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Oral administration of kefiran exerts a bifidogenic effect on BALB/c mice intestinal microbiota

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RESEARCH ARTICLE

Abstract

The activity of kefiran, the exopolysaccharide present in kefir grains, was evaluated on intestinal bacterial populations in BALB/c mice. Animals were orally administered with kefiran and *Eubacteria*, lactobacilli and bifidobacteria populations were monitored in faeces of mice at days 0, 2, 7, 14 and 21. Profiles obtained by Denaturing Gradient Gel Electrophoresis (DGGE) with primers for *Eubacteria* were compared by principal component analysis and clearly defined clusters, correlating with the time of kefiran consumption, were obtained. Furthermore, profile analysis of PCR products amplified with specific oligonucleotides for bifidobacteria showed an increment in the number of DGGE bands in the groups administered with kefiran. Fluorescent *In Situ* Hybridisation (FISH) with specific probes for bifidobacteria showed an increment of this population in faeces, in accordance to DGGE results. The bifidobacteria population was also studied on distal colon content after 0, 2 and 7 days of kefiran administration. Analysis of PCR products by DGGE with *Eubacteria* primers showed an increment in the number and intensity of bands with high GC content of mice administered with kefiran. Sequencing of DGGE bands confirmed that bifidobacteria were one of bacterial populations modified by kefiran administration. DGGE profiles of PCR amplicons obtained by using *Bifidobacterium* or *Lactobacillus* specific primers confirmed that kefiran administration enhances bifidobacteria, however no changes were observed in *Lactobacillus* populations. The results of the analysis of bifidobacteria populations assessed on different sampling sites in a murine model support the use of this exopolysaccharide as a bifidogenic functional ingredient.

Keywords: exopolysaccharides, kefir, *Lactobacillus*, *Bifidobacterium*, prebiotic

1. Introduction

Exopolysaccharides (EPSs) produced by lactic acid bacteria (LAB) offer an alternative source of natural carbohydrates for diverse applications in the food industry and contribute to the texture and stability of the food (Abraham *et al.,* 2010; Ruas-Madiedo *et al.,* 2008). In addition to the stabilising, emulsifying or gelling properties, these EPSs contribute to the health-promoting properties of food. This aspect was recently reviewed by Patten and Laws (2015) and Ryan *et al.* (2015), who pointed out the relevance of these biopolymers as functional components of food. The gut microbiota is considered as a symbiotic partner for the maintenance of health and its homeostasis depends on host characteristics, environmental conditions and day-to-day dietary changes (Scott *et al.,* 2013). Probiotics and prebiotics have successfully been incorporated in a wide variety of human functional foods as an alternative to modulate gut microbiota by diet (Al-Sheraji *et al.,* 2013; Patel *et al.,* 2012) thus improving host health (Geurts *et al.,* 2014; Rastall and Gibson, 2015). Scientific data on health benefits of prebiotics have been obtained using food ingredients belonging to 2 main groups: inulin-type fructans and galacto-oligosaccharides, which have proved to resist digestion and modulate intestinal microbiota (Robertfroid *et al.,* 2010; Sims *et al.,* 2014). Even though the list of prebiotic

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compounds is increasingly growing (Saad *et al.,* 2013), little is known about the prebiotic effect of non-digestible EPSs obtained from lactic acid bacteria. LAB EPSs, differ in sugar composition, degree of branching, molecular weight and are candidates to be used as carbon source by intestinal microbiota (Ruas-Madiedo *et al.*, 2008). Their role as fermentable substrates *in vitro* has been described, and depends on the physicochemical characteristics of each biopolymer (Dal Bello *et al.,* 2001; Das *et al.,* 2014; Grosu-Tudor *et al.,* 2013; Hongpattarakere *et al.,* 2012; Korakli *et al.,* 2002). Several reports demonstrate that EPSs produced by *Lactobacillus* or *Bifidobacterium* are promising as prebiotics *in vitro*; however, these effects have to be observed *in vivo* (Patten and Laws, 2015).

Kefiran, a branched hydrosoluble glucogalactan produced by LAB, is obtained from kefir fermented milk (Rimada and Abraham, 2003) or culture supernatants of *Lactobacillus kefiranofaciens* (Wang *et al.,* 2008) and is the main constituent of kefir grains (Rimada and Abraham, 2001). Kefiran can be used as a functional additive since some useful technological properties were demonstrated, such as improvement of viscoelastic properties on acid milk gels (Rimada and Abraham, 2006), gelling ability (Piermaria *et al.,* 2008) and formation of edible films (Piermaria *et al.,* 2011).

