Soybean-based functional food with vitamin B\textsubscript{12}-producing lactic acid bacteria

Verónica Molina\textsuperscript{a}, Marta Médici\textsuperscript{a}, Graciela Font de Valdez\textsuperscript{a,b}, María Pía Taranto\textsuperscript{a,}\textsuperscript{*}

\textsuperscript{a}Centro de Referencia para Lactobacilos (CERELA) – CONICET, Chacabuco 145, T4000LC San Miguel de Tucumán, Argentina
\textsuperscript{b}Cátedra de Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, San Miguel de Tucumán, Argentina

ARTICLE INFO

Article history:
Received 16 January 2012
Received in revised form
23 May 2012
Accepted 24 May 2012
Available online 27 June 2012

Keywords:
Functional food
Lactobacillus reuteri
Soy-based beverage
Vitamin B\textsubscript{12}

ABSTRACT

Consumption of soy products has increased greatly due to their beneficial health effect. However, soy does not contain vitamin B\textsubscript{12} (B\textsubscript{12}), an important water-soluble vitamin vital to prevent severe pathologies, some of which are irreversible. In this study a novel soymilk beverage containing a compound with B\textsubscript{12} activity-producer strain (\textit{Lactobacillus reuteri} CRL 1098) to prevent the pathologies caused by a B\textsubscript{12}-deficient diet was evaluated using an experimental murine model. Pregnant females were divided into four groups. Besides the B\textsubscript{12}-deficient and the control group, the animals in the remaining two groups received non-fermented soymilk and soymilk fermented with \textit{L. reuteri} CRL 1098 from the end of gestation to weaning. At the end of the trials, females and their corresponding offspring were sacrificed to determine haematological, immunological and histological parameters. The results showed that the administration of fermented soymilk prevented the development of all symptoms observed as a consequence of nutritional B\textsubscript{12} deficiency both in females mice and in their respective offspring. The design of soybean-based functional food biofortified with vitamin using a lactobacilli strain able to produce compounds with B\textsubscript{12} activity constitutes an interesting alternative therapy to prevent vitamin deficiency.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

The beneficial effect (probiotic) that some lactic acid bacteria (LAB) exert on the health of consumers has led to the developing of certain functional foods. Although the LAB are commonly associated with dairy products, this group of microorganisms also plays a role in other food systems such as sausages and drinks, food conservation, olive ripening and sourdough bread, among others. The versatility of LAB encourages scientists to search for new applications to obtain novel products for a continuously growing functional food market.

Besides dairy products, soy-based beverages are an interesting option in nutrition considering their high nutritional value, the quality of the protein and amino acids and the low production costs of soybeans. However, this substrate presents some disadvantages such as low vitamin content, mainly the water-soluble vitamin B\textsubscript{12} (Riaz, 2006) belonging to the B complex group. This vitamin 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. It is essential to maintain the integrity of the insulation sheath (myelin sheath) that surrounds the nerve cells (Miller, Korem, Almog, & Galboiz, 2005).

A diet sufficient in vitamin B\textsubscript{12} is essential to prevent severe pathologies, some of which are irreversible. It has been reported that vitamin B\textsubscript{12}-deficiency in the diet of pregnant females causes severe retardation of myelination in the nervous system and brain atrophy in the infants (Lovblad, Ramelli, &
During infancy, this deficiency may cause failure to thrive, irritability and delay of neurological development, convulsions, and even severe megaloblastic pancytopenia due to a delayed DNA synthesis and myelination defects (Grattan-Smith, Wilcken, Procopis, & Wise, 1997; Ramussen, Fernboff, & Scanlon, 2001; Taskesen, Yaramis, Pirinccioglu, & Ekici, 2011). Cobalamin is exclusively synthesized by certain bacteria and archaea. In cattle, sheep, and other ruminants, microorganisms present in the rumen can synthesize cobalamin. Humans, however, do not have such microflora in their small intestine and they must absorb the coenzyme from natural sources such as animal organs (especially liver and kidney), fish, eggs, and pharmaceutical products (Watanabe, 2007).

In a previous paper, we reported the ability of Lactobacillus reuteri CRL 1098 to produce a compound with vitamin B12 activity (Taranto, Vera, Hugenholtz, Font, & Sesma, 2003). This finding constitutes the first evidence of vitamin B12 production in LAB. Subsequently, Santos et al. (2007) confirmed that the corrinoid produced by this LAB strain under anaerobic conditions is a Co-[α-(7-adenyl)]-Co-cyanocobamide or pseudovitamin B12. Recently, Molina, Medici, Taranto, and Font de Valdez (2008b) demonstrated that the corrinoid produced by L. reuteri CRL 1098 is biologically active and effective in preventing the development of pathologies caused by nutritional vitamin B12 deficiency in both pregnant mice and their weaned young. Besides this attractive functional property, this strain also has important technological and probiotic characteristics, e.g., easy and low-cost biomass production; ability to grow on different food substrates; capacity to bear environmental stress and reduce cholesterol levels (Taranto, Font de Valdez, & Perez-Martinez, 1999, 2006).

