Inflammatory properties of almond milk fermented with potentially probiotic bacteria.

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ABSTRACT

A fermented product was developed by using almond “milk” produced by soaking and grinding almonds cv. Marcona by using different potentially probiotic bacteria. An in vitro digestion/Caco-2 cell model was used to evaluate the effect of not fermented almond milk (NFAM) and the fermented products on the mitochondrial enzymes activities (test MTT) of enterocytes. Otherwise, macrophages (RAW 264.7 cells) were challenged with the digests and the production of pro-inflammatory biomarkers (TNFα and IL-6) was determined by ELISA. A commercial milk-based infant formula (MBIF) providing B. bifidus was tested for comparison.

MTT conversion values were increased in cell cultures exposed to almond milks, fermented or not, but decreased in those incubated with the digests from MBIF. There was a significant (P<0.05) decrease in the production of TNFα by macrophages exposed to digests from fermented almond milks inoculated with B. bifidum CECT 870 (5.8 ng/ml) or B. longum CECT 4551 (4.2 ng/ml) relative to NFAM and MBIF, which caused similar TNFα concentrations in cell culture supernatants (7.3-7.9 ng/ml). Macrophage cultures exposed to NFAM exhibited the highest IL-6 production, and MBIF produced IL-6 concentrations similar to almond milk inoculated with L. plantarum. Almond milk inoculated with both bifidobacteria caused the lowest production of IL-6.

The results indicate that fermented almond milk favours energetic cell metabolism of enterocytes and had lower inflammatory potential than MBIF suggesting healthy benefits from those in managing cow-milk allergy/intolerance.
INTRODUCTION

Immunomodulatory effects of fermented soy- or dairy-milks and casein hydrolysates by lactic acid bacteria have been widely reported [1, 2]. However, there is a lack of data concerning milks from vegetal origin, and only recent data appeared concerning almond seeds [3, 4].

The aim of this study was to evaluate whether fermented almond milks by different potential probiotic bacteria affect energetic metabolism in intestinal cells and the production of proinflammatory biomarkers by immunocompetent cells to gain insights about the potential benefits for the consumer gut health.

MATERIAL AND METHODS

Preparation and fermentation of almond beverage. Almond beverage was produced by soaking and grinding Marcona almonds. The aqueous extraction was carried out in Sojamatic 1.5 equipment (Sojamatic®; Barcelona, Spain). The milky liquid obtained was then ultra-homogenized at 172 MPa (M-110P model; Microfluidics International Corporation, USA), heated at 121 ºC/15 min and fermented at 37 ºC/24 h with L. rhamnosus CECT 27 (s2), L. plantarum CECT 220 (s3), L. delbrueckii subs. Bulgaricus and S. thermophilus inoculated with cell suspensions (10^7-8 cells/ml) of either B. bifidum CECT 870 (s6) or B. longum CECT 4551 (s7).

Simulated gastrointestinal digestion. The human gastrointestinal digestion process was simulated as previously described [5]. As controls, non-fermented almond milk (NFAM) and a commercial milk-based infant formula (MBIF) were used.

Mitochondrial and Lysosomal enzyme activities. For the experiments intestinal epithelial (Caco-2) cells were used (5 days post-seeding) after an incubation period of 3 h. Mitochondrial activities were investigated by monitoring MTT (3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyl tetrazolium bromide) conversion. Lysosomal activities were investigated by using the neutral red (NR) (toluylene red; 3-amino-7-dimethylamino-2-methylphenazine hydrochloride) uptake assay. Control cells exposed to digests containing enzymes but not samples were used throughout each assay.

Analysis of pro-inflammatory markers. TNFα, IL-1β, and IL-6 (eBioscience) were determined by ELISAs, according to the instruction of the manufacturers, on exposed RAW 264.7 cell cultures after an incubation period of 3 h.
**Statistical analysis.** Each of the experiments was conducted in four independent replicates. One-way analysis of variance (ANOVA) and the Tukey post hoc test were applied. Statistical significance was established at \( P<0.05 \) for all comparisons. SPSS v.15 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

**RESULTS AND DISCUSSION**

A significant decline in pH from 6.6 to 4.6 after 20 h at 37°C of incubation with a corresponding increase in titratable acidity due to fermentation occurred in the developed product.