As a non-digestible polysaccharide, kefiran could reach the large intestine, thus eliciting biological effects. The effect of kefiran on immune cells balance (Medrano *et al.,* 2011) and cytokine profiles (Vinderola *et al.,* 2006) in BALB/c mice orally administered with kefiran, as well as *in vivo* antitumoral activity (Murofushi *et al.,* 1986; Shiomi *et al.,* 1982) were demonstrated.

Therefore, the aim of the present study was to gain insight on the effect of kefiran on intestinal and faecal bifidobacteria in a murine model to deepen the knowledge of this exopolysaccharide contribution on health promoting properties of kefir fermented milk.

2. Materials and methods

Isolation and purification of kefiran

Kefiran was isolated from CIDCA AGK1 kefir grains by ethanol precipitation according to Rimada and Abraham (2003). Obtained kefiran-containing solution was dialysed for 48 h at 4 °C against distilled water by using dialysis membranes with molecular weight cut-off of 1000 Da (Spectra/Por, The Spectrum Companies, Gardena, CA, USA). As described by Rimada and Abraham (2003), EPS concentration was determined by the anthrone method, absence of free sugars was verified by qualitative thin layer chromatography (TLC) on silica gel G type 60 plates (Merck, Darmstadt, Germany) and absence of proteins was verified by the Bradford method. Anthrone, Bradford and TLC reagents were obtained from Sigma (St. Louis, MO, USA). Pure EPS solution was lyophilised (Heto FD4; Heto-Holten, Allerød, Denmark) and stored at room temperature until use.

Animals

BALB/c female mice (6 to 8 weeks old) were purchased from the School of Veterinary Sciences, UNLP (La Plata, Argentina). Mice were housed in groups of 5 to 6 mice per cage and were maintained at controlled temperature (22-25 °C) and light-dark cycle (12 h + 12 h). Animals were handled in accordance with the EU Directive 2010/63/EU for animal experiments. Animal procedures were evaluated and approved by CICUAL Committee (*Comité Institucional para el Cuidado y Uso de los Animales de Laboratorio*) from the School of Veterinary Sciences, UNLP (La Plata, Argentina) (Protocol number: 35-1-13E).

Feeding procedures

Animals were divided in groups: control mice (animals fed with a balanced conventional diet and water *ad libitum*) and kefiran administered mice (animals fed with a balanced conventional diet and kefiran 300 mg/l administered *ad libitum* in the drinking water). Kefiran solutions were prepared by dissolving 300 mg of lyophilised kefiran in 1 litre of sterile drinking water. Fresh kefiran solutions were prepared every day.

Experimental design

Two different experimental designs were performed. In the first experiment, animals were randomly allocated to 2 groups of 6 mice per group (12 mice, n=6 per group). Each mouse was identified with a non-toxic permanent ink label. Kefiran solution was administered in drinking water of the treatment group (n=6) for 21 days (daily intake 0.75 to 1 mg kefiran per animal per day) whereas the control group (n=6) received only drinking water. Faecal samples were taken at days 0, 2, 7, 14 and 21 by placing each mouse in a plastic cage for up to 30 min. Faecal samples were collected in sterile weighted Eppendorf tubes and processed immediately or stored at -80 °C until analysis.

In the second experiment, animals were randomly allocated to 3 groups (15 mice, n=5 per group). One group was administered with kefiran solution for 2 days (n=5 mice). A second group was administered with kefiran solution for 7 days (n=5 mice) and the third group (control) received only water (n=5 mice). After 2 or 7 days of kefiran administration, mice were euthanised by CO_2 inhalation. Control mice were euthanised after 7 days of experiment. Content from the distal portion of the colon was aseptically removed, suspended in phosphate-buffered saline (PBS;

KH_2PO_4: 0.144 g/l; Na_2HPO_4: 0.795 g/l; NaCl: 9 g/l) and stored at -80 °C until analysis.

Microbiological analysis

Serial dilutions of faecal samples were plated in duplicate on MRS agar (Difco Laboratories, Detroit, MI, USA) supplemented with cysteine 0.05% (w/v). Plates were incubated at 37 °C for 48 h under anaerobic conditions (Anaero Pack-Anaero kit, Mitsubishi Gas Chemical CO Inc., Tokyo, Japan). Colonies were characterised by macroscopic morphology, Gram staining and catalase reaction. cfu/ml were expressed as means ± standard deviation (SD).

DNA isolation from faecal samples and intestinal content

500 mg of samples (faeces or intestinal content) were placed in 1 ml of PBS buffer. DNA was extracted and purified using the AccuPrep Stool DNA Extraction Kit (Bioneer, Daejeon, Republic of Korea) according to the manufacturer's protocol. The DNA obtained was stored in DNA-free tubes at -20 °C until use.

