
	DY	38 ± 3·9†
	PY	62 ± 3·6

*CF, B_{12}-sufficient females (control group); DF, B_{12}-defficient females; RF, B_{12}-deficient females treated with *Lactobacillus reuteri* CRL 1098; CY, weaned young coming from B_{12}-sufficient females; DY, weaned young from B_{12}-deficient females; RY, weaned young from B_{12}-deficient females, which were treated with *L. reuteri* CRL 1098.

†$P < 0{\cdot}05$ *vs* control group

Values are means ± SD. Female mouse group: $n = 5$ (each one); young groups: $n = 30$ (each one).

as a result of this kind of nutritional deficiency was also reported by other authors (Wagnon *et al.* 2005; Nakao *et al.* 2006).

The females that received the deficient diet plus *L. reuteri* CRL 1098 improved their general condition with weight gain and haematological values similar to those of the normal group females (control). The prevention of B_{12} deficiency in the pregnant females fed with the probiotic strain together with the deficient diet was evidenced particularly in the offspring. These weaned young showed normal haematological values and anthropometric parameters (weight and size) similar to the young from the normal females. The serum vitamin B_{12} level was similar to the values found in the normal young. It is important to emphasize here that the number of offspring was the same as in the case of the normal females. These results demonstrate clearly that the pseudovitamin B_{12} produced by *L. reuteri* CRL 1098 is biologically active for the host and that its efficacy is comparable with that of the commercial vitamin B_{12}.

Nutritional deficiencies during critical periods of gestation, neonatal maturation and weaning impair the normal development and differentiation of the immune system (Keusch 2003; Cunningham-Rundles *et al.* 2005). As the immune system is immature at birth, malnutrition in childhood can have long-term effects on health (Neumann *et al.* 2004). In our experimental model, vitamin B_{12} deficiency caused histological alterations in the small intestine and a decrease in the number of IgA-producing cells of the females and, consequently, in their offspring. Both alterations (intestinal damage and number of IgA^+ cells) were prevented in the pregnant females as well as their respective offspring by the consumption of *L. reuteri* CRL 1098 together with the B_{12}-deficient diet. These results confirm that vitamin B_{12} has immunomodulating functions as reported Bhaskaram (2002).

In conclusion, we demonstrated that the pseudovitamin produced by *L. reuteri* CRL 1098 has vitamin B_{12} activity. The consumption of the producer strain by pregnant females after weaning prevented the pathologies caused by cobalamin deficiency not only in the females but also in the breastfed young.

The prevention of a nutritional vitamin deficiency using a LAB strain has been demonstrated for the first time. The use of *L. reuteri* CRL 1098, a probiotic micro-organism, to develop natural foods bio-fortified with vitamin B_{12} would be an interesting biotechnological alternative for the functional foods market.

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