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Inhibitory Activity of Cheese Whey Fermented with Kefir Grains

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ABSTRACT

We investigated the chemical and microbiological compositions of three types of whey to be used for kefir fermentation as well as the inhibitory capacity of their subsequent fermentation products against 100 *Salmonella* sp. and 100 *Escherichia coli* pathogenic isolates. All the wheys after fermentation with 10% (wt/vol) kefir grains showed inhibition against all 200 isolates. The content of lactic acid bacteria in fermented whey ranged from 1.04×10^7 to 1.17×10^7 CFU/ml and the level of yeasts from 2.05×10^6 to 4.23×10^6 CFU/ml. The main changes in the chemical composition during fermentation were a decrease in lactose content by 41 to 48% along with a corresponding lactic acid production to a final level of 0.84 to 1.20% of the total reaction products. The MIC was a 30% dilution of the fermentation products for most of the isolates, while the MBC varied between 40 and 70%, depending on the isolate. The pathogenic isolates *Salmonella enterica* serovar Enteritidis 2713 and *E. coli* 2710 in the fermented whey lost their viability after 2 to 7 h of incubation. When pathogens were deliberately inoculated into whey before fermentation, the CFU were reduced by 2 log cycles for *E. coli* and 4 log cycles for *Salmonella* sp. after 24 h of incubation. The inhibition was mainly related to lactic acid production. This work demonstrated the possibility of using kefir grains to ferment an industrial by-product in order to obtain a natural acidic preparation with strong bacterial inhibitory properties that also contains potentially probiotic microorganisms.

Whey is the major by-product of the dairy industry, representing 85 to 90% of the milk volume and retaining 55% of the milk nutrients. The most abundant of these nutrients are lactose (4.5 to 5.0%, wt/vol), soluble proteins (0.6 to 0.8%, wt/vol), lipids, and mineral salts *(11).*

Because of economic and environmental pressures, most cheese manufacturing plants must now develop processes to recover all of the milk solids. At present, at most large cheese factories the whey is dried or fractionated into various components by membrane processes for use by food and pharmaceutical industries *(19).* However, cheese manufacturers from certain underdeveloped countries or small-scale cheese producers cannot afford the equipment needed for such processing; thus, the whey is used as a fertilizer and in animal feed or else is simply discarded. Therefore, the development of inexpensive alternative uses of this by-product that take advantage of whey's nutritional properties and thus increase its economical value is of interest to the dairy industry.

Kefir grains are composed mostly of proteins and polysaccharides and contain yeast and lactic acid bacteria that coexist in a symbiotic association *(8, 10).* The grains are traditionally used to ferment milk in order to produce a characteristic drink named kefir. The probiotic activity of kefir grains and microorganisms isolated from them has been widely studied. Among the effects associated with kefir consumption are stimulation of the immune system *(37, 39, 40),* inhibition of tumor growth *(15, 23),* antioxidant capacity *(12),* improvement of lactose-intolerance symptoms *(7, 14),* and reduction of cholesterol levels *(22).* Furthermore, an inhibitory activity by kefir against gram-negative and gram-positive foodborne bacterial pathogens has been demonstrated by several authors *(5, 9, 36, 38).*

The use of kefir grain microorganisms to ferment whey has been proposed to produce alcohol *(2),* polysaccharides *(33–35),* baker's yeast *(26),* cell proteins *(21, 25),* fermented beverages *(1, 3),* and a starter for cheese production *(20).* The fermentation of cheese whey by kefir grains to obtain a probiotic product, however, has not been reported. The aim of this work was therefore to characterize the products of fermentation of cheese whey by kefir grains in order to investigate possible inhibitory effects against *Salmonella* and *Escherichia coli* as an initial approach toward the generation of a potential new probiotic product.

MATERIALS AND METHODS

Cheese wheys and culture media. We used commercial nonfat milk (Sancor S.A., Santa Fe, Argentina) along with cheese wheys from three different sources: whey powder from Lactogal S.A. (Porto, Portugal) was reconstituted in water at 10% (wt/vol) before use (RWP), while liquid bovine whey (BW) and liquid ovine whey (OW), provided by Lacticínios Lda (Tolosa, Portugal), were used directly. With the exception of lower sodium and potassium levels in RWP and a higher lactic acid content in OW, the chemical compositions of the three cheese wheys were quite similar (Table 1).