Polymerase chain reaction-DGGE

The V3 region of the 16S rRNA gene (positions 341-534 in the *Escherichia coli* gene) was amplified by using primers 518R/341F-GC, LAC1/LAC2-GC and BIF164F/BIF662-GC, for *Eubacteria*, *Lactobacillus* and bifidobacteria, respectively (Table 1).

Polymerase chain reaction (PCR) mixture consisted of: 0.2 µM of each primer, 1.25 U of Taq polymerase (Inbio-Highway, Tandil, Argentina), 1.5 µl of PCR reaction buffer (100 mM Tris-HCl, 500 mM KCl, pH 9.0), 2.5 mM $MgCl_2$ and 0.2 mM of each dNTP. Three µl of the template DNA was added to 19 µl of the PCR reaction mixture in 0.2 ml DNA-free tubes. PCR reaction was conducted by using a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). The following amplification program was used: *Eubacteria*: 94 °C for 5 min, 30 cycles consisting of 94 °C 30 s, 60 °C 60 s, 72 °C 30 s, and 72 °C 5 min; *Lactobacillus*: 94 °C for 2 min, 35 cycles consisting of 94 °C 30s, 61 °C 60 s, 68 °C 60 s, and 68 °C 7 min; *Bifidobacterium:* 98 °C for 5 min, 40 cycles consisting of 94 °C 45 s, 52 °C 50 s, 72 °C 50 s, and 72 °C 7 min.

The PCR products were analysed by Denaturing Gradient Gel Electrophoresis (DGGE) using DGGE-2401 CBS equipment (CBS Scientific Co., Del Mar, CA, USA) in 15×20×0.075 cm gels. Samples were applied to 8% (w/v) polyacrylamide gels in Tris-acetate-EDTA buffer. Optimal separation for *Eubacteria* and *Lactobacillus* PCR products was achieved with 40-60% urea-formamide denaturing gradient (100% correspondent to 7 M urea and 40% v/v of formamide) and 45-65% urea-formamide for *Bifidobacterium* PCR products. Electrophoresis was performed at a constant voltage of 100 mV for 16 h at 60 °C. Gels were stained with SYBR® Gold Nucleic Acid Gel Stain (Invitrogen, Carlsbad, CA, USA) and visualised under UV light.

Table 1. Primers and DNA probes used in this study, sequences and references.[1]

Primer		Sequence (5´-3´)	Use	Reference
518R		ATTACCGCGGCTGCTGG	DGGE, total bacteria	Muyzer *et al.*, 1993
341F	with 5´-GC-clamp	CCTACGGGAGGCAGCAG		
BIF164F	with 5´-GC-clamp	GGGTGGTAATGCCGGATG	DGGE, *Bifidobacterium*	Satokari *et al.*, 2001
BIF 662		CCACCGTTACACCGGGAA		
LAC 1		AGCAGTAGGGAATCTTCA	DGGE, *Lactobacillus*	Walter *et al.*, 2001
LAC 2	with 5´-GC-clamp	ATTYCACCGCTACACATG		
DNA probes		**Sequence (5´-3´)**	**Use**	**Reference**
Eub 338	labelled with FITC marker	GCTGCCTCCCGTAGGAGT	FISH, total bacteria	Amann *et al.*, 1990
NON 338	labelled with FITC marker	ACATCCTACGGGAGGC	FISH, non-bacteria	Wallner *et al.*,1993
Bif 164	labelled with AlexaFluor 647 marker	CATCCGGCATTACCACCC	FISH, *Bifidobacterium*	Satokari *et al.*, 2001
Lab 158	labelled with AlexaFluor 532 marker	GGTATTAGCAYCTGTTTCCA	FISH, *Lactobacillus*	Harmsen *et al.*, 2002

[1]DGGE = denaturing gradient Gel Electrophoresis; FISH = fluorescent *in situ* hybridisation.

Identification of DGGE bands

For sequencing, DGGE bands were excised from the gels, suspended in 50 µl TE buffer (50 mM Tris-HCl, 5 mM EDTA, pH 8.0), and incubated at 4 °C overnight for DNA diffusion. This solution was used to amplify the PCR products with the same primers but without the GC clamp. The amplicons were checked for purity by DGGE analysis under the conditions described above. Amplified DNA of the original sample was used as control. The direct sequencing of PCR products was performed on a 3730XLs 23 ABI DNA sequencer by Macrogen (Seoul, Rep. of Korea) and the resulting sequences were compared with those in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) through the BLAST program.