The aim of this study was to develop a soy-bean-based functional food using L. reuteri CRL 1098 – a cobalamin producer lactobacilli – and to evaluate the efficiency of the biofortified soy milk obtained to prevent the symptoms produced by nutritional vitamin B12 deficiency in pregnant females and their weaning offspring by using a previously standardized experimental murine model (Molina, Medici, Taranto, & Font de Valdez, 2008a).

2. Materials and methods

2.1. Microorganisms and growth conditions

The strain used in this study, L. reuteri CRL 1098, belongs to Centro de Referencia para Lactobacilos culture collection (CRL) (CERELA-CONICET, Tucumán, Argentina). Before experimental use, cultures were grown in sterile MRS broth (De Man, Rogosa, & Shape, 1960) and incubated at 37 °C for 16 h.

2.2. Preparation of fermented soymilk

A commercial soymilk (SM) (kindly provided by Refinería de Maíz, UNILEVER) was used as a substrate. The composition (per 100 ml) is as follows: protein, 2.6 g; carbohydrates, 4 g; lipids, 1.5 g; fibre, 0.6 g; Ca, 48 mg; Fe, 0.84 mg; P, 48 mg, and Mg, 18 mg; pH 7.3. Sterile SM (115 °C for 20 min) was cooled to 37 °C, inoculated (1%, v/v) with L. reuteri CRL 1098, and incubated at 37 °C for 6 h. The fermented SM had a final pH of 6.8 and a total colony counts of 1.6 × 10⁷ cfu/ml. The concentration of vitamin B12 in the SM fermented was determined with a quantitative bioassay that is used for the assessment of B12 in food (Kelleher & Broin, 1991). Briefly, L. delbrueckii subsp. lactis ATCC 7830, a strain that requires cobalamin, was used to evaluate this vitamin content in the fermented and unfermented SM. Quantification analyses using a cyanocobalamin standard curve indicated that the fermented SM contains approximately 20 μg of cobalamin/litre. The vitamin was not detected in the unfermented SM (control).

2.3. Animals

Six-week old pregnant female BALB/c mice (fourteen-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 × 30 × 15 cm) and maintained at 20 ± 2 °C with a 12-h light/dark cycle.

The animals were randomly allocated to four main groups (each of five mice) as follows: B12-deficient females that received a B12-deficient diet (DF group); B12-sufficient females that received a B12-deficient diet plus soy milk supplemented with 1.3 μg per kg of diet of commercial vitamin B12 (Parafarm, Buenos Aires, Argentina) (CF group, control group); B12-deficient females that received a B12-deficient diet plus non-fermented soymilk (SF group); B12-deficient females that received a B12-deficient diet plus soymilk fermented with L. reuteri CRL 1098 (10⁷ cells/day/mouse) (SRF group). The B12-deficient diet used in this study was provided by Biomedical Inc/ ICN (Irvine, CA, USA). The animals were allowed free access to the diets and water for 30 days from the middle of gestation (day 11th from mating) up to weaning (day 21st after offspring birth).

The administration of soy milk (B12-supplemented, fermented, and non-fermented) to females was carried out by gavage (0.5 ml/day/mice).

The females belonging to the CF and SRF groups gave birth to ca. ten young (CY and SRY group, respectively) while the females in the DF and SF groups gave birth to ca. five B12-deficient young (DY and SY group, respectively). The young remained with their mothers until weaning and were selected at random for the studies without considering sex. Females (DF, CF, SF, and SRF groups) continued to receive their corresponding diets during the suckling period. Feed intake (5.2 ± 0.6 g feed/day) was similar in all groups. Offspring received only maternal milk.

The body weight of the females was recorded at 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). Results were expressed in grams (g). All determinations in females (mothers) and offspring were performed in five and ten mice/group, respectively, for statistical validation. Determinations in the offspring were carried out during the weaning period (21-days old young).

2.4. Blood and organ collection

At the end of the trials, the females in each group and their corresponding offspring were anesthetized with an intraperi-
tonal injection of ketamin (5%) – xylacin (2%) (2.0 ml/kg animal weight; 20:1 v/v) (Bayer S.A) and bled by cardiac puncture. Blood was transferred into tubes with ethylenediaminetetraacetic acid (EDTA) solution (anticoagulant) to determine haematological parameters and into plastic centrifuge tubes for determination of vitamin B₁₂ by immunoassay.