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TABLE 1. *Chemical composition of cheese wheys from different sources*

	Cheese whey[a]:		
	RWP	BW	OW
Proteins (g/100 g)	1.0	1.3	1.6
Lipids (g/100 ml)	<0.3	0.4	0.7
Lactose (g/100 g)	6.5	7.1	4.6
Moisture (g/100 ml)	91.3	88.7	90.7
Ash (g/100 ml)	0.8	1.7	1.6
Sodium (g/100 g)	0.06	0.43	0.44
Magnesium (mg/100 g)	100.6	114.1	112.7
Potassium (mg/100 ml)	239.4	3,240.5	1,625.6
Calcium (mg/liter)	549.6	564.1	495.7
Phosphorus (g/100 ml)	<0.01	<0.01	<0.01
pH	6.34	6.38	6.43
Lactic acid (g/100 ml)	0.05	0.04	0.22

[a] RWP, reconstituted whey powder; BW, liquid bovine whey; OW, liquid ovine whey.

Müller-Hinton broth, Müller-Hinton agar, tryptone bile x-glucuronide agar, de Man Rogosa Sharpe (MRS) agar, xylose lysine deoxycholate agar, rose bengal chloramphenicol agar, and buffered peptone water (BPW) were obtained from Biokar Diagnostics (Beauvais, France).

Microorganisms. Pathogenic isolates of *Salmonella* sp. and *E. coli* were provided by Controlvet S.A. (Tondela, Portugal).

One hundred *E. coli* isolates were isolated from organs (liver, spleen, lungs) of infected commercial chickens and identified according to Portuguese Norm (NP) 2308:1986 *(28)*. *E. coli* 2710 was likewise isolated from an infected commercial chicken with typical lesions of colibacillosis.

S. enterica strains were isolated from food samples or infected chickens and identified according to ISO 6579:2002/A1:2007 *(16)*. Of 100 isolates, 49 could not be typed, 21 belonged to *Salmonella enterica* serovar Typhimurium, 23 to *Salmonella enterica* serovar Enteritidis, and 7 to *Salmonella enterica* serovar Infantis. *Salmonella enterica* serovar Enteritidis 2713 was isolated from an infected commercial chicken with lesions typical of *Salmonella* infection and typed by means of PCR through the use of specific primers.

Cheese whey fermentation. Whey was fermented by procedures involving three different starters: kefir grains (i), whey culture (ii), and milk culture (iii). (i) Kefir grains were added to whey at concentrations of either 1 or 10% (wt/vol). After fermentation for 24 h at 20°C the kefir grains were separated from the fermented whey by filtration through a plastic sieve. (ii) A previous whey culture was inoculated into fresh whey at a final concentration of 10% (vol/vol), and the mixture was incubated for 24 h at 20°C. (iii) A previous milk culture was inoculated into fresh whey at a final concentration of 10% (vol/vol) and incubated for 24 h at 20°C.

The whey and milk starter cultures were obtained by the respective fermentation of whey and milk with 10% (wt/vol) kefir grains for 24 h at 20°C followed by removal of the grains. Kefir grains CIDCA AGK10 came from the collection of the Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA, La Plata, Argentina). The consistency of the quality of the kefir grains throughout the assays was corroborated by measuring their microbiological composition and increase in biomass.

Enumeration of viable microorganisms in fermentation products. The numbers of viable bacteria and yeasts in the fermentation products were determined by plating serial dilutions in tryptone (1 g/liter) on agar plates. Differential counting was performed on MRS agar for lactic acid bacteria and on rose bengal chloramphenicol agar for yeasts. Results were expressed as the number of CFU per milliliter in the fermented products.

Chemical measurements. The chemical composition of the different fermentation products was analyzed. The proteins were determined by the Kjeldahl method as indicated in ISO 5983-2 *(17)* and the lipids according to NP 876:2001 *(31)*, while lactose was quantified by high-performance liquid chromatography (HPLC) as described by Chavez-Servín et al. *(6)*. The separation was effected on a C_{18} 5-μm (150 by 4.0 mm) column, at a flow rate of 1.0 ml/min with 1% (vol/vol) acetic acid–acetonitrile, 95:5, as the mobile phase. The detection was done with a wavelength detector at 273 nm. The water content of samples was determined by drying at 103 ± 2°C to constant weight (according to NP 875 *(29)*). The ash was analyzed using a muffle furnace at 550°C after previous vaporization of water and volatile materials (according to NP 872 *(27)*). Sodium, magnesium, potassium, and calcium were quantified by atomic absorption spectrometry after ISO 6869 *(18)*. Phosphorus was measured spectrophotometrically by absorbance at 430 nm as indicated in NP 874 *(30)*. Organic acids were determined both qualitatively and quantitatively by HPLC. Species separation was carried out on a PRP-X300 7-μm (150 by 4.1 mm) column (Hamilton, Bonaduz, Switzerland), and the organic acids were detected in the UV at 210 nm. Acid identification was based on comparing the retention times within a sample to those of a standard acid mixture. For sample preparation, 1 ml of water plus 4 ml of acetonitrile were added to 1 g of fermentation product, and after homogenization by vortexing, the mixture was centrifuged at 10,000 × *g* for 5 min. The resulting supernatant was filtered through a 0.45-μm nitrocellulose membrane (Orange Scientific, Braine-l'Alleud, Belgium). Twenty microliters of the resulting filtrate was injected into the chromatograph, and analysis was performed at a flow rate of 0.6 ml/min with 0.024 M sulphuric acid as the mobile phase.