Enumeration of *Eubacteria* and bifidobacteria in faecal samples by FISH and flow cytometry

The process of sample fixation and labelling was carried out according to Snart *et al.* (2006). Probe Eub338 was used as the positive control (total *Eubacteria* group). Probe NON338 was used as the negative control. Both were covalently linked at the 5´ end to fluorescein isothiocyanate (FITC). Probe Bif164, specific for the *Bifidobacterium* group, was linked at the 5´end to AlexaFluor 647. Probe Lab 158, specific for the *Lactobacillus* genus was linked at the 5´end to AlexaFluor 532. All of the probes were purchased from Invitrogen (Eugene, OR, USA). The nucleotide sequences of probes used in this work are listed in Table 1.

Flow cytometry was performed in a FACS-Calibur flow cytometer (Becton and Dickinson, San Jose, CA, USA). A total of 50,000 events per sample were collected. Data were analysed by using the CellQuest Pro 5.2.1 software (BD Biosciences). Results were expressed as the ratio between positive events for *Bifidobacterium* probe and positive events for total *Eubacteria* probe for each sample (% bifidobacteria).

Statistical analysis

Gel analysis was performed by using GelCompar II software package (Applied Maths, Kortrijk, Belgium). The matrix of *Jaccard* coefficient was clustered by the unweighted average linkage method (UPGMA). Principal component analysis was performed by using InfoStat software version 2008, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). Plate count results and FISH-cytometry data were analysed by using Systat-12 (SYSTAT, Inc., Evanston, IL, USA). Statistical significant differences were calculated by the t-test ($P \leq 0.05$).

3. Results

Kefiran administration did not modify water, food intake nor weight gain of mice, as compared with control group. Average weight increment was 1.07 g and 1.04 g for control and kefiran-administered group, respectively, after 21 days of experiment. The number of Gram positive, catalase negative rod-shaped bacteria in faeces of kefiran administered mice increased from $1.64\times10^{8}\pm1.48\times10^{7}$ cfu/ml at day 0 to $4.25\times10^{9}\pm5.30\times10^{8}$ cfu/ml at day 21. In contrast, the same population present in faeces of control mice decreased from $1.78\times10^{8}\pm9.9\times10^{6}$ cfu/ml at day 0 to $6.65\times10^{7}\pm4.95\times10^{6}$ cfu/ml at day 21 ($P \leq 0.05$).

The evolution of faecal microbiota at 0, 2, 7, 14 and 21 days was monitored by PCR-DGGE. Profiles resulting from amplification with *Eubacteria* primers of DNA extracted from faecal samples of each mouse were analysed by principal component analysis. As shown in Fig 1, 4 well defined clusters were observed: controls, K0-K2, K7-K14 and K21 depending on the days of kefiran administration. These clusters were consistently found in PC1 vs PC2 and PC1 vs PC3 plots (Figure 1A and B). These findings indicate a divergent evolution of the intestinal microbiota in control and kefiran-administered mice. The difference between groups as a function of time was more evident in PC3 vs PC2 plot (Figure 1C) where kefiran administered mice for 21 days (K21) was clearly separated from the other groups.

Samples of DNA purified from distal colon content of kefiran-administered and control mice were amplified with specific primers for *Eubacteria*. DGGE profiles are shown in Figure 2A. An increase in the number of bands was observed in kefiran-administered mice at days 2 and 7 as compared with control mice that did not received kefiran. Analysis of DGGE profiles by using the *Jaccard* coefficient and UPGMA method shows 2 main clusters (Figure 2B). Cluster I includes animals treated with kefiran for 7 days, whereas cluster II includes controls and animals administered with kefiran for 2 days. These results are in agreement with those obtained from faeces analysis (Figure 1).

Sequences of all DGGE bands obtained were compared to those present in the GenBank database. It was found that most of the sequences did not belong to any known bacterial species, or belonged to rRNA 16S regions of non-cultivable bacteria. Identified sequences are indicated by letters in Figure 2A and homology is listed in Table 2. Several genera were identified in intestinal content of all the mice. Interestingly, the 'j' band, which sequence presented a high homology with rRNA 16S belonging to *Bifidobacterium* genus (Figure 2A and Table 2), was present only in kefiran-administered mice at days 2 and 7.

DNA obtained from distal colon content and faecal samples was amplified with specific oligonucleotides