**Bacterial fermentation effects on TNFα production.** Cells treated with the dialyzable fractions from samples fermented with either *L. rhamnosus* CECT 278 (s2) or *L. plantarum* CECT 220 (s3) did not change \( (P>0.05) \) TNFα production from controls (Fig. 1). Otherwise, only cells exposed to dialysates from samples fermented with *L. rhamnosus* CECT 278 exhibited lower IL-6 concentrations than controls. Fermentation with the bifidobacteria (s6, s7) had a positive effect decreasing \( (P<0.05) \), up to 50\%, the production of TNFα relative to controls. When considering IL-6 production, cell cultures challenged to fermented samples inoculated with either *B. bifidum* CECT 870 (s6) or *B. longum* CECT 4551 (s7) exhibited lower production of IL-6 than those inoculated with lactobacilli.

The bacterial fermentation effects observed could have important consequences on the intestinal barrier function because TNFα plays crucial roles increasing paracellular permeability impairing tight junction functionality [6] and leukocytes infiltration in intestinal wall [7]. In addition, IL-6 is a key mediator of a multifunctional proinflammatory cytokines, and the major cytokine produced by activated mast cells. Almonds are known to have several nutritional benefits and it has been recently suggested their potential prebiotic, increasing numbers of bifidobacteria, associated to almond lipids available for fermentation [8]. This effect could explain, at least in part the observed results.
Figure 1. Production of pro-inflammatory markers, TNFα and IL-6, on exposed RAW 264.7 cell cultures to digests from non-fermented (control), commercial milk-based infant formula (MBIF) and fermented samples with L. rhamnosus (s2), L. plantarum (s3), yogurt cultures + B. bifidum (s6), yogurt cultures + B. longum (s7).

Bacterial fermentation effects on energetic metabolism of intestinal cells. The cytotoxicity of ferments to intestinal epithelial (Caco-2) cells was determined, monitoring the mitochondrial enzyme (test MTT) and endo/lysosomal (test NR) activities (Fig. 2). Both metabolic assays showed that none of the dialysates exposed to cell cultures caused toxic effects as concluded from MTT and NR values (%) similar (P>0.05) or higher (P<0.05) than those calculated for controls.

Only were detected significant (P<0.05) differences in mitochondrial enzyme activities coupled to the cellular energetic metabolism. MTT values showed that dialysates from samples fermented with L. rhamnosus CECT 278 (s2) or L. plantarum CECT 220 (s3) had a positive effect on energetic cell metabolism similar to that of non fermented almond milk. These results evidence the non cytotoxic effect to intestinal cells of the fermented almond milks. Cell cultures exposed to dialysates from samples fermented with either B. bifidum CECT 870 (s6) or B. longum CECT (s7) exhibited similar MTT conversion percentages suggesting similar effects on energetic cell metabolism. Interestingly, the stimulatory capacity of fermented almond milks on energetic cell metabolism resulted higher than observed in cell cultures challenged to
the dyalysates from the MBIF. Increases in MTT conversion values have been associated to the production of reducing equivalents preserving alterations in the cellular redox status [9]. It has been reported that almond extracts are a source of bioactive polyphenols with antioxidant activity [10]. These almond-derived components with functional characteristics could also be present in the fermented samples and may explain the effects observed.

![Figure 2](image-url)

**Figure 2.** MTT conversion and neutral red (NR) uptake values in intestinal epithelial (Caco-2 cells) exposed to digests from non-fermented almond milk (NFAM), commercial milk-based infant formula (MBIF) and fermented samples with *L. rhamnosus* (s2), *L. plantarum* (s3), yogurt cultures + *B. bifidum* (s6), yogurt cultures + *B. longum* (s7).

**CONCLUSIONS**

The results indicate that fermented almond milk favours energetic cell metabolism of enterocytes and had lower inflammatory potential, when inoculated with with either *B. bifidum* CECT 870 or *B. longum* CECT 4551, than the commercial MBIF containing bifidobacteria. These results suggest health benefits from fermented almond milks that may be helpful in managing cow-milk allergy/intolerance.
References