Supernatant preparation. Supernatants of fermented cheese whey were obtained by centrifugation at 13,000 × *g* for 15 min. They were then filtered through a 0.45-μm membrane (Orange Scientific). The resulting sterile filtrates were stored at −20°C until use.

By the same procedure, supernatants were obtained from fermented RWP alkalinized with 2.5 M NaOH (Merck, Darmstadt, Germany) to a pH of 4.5 and 7, fermented RWP heated for 20 min at 100°C, fresh RWP acidified with 2 M HCl (Merck) to a pH of 3.5, and RWP supplemented with D,L-lactic acid (Sigma Chemical Co., St. Louis, MO) to give the same lactate concentration as had been measured in the fermentation products.

Agar well diffusion assay. The inhibitory activity of supernatants against pathogenic microorganisms was determined by the agar well diffusion assay. Stated in brief, a suspension of target bacteria containing 10^8 CFU/ml was swabbed in three directions over standard 9-cm Petri plates containing 12 ml of Müller-Hinton agar. Wells (5-mm diameter) were uniformly bored in the agar, and 40 μl of a given supernatant, obtained as described above, was dispensed into each well. The plates were then incubated at 37°C for 24 h and the diameters of the inhibition zones around the wells measured with a vernier caliper (millimeter scale). The results were expressed as the average of three measurements in different directions.

Determination of MICs and MBCs. Fermented cheese whey supernatants were diluted in BPW at concentrations of 20, 30, 40, 50, 60, and 70% (vol/vol). One milliliter of each dilution was inoculated with 50 μl of pathogen suspension containing 10^7 CFU/ml. All suspensions were incubated at 37°C for 24 h, and pathogen growth was detected by turbidity. The MIC was considered the lowest percent concentration of supernatant that inhibited the visible bacterial growth completely.

Aliquots of suspensions without turbidity were subcultured to Müller-Hinton agar. The MBC was defined as the lowest percent concentration of supernatant that prevented bacterial growth.

Effect of fermented cheese whey supernatants on *E. coli* and *Salmonella enterica* serovar Enteritidis growth. The supernatant of cheese whey fermented with 10% (wt/vol) kefir grains was diluted in BPW to reach concentrations of 10, 20, 30, and 40% (vol/vol). Five milliliters of each dilution was inoculated with 50 μl of suspensions of the *Salmonella enterica* serovar Enteritidis isolate 2713 or the *E. coli* isolate 2710, each containing 10^8 CFU/ml, and then these were uniformly mixed and incubated at 37°C. Samples were collected at 15-min intervals, and the optical density at 600 nm was measured in a SmartSpec Plus spectrophotometer (BioRad Laboratories, Richmond, CA).

Survival of pathogens in fermented cheese whey supernatants. We determined the survival of the *Salmonella enterica* serovar Enteritidis isolate 2713 and the *E. coli* isolate 2710 in supernatants of RWP fermented with 10% (wt/vol) kefir grains. After an overnight culture the pathogenic bacteria were recovered by centrifugation, and the pellet was suspended in the same volume of either pure whey supernatant or whey supernatant diluted in BPW at 60% (vol/vol). At predetermined intervals, aliquots were removed, serially diluted in 0.1% (vol/vol) tryptone, and plated on Müeller-Hinton agar. Colony counts were performed after incubation at 37°C for 48 h.

Reduction of the concentration of *E. coli* or *Salmonella* sp. in cheese whey by fermentation with kefir grains. Either *E. coli* 2710 or *Salmonella enterica* serovar Enteritidis 2713 was inoculated into fresh cheese whey to final concentrations of 10^2, 10^3, 10^4, 10^5, and 10^6 CFU/ml. Kefir grains (10%, wt/vol) were then added, and fermentation was conducted at 20°C for 24 h. Both uncontaminated and contaminated cheese wheys without kefir grains were used as controls.