Freshly excised small intestine was removed and processed for paraffin inclusion following the technique developed by Saint-Marie (1962).

2.5. Haematological parameters and serum vitamin B₁₂ levels determination

Haematokrit (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 colourimetric assays.

For examination of reticulocytes (% 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. The Ret were counted under a microscope (1000X magnification) in ten areas of the stained smears, corresponding to approximately 1000 red blood cells. Results are expressed as percentage of total red blood cells.

Vitamin B₁₂ concentration was measured in serum samples by electrochemiluminescence immunoassay (ECLIA) on a Roche Elecsys 2010 automatic analyzer (Roche Diagnostics, Basel, Switzerland) at the Laboratory for High Complex Clinical Analysis – Quevedo S.R.L. (Tucumán, Argentina). Results are expressed as pg/ml.

2.6. Histological studies

The small intestine was removed at the end of each treatment and processed by modified Saint-Marie’s technique (1962). Briefly, tissues were fixed in 10% formalin in phosphate saline solution (PBS) for 48 h at room temperature and then dehydrated in successive alcohols baths (40%, 50%, 70%, 96% and 100%) for 20 min each alcohol. Finally, samples were cleared by passing 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.

2.7. Determination of IgA-producing cells in the small intestine

The number of IgA-producing (IgA+) cells was determined on histological slices of samples from the ileal region near Peyer’s patches by direct immunofluorescence test (DIFT) (Vinderola, Perdiguón, Duarte, Farnworth, & Matar, 2006). The test was performed by using γ-chain specific FITC conjugated anti-mouse IgA (Sigma–Aldrich, St. Louis, MO, USA). Deparaffinised historical samples were incubated with the antibody dilution (1/100) in PBS (Phosphate Buffer Sodium 0.1 M, pH 7) solution for 30 min at 37 °C. Samples were then washed three times with PBS solution and examined by using a fluorescent light microscope. Results were expressed as the number of IgA+ cells (positive: fluorescent cell) per 10 fields (magnification 100X). Results were the mean of three histological slices per animal.

2.8. Statistical analysis

A Student’s test was used to compare the data from vitamin B₁₂ deficient groups with the control group (females and offspring). 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.

3. Results

3.1. Animal weight

The body weight of mouse females and their corresponding offspring are shown in Table 1. Females that received the L. reuteri CRL 1098-fermented soymilk (SRF Group) and the B₁₂ deficient diet showed a body weight similar to normal animals (CF group) that received soymilk supplemented with commercial vitamin B₁₂. In contrast, body weight was significantly lower in deficient females (DF group) and this smaller development as a consequence of the B₁₂-deficit was also found in the females that received the unfermented soymilk with the deficient diet (SF group).

On the other hand, the weaned young of females that received the deficient diet and the fermented soymilk (SRY group) reached a higher body weight than the offspring from

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>DF</td>
<td>24.3 ± 1.6a</td>
</tr>
<tr>
<td>CF</td>
<td>37.5 ± 0.6b</td>
</tr>
<tr>
<td>SF</td>
<td>28.7 ± 1.5a</td>
</tr>
<tr>
<td>SRF</td>
<td>36.2 ± 1.2b</td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>DY</td>
<td>8.9 ± 0.8a</td>
</tr>
<tr>
<td>CY</td>
<td>13.1 ± 1.3a</td>
</tr>
<tr>
<td>SY</td>
<td>9.6 ± 1.2a</td>
</tr>
<tr>
<td>SRY</td>
<td>12.5 ± 0.9b</td>
</tr>
</tbody>
</table>

Values are means ± SD. Females mouse group: n = 5 (each one); young groups: n = 30 (each one). Values with different superscript letters (a and b) indicate significant differences among groups (p < 0.05). DF, B₁₂-deficient females; CF, females receiving a B₁₂-deficient diet plus soymilk supplemented with commercial vitamin B₁₂ (control group); SF, females receiving a B₁₂-deficient diet plus non-fermented soymilk; SRF, females receiving a B₁₂-deficient diet plus soymilk fermented with L. reuteri CRL 1098; DY, weaned young coming from B₁₂-deficient females; CY, weaned young coming from B₁₂-deficient females fed with unfermented soymilk; SY, weaned young coming from B₁₂-deficient females fed with the soymilk fermented with L. reuteri CRL 1098.
females fed a deficient diet plus water or unfermented soymilk (DY and SY group, respectively). The values obtained for the SRY group were statistically similar ($p > 0.05$) to those found in normal young from control females (CY group) that received soymilk supplemented with commercial vitamin B$_{12}$.