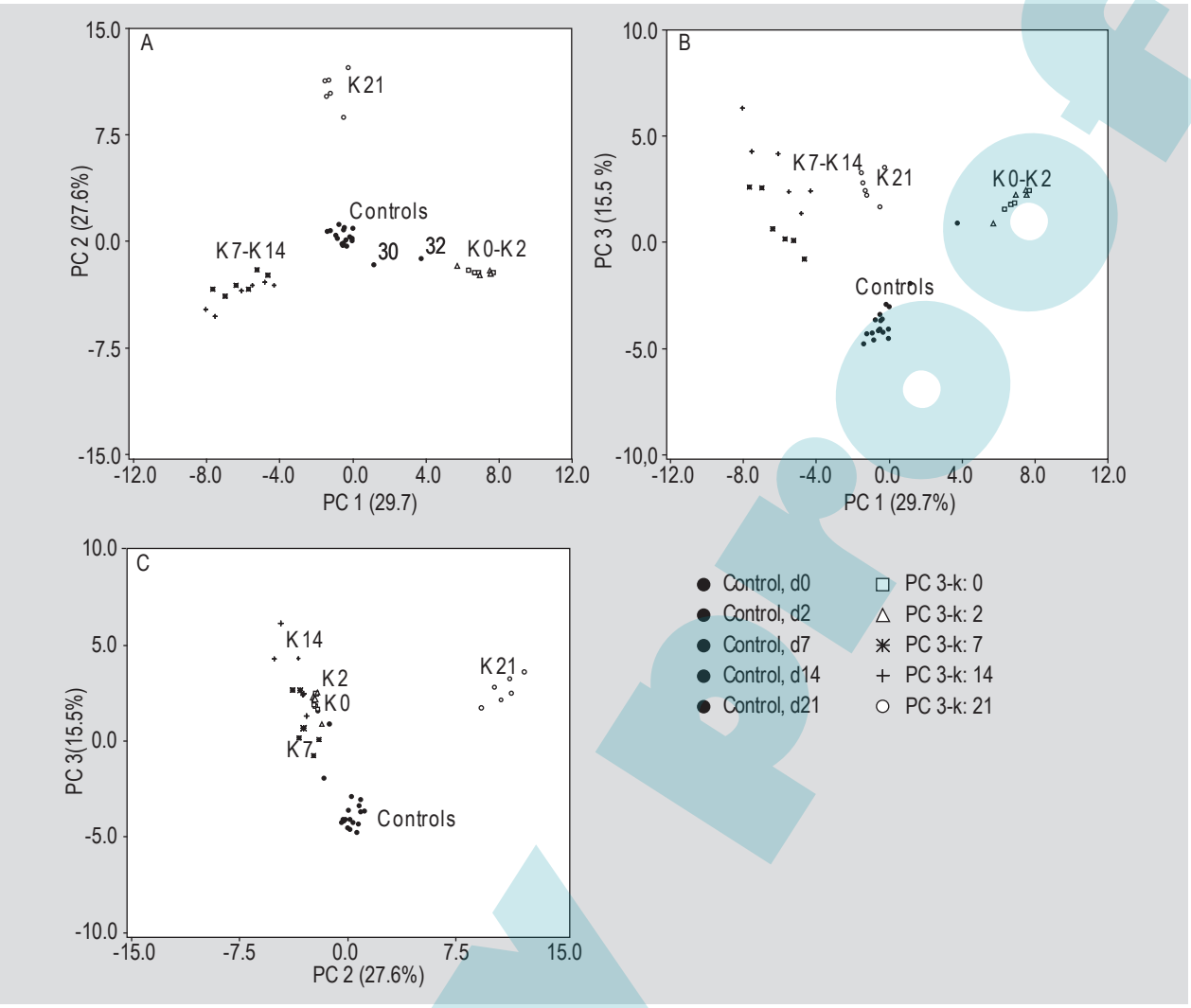

Figure 1. Principal component analysis of denaturing gradient gel electrophoresis profiles resulting from the amplification of the DNA purified from faecal samples with primers specific for total bacteria (518R and 341F-GC). Percentages of the total variation associated to each principal component are indicated in brackets. A, B and C represent the relation of the principal components analysed. (A) PC2 vs PC1; (B) PC3 vs PC1; (C) PC3 vs PC2.

Table 2. Percentage (%) similarity of partial 16S rDNA sequences to their closest relatives in the NCBI nucleotide-sequence database.

Band	Closest relative	Identity (%)
a	*Dehalococcoides* sp.	99
b	*Gardnerella vaginalis*	79
c	*Acidovorax radicis*/*Verminephrobacter* sp./*Acidovorax delafieldii*	89
d	*Bacteroides capillosus*	86
e	*Dehalogenimonas lykanthroporepellens*	84
f	*Clostridium difficile*	100
g	*Alistipes putredinis*	96
h	*Bacteroides* sp.	98
i	*Listeria monocytogenes*	81
j	*Bifidobacterium* sp.	97