The survival of the pathogenic bacteria after 24 h of incubation was examined by assaying viable counts on selective culture media. The differential counting of *E. coli* was performed after an initial mixing with tryptone bile x-glucuronide agar, and blue colonies throughout the volume were finally counted after 24 h of incubation at 44°C. The concentration of viable *Salmonella* sp. was determined on xylose lysine deoxycholate agar, and the characteristic black colonies were counted after 24 h of incubation at 37°C. The experiment was performed in triplicate.

The survival of the bacterial pathogens after fermentation was evaluated by the same procedure with initial pathogen concentrations at 10^2 and 10^4 CFU/ml for the isolates *E. coli* 2760, *E. coli* 2622, *E. coli* 2751, *E. coli* 2846, *Salmonella* sp. 54, *Salmonella enterica* serovar Typhimurium 45, *Salmonella enterica* serovar Infantis 2729, and *Salmonella enterica* serovar Enteritidis 735.

Statistical analysis. The differences in microbiological composition and in the diameters of the inhibition zones were tested for statistical significance at $P < 0.05$ by analysis of variance (ANOVA). The normal distribution of data was determined by the Shapiro-Wilk test. The results were expressed as means ± standard deviations (±SD) of at least three separate duplicate experiments.

TABLE 2. *Microbiological composition of fermentation products*

Inoculum	Whey[a]	LAB (CFU/ml)[b]	Yeasts (CFU/ml)
Kefir grains	RWP	$1.04 \pm 0.6 \times 10^7$ a[c]	$4.23 \pm 1.5 \times 10^6$ a
	BW	$1.05 \pm 0.3 \times 10^7$ a	$2.05 \pm 1.4 \times 10^6$ ab
	OW	$1.17 \pm 0.4 \times 10^7$ ab	$2.70 \pm 1.6 \times 10^6$ ab
Whey culture	RWP	$1.50 \pm 0.3 \times 10^8$ b	$1.10 \pm 0.5 \times 10^7$ b
Milk culture	RWP	$7.70 \pm 1.3 \times 10^8$ c	$7.50 \pm 3.0 \times 10^5$ c

[a] RWP, reconstituted whey powder; BW, liquid bovine whey; OW, liquid ovine whey.
[b] LAB, lactic acid bacteria.
[c] Within columns different letters indicate statistically different data ($P < 0.05$) according to the Tukey test.

RESULTS

Microbiological and chemical composition of fermented products. The quantity of yeasts and lactic acid bacteria in the different wheys after fermentation by kefir grains was not significantly different ($P > 0.05$). The content of lactic acid bacteria in these products ranged from 1.04×10^7 to 1.17×10^7 CFU/ml and that of yeasts from 2.05×10^6 to 4.23×10^6 CFU/ml (Table 2).

In contrast, when whey and milk cultures were used as starters instead of kefir grains, the content of lactic acid bacteria was significantly different ($P < 0.05$), with counts of at least one log order higher. Moreover, the concentration of yeasts was significantly higher ($P < 0.05$) in the product obtained with the whey culture and significantly lower ($P < 0.05$) in that obtained with the milk starter with respect to the corresponding CFU in the three wheys fermented with kefir grains.

In terms of chemical composition, the fermentation products did not differ substantially from the corresponding unfermented cheese whey except with respect to the lactose and lactic acid contents (Table 3 versus Table 1). During fermentation, the lactose content of the whey decreased by 41 to 48%, while the lactic acid levels reached 0.84 to 1.20% of the total product mixture when fermentation was started by kefir grains (Table 3).

Inhibitory activity of fermented whey. The inhibitory activity of the fermented products against 100 isolates of *Salmonella* sp. and a further 100 isolates of *E. coli* was tested by the agar well diffusion assay (Table 4). All fermented products obtained with 10% (wt/vol) kefir grains inhibited all the isolates evaluated, but significant differences ($P < 0.05$) were observed in the mean diameters of the inhibition zones when different sources of whey were fermented. In addition, each fermentation product exhibited a significantly greater ($P < 0.05$) inhibition of *Salmonella* sp. than of *E. coli*. By contrast, cheese whey fermented by 1% (wt/vol) kefir grains and by whey and milk starter cultures showed no inhibition in this agar well diffusion assay.