3.2. Haematological determinations

The haematological values of females and their corresponding offspring are shown in Table 2. Vitamin B$_{12}$ deficiency caused a decrease in the values of all haematological parameters evaluated (DF group) and this diminution was not normalized when the animals received the unfermented soymilk with the deficient diet (SF group). On the contrary, the females that received the deficient diet supplied with soymilk fermented with L. reuteri CRL 1098 displayed a haematological pattern similar to the one in the normal animals (CF group) that received commercial vitamin B$_{12}$. No statistically significant differences ($p > 0.05$) in the total number of leukocytes between the B$_{12}$-deficient and the B$_{12}$-sufficient groups (females and young) were observed (data not shown).

3.3. Determination of serum vitamin B$_{12}$ levels

The weaned young proceeding to the deficient females treated with soymilk fermented with L. reuteri CRL 1098 (SRY group) showed values of serum vitamin B$_{12}$ similar ($p > 0.05$) to those found in the offspring of the normal females that received soymilk supplemented with commercial vitamin B$_{12}$ (Table 3). In contrast, the consumption of unfermented soymilk by deficient females did not normalize the serum vitamin B$_{12}$ levels in their corresponding offspring (SY group), which showed values similar to those found in the weaned young of females that only received the deficient diet (Table 3).

3.4. Determination of IgA-producing cells in the small intestine

A remarkable decrease in the number of IgA-producing (IgA+) cells in the small intestine of deficient females in the DF and SF groups was observed. In contrast, the females that received soymilk fermented with L. reuteri CRL 1098 (SRF group) showed IgA+ cells numbers similar to those of the normal females that received a commercial vitamin B$_{12}$ (CF group). The analysis of pups samples showed that though the SRY group did not reach the values found in the control young (CY), the same ones were significantly higher than those obtained for the offspring belonging to the DY and SY groups (Table 4B).

Besides the decrease in the IgA+ cells number caused by nutritional B$_{12}$ deficiency, the small intestine of females in the DF and SF groups showed severe alterations in the gut mucosa (size of villi, oedema and leukocyte infiltrations). In contrast, the females that received soymilk fermented with L. reuteri CRL 1098 together with the deficient diet presented a normal histological structure of the small intestine, similar to the control animals that received soymilk supplemented with commercial vitamin B$_{12}$.

A similar behaviour was found in the corresponding young groups.

4. Discussion

The development of functional foods (FF) constitutes an important alternative to improve the quality of the diet

<p>| Table 2 – Effect of the oral administration of soymilk fermented with Lactobacillus reuteri CRL 1098 on the haematological parameters of murine females (A) and their corresponding offspring (B). |</p>
<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Hto (%)</th>
<th>Ret (%)</th>
<th>Hb (g dl$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF</td>
<td>37.8 ± 2.6$^a$</td>
<td>2.7 ± 0.3$^a$</td>
<td>11.8 ± 1.6$^a$</td>
</tr>
<tr>
<td>CF</td>
<td>50.2 ± 5.6$^a$</td>
<td>4.8 ± 0.5$^a$</td>
<td>15.0 ± 2.4$^a$</td>
</tr>
<tr>
<td>SF</td>
<td>38.4 ± 3.2$^a$</td>
<td>2.8 ± 0.2$^a$</td>
<td>12.1 ± 1.5$^a$</td>
</tr>
<tr>
<td>SRF</td>
<td>48.6 ± 5.1$^a$</td>
<td>4.2 ± 0.7$^a$</td>
<td>14.5 ± 2.1$^a$</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DY</td>
<td>35.8 ± 3.3$^a$</td>
<td>2.2 ± 0.2$^a$</td>
<td>8.2 ± 1.5$^a$</td>
</tr>
<tr>
<td>CY</td>
<td>48.2 ± 2.5$^a$</td>
<td>4.3 ± 0.4$^a$</td>
<td>12.0 ± 1.2$^a$</td>
</tr>
<tr>
<td>SY</td>
<td>38.0 ± 1.9$^a$</td>
<td>2.3 ± 0.4$^a$</td>
<td>8.5 ± 1.3$^a$</td>
</tr>
<tr>
<td>SRF</td>
<td>45.2 ± 3.3$^a$</td>
<td>3.9 ± 0.3$^a$</td>
<td>10.8 ± 2.1$^a$</td>
</tr>
</tbody>
</table>

Values are means ± SD. Females mouse group: n = 5 (each one); young groups: n = 30 (each one). Values with different superscript letters (a and b) indicate significant differences among groups ($p < 0.05$).