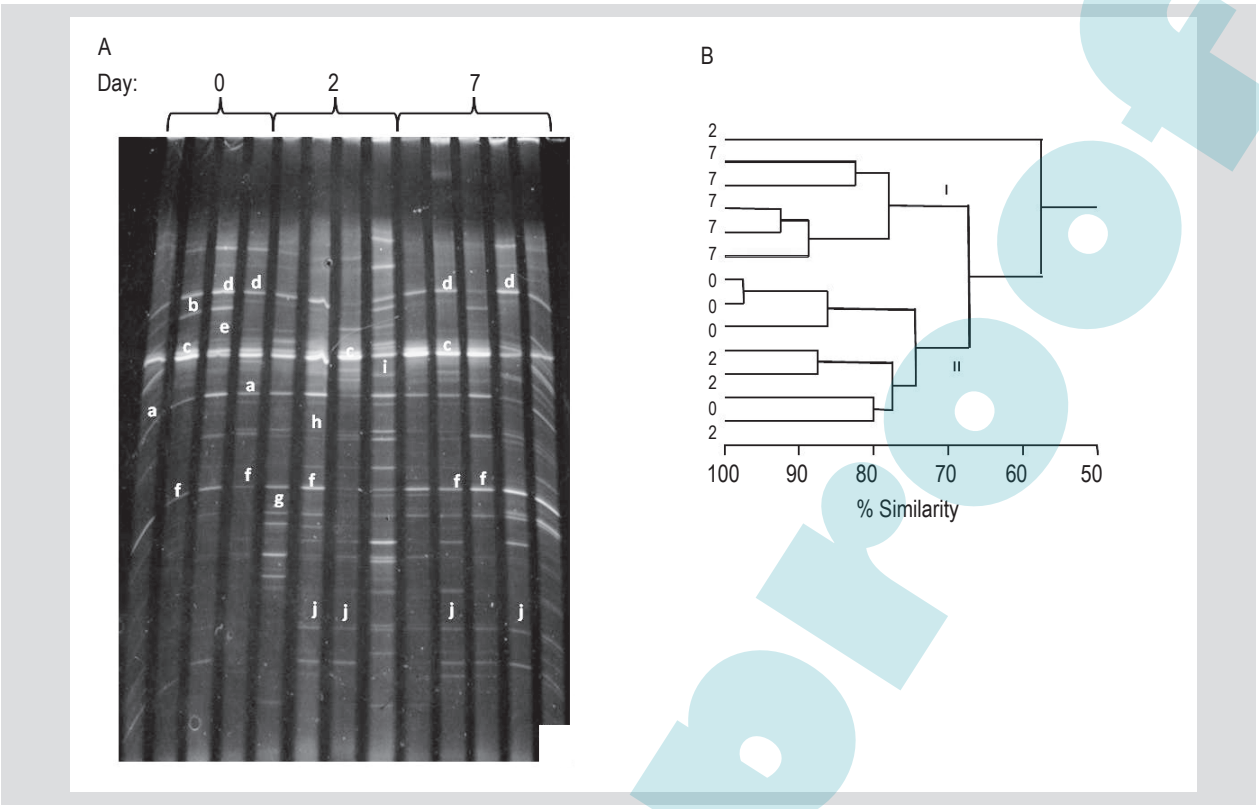

Figure 2. (A) Denaturing gradient gel electrophoresis (DGGE) profiles resulting from the amplification of DNA obtained from distal colon content of control mice and kefiran-administered mice for 2 and 7 days. The amplification was performed by using universal primers specific for total bacteria (518R and 341F-GC). Letters (from *a* to *j*) indicate the bands that matched with sequences present on the GenBank database listed in Table 2. (B) Dendrogram obtained from the amplification of DNA obtained from distal colon content of control mice and kefiran-administered mice for 2 and 7 days (Figure 4A). DGGE profiles were analysed by *Jaccard* coefficient and UPGMA cluster analysis.

for *Bifidobacterium* and *Lactobacillus* genera. Figure 3 shows DGGE profiles of amplicons resulting from DNA obtained from distal colon content of control mice and kefiran-administered mice for 2 and 7 days amplified with specific oligonucleotides for *Bifidobacterium*. It can be noted that in DDGE patterns from samples of control mice that did not consume kefiran there are low number of DGGE bands with the exception of 2 individuals, while new bands were observed in DGGE profiles of kefiran-administered mice (for 2 and 7 days) (Figure 2). Taking into account that each DGGE band could be ascribed to different *Bifidobacterium sp*., the occurrence of new bands on DGGE profiles could indicate an increment in the number and diversity of bifidobacteria population. Results obtained with faecal samples were in accordance to these findings (data not shown).