Fermented cheese whey maintained its inhibitory activity after heat treatment. The mean inhibition zone diameters of whey fermented with 10% (wt/vol) kefir grains and heated

TABLE 3. *Chemical composition of fermentation products*

Inoculum:	Kefir grains			Whey culture	Milk culture
Cheese whey[a]:	RWP	BW	OW	RWP	RWP
Proteins (g/100 g)	0.93 ± 0.06	1.35 ± 0.07	1.25 ± 0.49	1.05 ± 0.07	1.25 ± 0.07
Lipids (g/100 ml)	<0.3	0.4	0.4	<0.3	<0.3
Lactose (g/100 g)	3.36 ± 1.92	3.81 ± 1.26	2.71 ± 0.45	3.38 ± 1.29	4.12 ± 1.66
Moisture (g/100 ml)	93.23 ± 1.01	89.5 ± 0.14	91.65 ± 0.21	91.95 ± 0.21	91.6 ± 0.14
Ash (g/100 ml)	0.69 ± 0.02	1.85 ± 0.07	1.65 ± 0.07	0.55 ± 0.21	0.75 ± 0.07
Sodium (g/100 g)	0.05 ± 0.01	0.44 ± 0.02	0.46 ± 0.01	0.06 ± 0.01	0.05 ± 0.01
Magnesium (mg/100 g)	88.2 ± 23.7	103.9 ± 16.3	106.4 ± 16.9	92.2 ± 21.3	97.3 ± 14.0
Potassium (mg/100 ml)	220.1 ± 8.59	290.5 ± 67.8	159.0 ± 16.8	219.8 ± 24.4	223.6 ± 15.7
Calcium (mg/liter)	479.8 ± 90.4	579.1 ± 23.4	554.5 ± 17.3	442.6 ± 105.8	570.7 ± 13.1
Phosphorus (%)	<0.01	<0.01	<0.01	<0.01	<0.01
pH	3.65 ± 0.09	3.54 ± 0.06	3.39 ± 0.04	5.05 ± 0.35	4.46 ± 0.01
Lactic acid (%)	0.84 ± 0.03	0.99 ± 0.04	1.20 ± 0.05	0.17 ± 0.07	0.55 ± 0.02

[a] RWP, reconstituted whey powder; BW, liquid bovine whey; OW, liquid ovine whey.

were 9.5 ± 0.7 mm and 9.1 ± 0.8 mm for *Salmonella* sp. and *E. coli,* respectively. These values were not significantly different ($P > 0.05$) from those of the same untreated product (Table 4), indicating that the inhibitor was not thermolabile.

When the fermentation product was alkalinized to pH 4.5 or 7, the inhibitory activity was lost; thus, the inhibition does require a relatively low pH. The pH per se, however, did not inhibit the isolates of *Salmonella* sp. and *E. coli* evaluated, since unfermented whey acidified with HCl to a pH of 3 produced no inhibition of pathogen growth. In contrast, the diameters of the inhibition zones produced by fermented whey (Table 4) and by artificially acidified whey containing the same concentration of lactic acid (9.3 ± 0. 7 mm for *Salmonella* sp. and 8.8 ± 0.6 mm for *E. coli*) were not significantly different ($P > 0.05$).

TABLE 4. *Antimicrobial activity of different fermented cheese whey against* Salmonella *sp. and* E. coli

Inoculum	Cheese whey[a]	Mean (±SD) diam of inhibitory zone (mm)[b]	
		Salmonella sp. (n = 100)[c]	*E. coli* (n = 100)
Kefir grains 10% (wt/vol)	RWP	9.20 (±1.06) A a	8.92 (±0.82) A b
	BW	10.37 (±0.69) B a	9.69 (±0.59) B b
	OW	10.37 (±0.61) B a	9.97 (±0.64) C b
Kefir grains 1% (wt/vol)	RWP	NI[d]	NI
	BW	NI	NI
	OW	NI	NI
Whey culture	RWP	NI	NI
Milk culture	RWP	NI	NI

[a] RWP, reconstituted whey powder; BW, liquid bovine whey; OW, liquid ovine whey.

[b] Uppercase letters compare the sensitivities of the two indicator microorganisms to each of the three cheese wheys fermented with 10% (wt/vol) kefir grains. Lowercase letters compare the growth inhibition of *Salmonella* sp. versus *E. coli* evoked by each fermentation product. The mean diameters followed by the same letter did not differ significantly ($P > 0.05$).

[c] *n,* number of tested isolates.

[d] NI, no inhibition zone was detected.

For all the *E. coli* isolates evaluated, the MICs of the wheys obtained from the three sources and fermented with 10% (wt/vol) kefir grains were 30%. For all *Salmonella* sp. isolates tested, the MICs of fermented BW and OW were likewise 30%; however, the MICs of the fermented RWP were 30% for 10 isolates and 40% for another 10 isolates.

The MBCs of the wheys fermented with 10% (wt/vol) kefir grains varied according to the pathogenic isolate tested (Table 5). The MBC of fermented BW and OW was between 40 and 60% for most of the *Salmonella* sp. isolates and for all the *E. coli* isolates. With the fermented RWP, the MBC was between 50 and 70% for most of the isolates of both genera.