<p>| Table 3 – Vitamin B$_{12}$ serum concentration in weaned young. |</p>
<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Vitamin B$_{12}$ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DY</td>
<td>275 ± 35$^a$</td>
</tr>
<tr>
<td>CY</td>
<td>735 ± 24$^b$</td>
</tr>
<tr>
<td>SY</td>
<td>265 ± 13$^a$</td>
</tr>
<tr>
<td>SRY</td>
<td>588 ± 24$^b$</td>
</tr>
</tbody>
</table>

Values are means ± SD. Groups: n = 30 (each one). Values with different superscripts are significantly different ($p < 0.05$).

DY, weaned young coming from B$_{12}$-deficient females; CY, weaned young coming from B$_{12}$-sufficient females; SY, weaned young coming from B$_{12}$-deficient females fed with unfermented soymilk; SRY, weaned young coming from B$_{12}$-deficient females fed with soymilk fermented with L. reuteri CRL 1098.
considering that food selection can exert a positive influence on well-being and health. The development of foods fermented with selected Lactobacillus and Bifidobacterium strains constitutes an important sector in the FF market. At present, the development of dairy products using LAB probiotics is an important topic with relevant implications in the industrial and commercial areas. Nevertheless, one of the current trends in the FF industry is the diversification of the substrate used as a vehicle of the probiotic microorganisms to replace the animal origin vehicles by others of vegetable origin. In this way, the use of oat (Angelov, Gotcheva, Kuncheva, & Hristozova, 2006), millet (Lei, Friis, & Michaelsen, 2006) and cereal extracts (Charalampopoulos, Pandiella, & Webb, 2003) as alternative fermentation substrates for BAL has been reported. Within the potential vegetable vehicles, soybean constitutes an interesting alternative considering its valuable nutritional characteristics (Sarkar & Li, 2003; Squadrito et al., 2003). It is known that the organoleptic and health beneficial properties of soybean-based products can be improved by using selected probiotic bacteria for the development of fermented products (Wang, Yu, & Chou, 2006). However, soybean lacks essential micronutrients such as vitamin B12. In the present work, we designed a soy-based functional food with pseudovitamin B12-producing LAB (L. reuteri CRL 1098) to restore the lack of vitamin B12 in this vegetable food.

During pregnancy and lactation, micronutrient deficiencies are one of the major complications promoting infection processes due to the high nutritional requirements needed to support foetal and infant growth as well as maternal metabolism. Since nutritional factors during early infancy cause short and long term effects, the nourishment of mothers, breast-fed babies and small children has great biological importance. According to the experimental animal model described by Molina et al. (2008a), a vitamin B12-deficient diet during gestation and lactation causes several clinical and haematological alterations in mouse dams and their corresponding offspring. In this work, both the females and their offspring that received unfermented soy milk presented the typical symptoms of vitamin B12 depletion. Moreover, the consumption of soymilk fermented with L. reuteri CRL 1098 for mice feeding with a B12-deficient diet prevented the development of all manifestations that characterize this nutritional deficiency. The females fed with the fermented product and their offspring showed absence of anaemia and normal body development similar to normal animals. These results would demonstrate that the soymilk was bio-fortified in situ and that its consumption had a biologically positive effect on the host.

On the other side it has also been established that nutritional deficiency is commonly associated with an impaired immune response (Jayarajan & Daly, 2011). Non-specific mechanisms as intestinal microbiota balance, anatomical barriers (mucosa and epithelium) and secretory substances (lysozymes and mucus) are affected by malnutrition (Erickson, Medina, & Hubbard, 2000). Some authors (Gauffin Cano & Perdigón, 2003; Maldonado Galdeano et al., 2011) reported that some of these alterations can be corrected by the administration of selected LAB strains. In this work we demonstrated that the administration of soymilk fermented with L. reuteri CRL 1098 reduced the histological and immunological intestinal alterations caused by B12 nutritional deficiency obtaining IgA+ cells values close to the control animals in females receiving the fermented soymilk and their respective weaned young. These results would prove the bioavailability of the vitamin produced by this strain.

Considering that actually the consumption of soybean is important and that LAB are GRAS (generally regarded as safe) microorganisms, the design of a soybean product bio-fortified with vitamin B12 will contribute to modify the nutritional habits of the population and to diversify the local diet, obtaining all the advantages of a complete vegetable food.

Acknowledgement

This project was supported by grants of CONICET, FONCYT and CIUNT from Argentina.

REFERENCES