In order to gain insight on the evolution of the population of bifidobacteria due to kefiran administration, *Bifidobacterium* in faecal samples were labelled with specific FISH probes and were analysed by flow cytometry. As depicted in Figure 4A, there is a significantly (P=0.004) increase of the ratio of bifidobacteria in kefiran-administered mice after 21 days whereas no differences were found on control mice

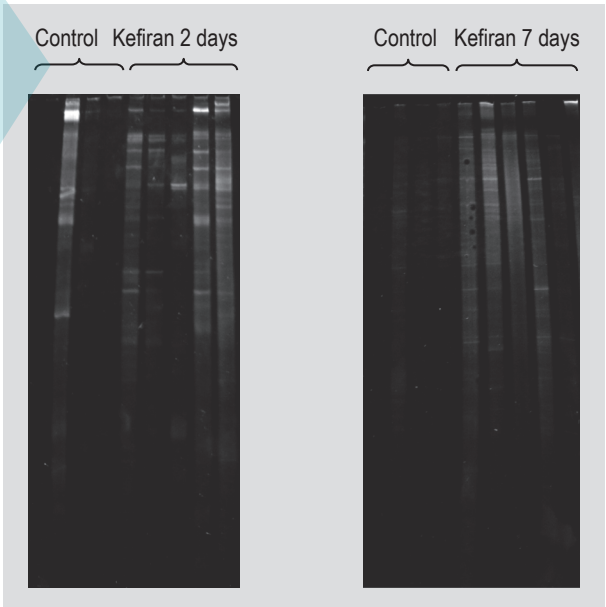

Figure 3. Denaturing gradient gel electrophoresis profiles resulting from the amplification with specific bifidobacteria oligonucleotides (Bif164F and Bif662-GC) of the DNA purified from distal colon content obtained from control mice and kefiran-administered mice for 2 and 7 days.

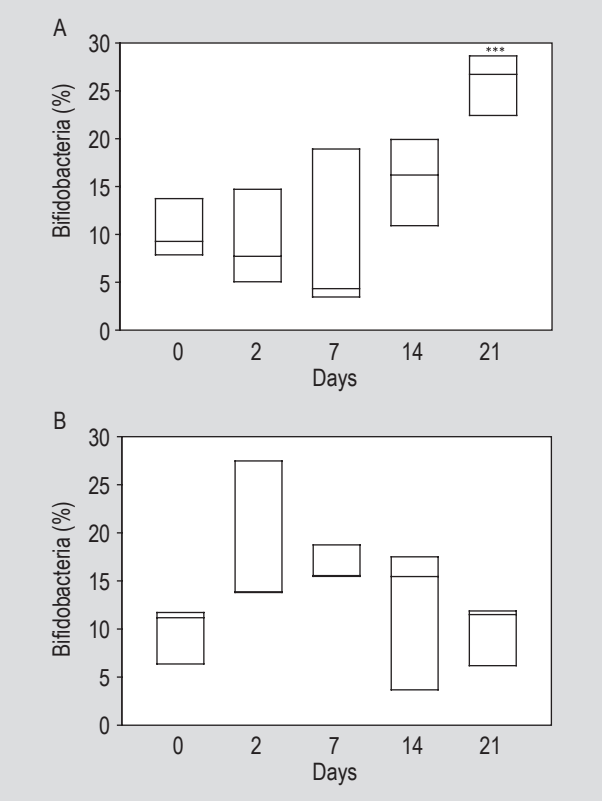

Figure 4. Relative abundance of bifidobacteria present in faecal samples (taken on days 0, 2, 7, 14 and 21) of kefiran-administered mice (A) and control mice (B). Quantification was performed by fluorescent *in situ* hybridisation and flow cytometry by using a specific primer for bifidobacteria (Bif164) related to total *Eubacteria* (Eub338). Presented results are the maximum, minimum and mean values obtained from kefiran-administered mice and control mice. * indicates significant differences between 0 and 21 days of kefiran consumption ($P \leq 0.005$).**

that did not receive kefiran (Figure 4B). It is noteworthy that neither qualitative (DGGE profiles analysis) nor quantitative (FISH-cytometry) analysis revealed significant changes in *Lactobacillus* population between kefiran-administered and control mice (data not shown).

4. Discussion

The usual target microorganisms for prebiotic approaches are *Bifidobacterium* or *Lactobacillus*, since they represent one of the groups of commensal beneficial microorganisms inhabiting the human and animal gastrointestinal tract, and they are traditionally associated with a broad range of beneficial effects on host's health (Dewulf *et al.*, 2012; Everard *et al.*, 2014; Tojo *et al.,* 2014). In the present work we demonstrated that orally administered, kefiran is able to change intestinal and faecal microbiota of BALB/c mice, by increasing the number of bifidobacteria populations. However, no changes were observed in *Lactobacillus* populations.