We next characterized the growth inhibition of *E. coli* and *Salmonella* sp. in culture media containing different concentrations of fermented cheese whey supernatant (Fig. 1A and 1B). When the supernatant was added to the culture medium at a concentration of 20% (vol/vol), a significant reduction in the growth occurred with respect to that of the control; while at concentrations of 30% (vol/vol) or higher no pathogen growth whatsoever was detected.

TABLE 5. *MBC of cheese whey fermentation products on* Salmonella *sp. and* E. coli

	Salmonella sp.			*E. coli*		
MBC (%)	RWP[a]	BW	OW	RWP	BW	OW
30	2/21[b]	—	1/10	1/20	—	—
40	2/21	2/10	4/10	—	1/11	1/10
50	4/21	4/10	2/10	7/20	7/11	7/10
60	3/21	4/10	3/10	6/20	3/11	2/10
70	9/21	—	—	4/20	—	—
90	1/21	—	—	2/20	—	—

[a] The columns correspond to supernatants of different cheese wheys fermented with 10% (wt/vol) kefir grains: RWP, reconstituted whey powder; BW, liquid bovine whey; OW, liquid ovine whey.

[b] Number of strains of *Salmonella* sp. or *E. coli* showing a certain MBC/number of tested isolates.

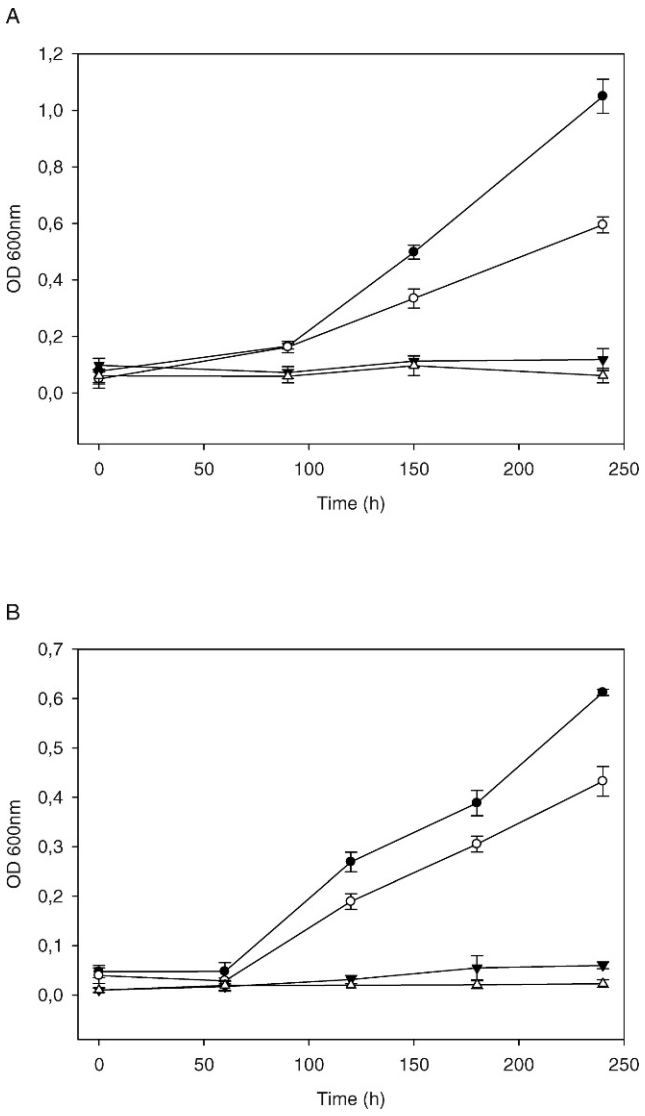

FIGURE 1. *Growth of* E. coli *2710 (A) and* Salmonella enterica *serovar Enteritidis 2713 (B) in presence of fermented cheese whey supernatant. (●) Control without supernatant, (○) with 20% (vol/vol) supernatant, (▼) with 30% (vol/vol) supernatant, and (△) with 40% (vol/vol) supernatant. Error bars indicate the standard deviation among three independent trials.*