Principal component analysis of DDGE profiles demonstrated that kefiran administration lead to a consistent shift in the composition of faecal microbiota, while no such change was observed on control mice, since faecal microbiota begins to evolve differently depending on the administration or not of kefiran. It is noteworthy that control mice assembled in a definite cluster, irrespective of the day of assay when PCA was performed. On the contrary, PCA analysis of DGGE profiles of kefiran-administered mice allowed to distinguish well-defined clusters that were consistent to kefiran administration time, suggesting that kefiran is able to elicit a modification of intestinal microbiota along time, despite of inter individual variation. The fact that control group did not cluster together with K0-K2 group could be ascribed to the natural evolution of intestinal microbiota due to change of animals from breeding to experimental areas. Indeed, experiments started after one week adaptation of mice to the new environment into 2 separated cages according to treatments.

Even though faecal microbiota does not strictly reflect the whole gut ecosystem, it is considered as an acceptable surrogate of the bacterial content of distal colon. This approach allows studying higher number of individuals per group due to the diminution of euthanatised animals (Yasuda *et al.,* 2015). The experimental model used in the present study, in which 2 different sampling sites were used (distal colon content and faeces) showed that results were qualitatively comparable. The correlation between microbiota of faeces and distal colon was also reported for humans (Macfarlane *et al.,* 1998) and *Rhesus macaque* model (Yasuda *et al.,* 2015).

According to our results, no stimulation of *Lactobacillus* population was observed in faecal or intestinal samples. This may be due to the relative high abundance of this genus in mouse gut microbiota (Nguyen *et al.,* 2015). The modification of the total faecal microbiota triggered by kefiran correlates with an increment on bifidobacteria population. These findings are probably due to the competitive success of *Bifidobacterium* spp. in the intestinal lumen of kefiran-administered mice. The more pronounced effect of kefiran on *Bifidobacterium* could be due to their less abundance in the microbiota of mice (Nguyen *et al.,* 2015).

Available scientific data largely connects intestinal bifidobacteria increment with the enhancement of mucosal associated immune response and protection against infectious diseases (Fukuda *et al.,* 2011; Prasanna *et al.,* 2014; Robertfroid *et al.,* 2010; Russell *et al.,* 2011; Scott *et al.,* 2013). In this context, it should be noted that elderly people and patients suffering from chronic intestinal inflammation have low numbers of bifidobacteria in their intestine (Litch *et al.*, 2012) that could be increased by kefiran consumption.

Therefore, modulating the intestinal bifidobacteria population has often been considered as a target for dietary interventions thus providing the rational for the use of microorganisms of the genus *Bifidobacterium* as probiotics (Tojo *et al.,* 2014). The results obtained allow us to conclude that administration of kefiran provides an alternative for stimulation of bifidobacteria by dietary intervention.

In accordance to our findings, many *in vitro* and *in vivo* studies performed by other authors demonstrated that some EPSs produced by different lactic acid bacteria were able to exert a bifidogenic effect (Dal Bello *et al.*, 2001; Das *et al.,* 2014; Hongpattarakere *et al.*, 2012; Korakli *et al.*, 2002). Nevertheless, not all the EPS produced by microorganisms were able to exert modifications in faecal microbiota in murine models. Lindström *et al.* (2013) found that oral administration of the EPS produced by *Pediococcus parvulus* to mice decreases both microbial diversity and bifidobacteria population. The opposite findings described in different reports could be explained because the prebiotic effect is related to the chemical structure and glycosidic linkages of each polymer (Ruas-Madiedo *et al.,* 2008).

Previous results demonstrated that oral administration of kefiran in animal models produce different positive effects. However, kefiran mechanisms of action have not been elucidated yet. It is noteworthy that, by using the same experimental model than in the present work, we previously found a significant increment in the number of goblet cells in the intestine of kefiran-administered mice (Medrano *et al.,* 2011). Since mucins are used by *Bifidobacterium bifidum* as a source of nutrients (Pokusaeva *et al.,* 2011), the increment in bifidobacteria population induced by kefiran administration shown in the present study could also be related to the increment in mucin intestinal availability. In this regard, it was demonstrated that kefiran is able to induce the expression of gene related to carbohydrates transport and metabolism on *B. bifidum* PRL2010 *in vitro* (Serafini *et al.*, 2014).

Taking into account the above mentioned results, it is tempting to speculate that the biological response elicited *in vivo* by kefiran could be ascribed, at less in part, to the bifidogenic effect. Kefiran ability to interact with cultured human enterocytes should not be ruled out as a relevant step for the biological effect (Medrano *et al.,* 2009).

In conclusion, in the present work, it was clearly demonstrated that, orally administered, kefiran is able to induce changes on intestinal microbiota of BALB/c mice exerting a bifidogenic effect. The health promoting activity of kefiran previously reported could be associated to the increase of intestinal bifidobacteria. These results provide scientific evidence for application of kefiran as a potential prebiotic ingredient in functional foods and contribute to better understanding the beneficial effect of kefir fermented milk.

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