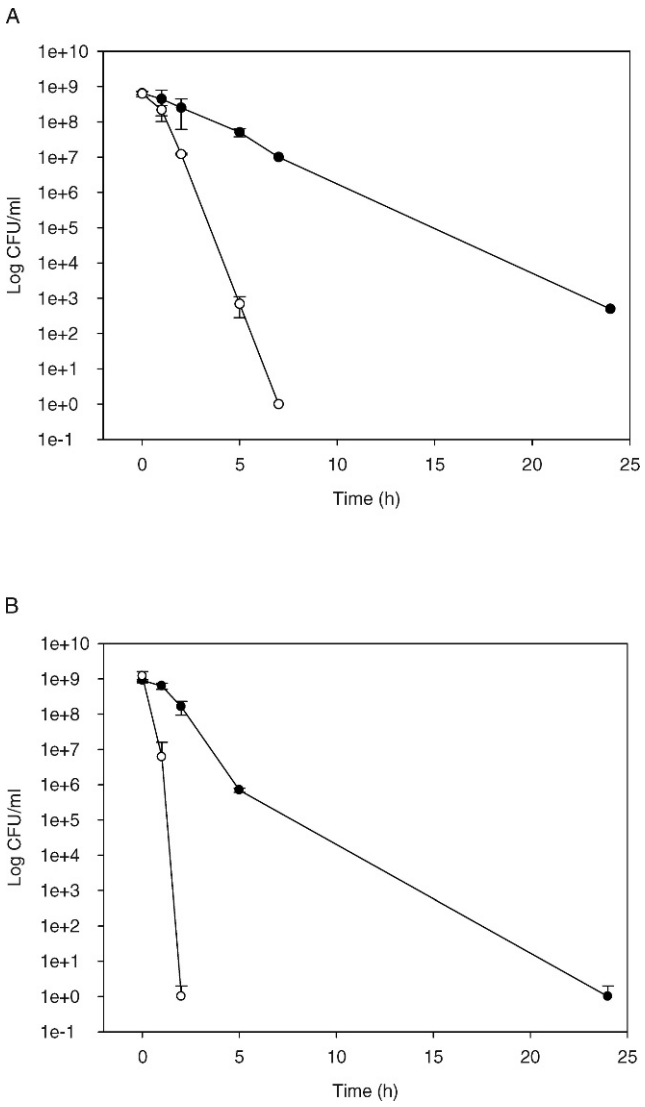

FIGURE 2. *Decrease in the viability of* E. coli *2710 (A) and* Salmonella enterica *serovar Enteritidis 2713 (B) in contact for 24 h with supernatant of cheese whey fermented by 10% (wt/vol) kefir grains. (○) Pure supernatant and (●) supernatant diluted in BPW to a concentration of 60% (vol/vol). Error bars indicate the standard deviation.*

Figure 2 shows the effect of fermented cheese whey supernatant on the viability of *E. coli* and *Salmonella* sp. When *E. coli* 2710 was exposed to 60% (vol/vol) supernatant, the pathogen concentration decreased from 10^9 to 10^7 CFU/ml after 7 h, and only 10^2 CFU/ml survived after 24 h of incubation. With the undiluted supernatant, after a 7-h incubation no CFU were detectable by the methodology used (Fig. 2A). The viability of *Salmonella enterica* serovar Enteritidis 2713 in the presence of 60% (vol/vol) supernatant declined by 3 log after 5 h, whereas after 24 h of incubation no surviving bacteria were observed. With the undiluted whey fermentation supernatant, no CFU were detectable after a mere 2 h of incubation (Fig. 2B).

Reduction of bacterial pathogens in cheese whey after fermentation with kefir grains. In order to elucidate the effect of kefir grain fermentation on the survival of possible whey-contaminating pathogens, the reduction in the concentration of *Salmonella* sp. and *E. coli* was evaluated (Table 6). Regardless of the initial concentration (10^2 to 10^6 CFU/ml), both pathogens reached concentrations exceeding 10^6 CFU/ml after 24 h of incubation in fresh whey. When the whey containing *Salmonella enterica* serovar Enteritidis 2713 at concentrations lower than 10^5 CFU/ml was fermented with kefir grains, the pathogen was not recovered in the reaction product mixture. When, however, the pathogen's initial concentration in whey reached 10^6 CFU/ml, the viability still decreased by 4 log cycles during fermentation. Of the other four *Salmonella* sp. isolates tested, no survivors remained after fermentation at initial concentrations of 10^2 CFU/ml or 10^4 CFU/ml. The fermentation of cheese whey containing 10^2 CFU/ml of *E. coli* 2710 resulted in a complete loss of the pathogen's viability. In whey with higher initial concentrations of *E.*

TABLE 6. *Reduction of the concentration of* Salmonella enterica *serovar Enteritidis and of* E. coli *in cheese whey after fermentation with kefir grains*

Initial concn (CFU/ml)	*Salmonella enterica* serovar Enteritidis 2713		*Escherichia coli* 2710	
	Concn in fermented whey (CFU $\times 10^{-2}$/ml)	Concn in nonfermented whey (CFU/ml)	Concn in fermented whey (CFU $\times 10^{-2}$/ml)	Concn in nonfermented whey (CFU/ml)
1×10^2	<0.3	1.1×10^6	<0.3	6.0×10^7
1×10^3	<0.3	5.0×10^6	0.2 ± 0.3	1.8×10^8
1×10^4	<0.3	7.2×10^7	1.2 ± 0.8	3.1×10^8
1×10^5	<0.3	1.5×10^9	22.0 ± 31.0	3.5×10^8
1×10^6	1.7 ± 2.9	5.0×10^9	ND[a]	ND

[a] ND, not determined.

coli, the fermentation resulted in a reduction in viable bacterial counts by 2 log cycles. The same results were found for the other four *E. coli* isolates tested.

DISCUSSION

The disposal of cheese whey represents an environmental problem of concern for the dairy industry. Furthermore, the carbohydrate reservoir of whey in the form of lactose and the presence of other nutrients essential for microbial growth make this by-product a potential substrate for kefir grain fermentation. The fermentation product could potentially be used as a probiotic, thus providing a rationale for recycling whey. This work characterized different kefir grain fermentation products and investigated their inhibitory activity.

The microbiological composition of different wheys fermented with 10% (wt/vol) kefir grains was determined and found not to be affected by the whey employed as the source. Fermented wheys have a lower content of lactic acid bacteria and a higher level of yeasts than concentrations previously described for kefir milk *(10).* A similar trend had been reported by Rimada and Abraham *(33)* for deproteinized milk whey after fermentation with kefir grains.

As for chemical composition, the major variation during fermentation is the conversion of lactose to lactic acid. The data from this study are also in agreement with those reported by Assadi et al. *(1),* who described a lactose consumption of 30% (wt/vol) and a lactic acid production up to 0.83% of the reaction products in whey fermented for 24 h with 2% (wt/vol) kefir grains. On the other hand, the differences in chemical composition among the fermentation products depend principally on the type of whey employed as the substrate.

We demonstrated the ability of whey fermented with 10% (wt/vol) kefir grains to inhibit the growth of *E. coli* and *Salmonella* sp. and also evaluated the use of milk culture, whey culture, or 1% (wt/vol) kefir grains as starters in order to avoid excessive quantities of grains in large-scale industrial production. These latter fermentation products, however, have low lactic acid concentrations; therefore, they proved to lack inhibitory activity.

The results obtained in this work indicate that the inhibitory activity of fermented whey is largely a result of high levels of lactic acid. Fermentation products with higher lactic acid contents were more inhibitory in agar well diffusion assays. In addition, we demonstrated that nonfermented whey supplemented with lactic acid exhibits the same inhibitory potency as fermented whey containing an equal content of lactate. The use of lactic acid is one of the oldest methods employed to inhibit the growth of gram-negative and gram-positive bacteria *(13).* Such inhibition is due mainly to the undissociated form of this organic acid, which can traverse bacterial membranes, lower the intracellular pH, and, in so doing, adversely affect several metabolic functions along with cellular osmolarity *(4, 32).* Östling and Lindgren *(24)* reported that the MIC for undissociated lactic acid was 7 mM for a single isolate of *Salmonella* sp. and for four isolates of *E. coli*. Our study showed that the MICs for 20 isolates of *E. coli* and 20 of *Salmonella* sp. were 30 or 40% of the concentration of the fermentation product, at a concentration of undissociated lactic acid between 5 and 9 mM. The bacterial growth kinetics in medium supplemented with fermented cheese whey supernatant also confirmed that result.

We found differences in the MBCs of the whey fermentation supernatants among the various pathogenic isolates. The pronounced bactericidal effect on *Salmonella* sp. and *E. coli* was most greatly evidenced by the results of the survival assay, in which pathogens lost their viability after 2 to 7 h of incubation.

In addition to the bactericidal efficacy of the fermentation products, the pathogenic bacteria became dramatically reduced in number in whey during the fermentation process itself. Indeed, for a population to survive that process, the initial concentration had to be greater than 10^4 CFU/ml for *Salmonella* sp. and 10^2 CFU/ml for *E. coli*. Therefore, the fermentation product itself is pathogen free and thus cannot be a vehicle for harmful foodborne microorganisms after the fermentation process.

The use of whey to prepare a naturally fermented acidic product having strong growth-inhibitory activity against *Salmonella* sp. and *Escherichia* sp. would provide probiotic kefir-derived microorganisms directly to the consumer (animal or human) and would be an innovative way to recycle that by-product. Also, the industrial production would run a low risk of contamination as a result of the product's inherent ability to inhibit contaminating microorganisms. We consider that cheese whey fermented with kefir grains could be potentially used as a probiotic product in the near future.

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