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Abstract

Fungal contamination negatively affects the production of cereal foods such as *arepa loaf*, an ancient corn bread consumed daily in several countries of Latin-America. Chemical preservatives such as potassium sorbate are applied in order to improve the *arepa*'s shelf life and to reduce the health risks. The use of natural preservatives such as natural fermented products in food commodities is a common demand among the consumers. Kefir is a milk fermented beverage obtained by fermentation of kefir grains. Its antibacterial and probiotic activity has been exhaustively demonstrated.

Our objectives were to determine the antifungal effect of kefir fermented milk on *Aspergillus flavus* AFUNL5 *in vitro* and to study if the addition of kefir fermented milk to *arepas* could produce shelf life improvement. We determined the antifungal effect on solid medium of kefir cell-free supernatants (CFS) obtained under different fermentation conditions. Additionally, we compared the antifungal effect of kefir CFS with that obtained with unfermented milk artificially acidified with lactic plus acetic acids (lactic and acetic acids at the same concentration determined in kefir CFS) or with hydrochloric acid. Finally, kefir was added to the corn products either in the loaf recipe (kefir-baked *arepas*) or sprayed onto the baked-loaf surface (kefir-sprayed *arepas*). The loaves' resistance to natural and artificial fungal contamination and their organoleptic profiles were studied.

The highest fungal inhibition on solid medium was achieved with kefir CFS produced by kefir grains CIDCA AGK1 at 100 g/L, incubated at 30 ºC and fermented until pH 3.3. Other CFS obtained from different fermentation conditions achieved less antifungal activity than that mentioned above. However, CFS of milk fermented with kefir grains, until pH 4.5 caused an increase of growth rates. Additionally, CFS produced by kefir grains CIDCA AGK1 at 100 g/L, incubated at 30 ºC and fermented until pH 3.3 achieved higher antifungal activity than CFS from artificially acidified milk with organic acids (CFS L+A) at the same concentration of kefir CFS. Besides, CFS from milk acidified with hydrochloric acid (CFS HCl) showed no fungal inhibition. On the other hand, kefir-baked *arepas* exhibited significant resistance to natural and artificial fungal contamination. Finally, both kefir-baked and kefir-sprayed *arepas* retained the organoleptic characteristics of the traditional corn product, but with certain tastes imparted by the kefir fermentation. This

work constitutes the first study on fungal inhibition by kefir-fermented milk extending to the protection of corn products of mass-consumption and the possible application as a food preservative.

Keywords: kefir, *arepa,* antifungal, *Aspergillus flavus*, organoleptic profile.

1. Introduction

Maize is an ancient grain that has been the staple food in many Latin-American countries since ancient times. Maize is the leading cereal crop in terms of worldwide production, used for various food and feed products. Maize is transformed into various food products including breakfast cereals (as the *arepa*), snacks, yeast and chemically leavened bakery items, corn syrups, beer and distilled spirits, and an array of nixtamalized products such as tortillas and chips that are gaining relevance throughout the globe. The nutritional qualities of these industrialized and traditional foods greatly contribute to the food cultures of many civilizations throughout the world (Serna-Saldivar, 2016).

Food contamination by *Aspergillus flavus* or *A. parasiticus* and their mycotoxins causes losses particularly in grains and cereal derivatives (Pitt and Hocking, 1999). Warm and humid subtropical and tropical conditions are ideal for colonization and dominance of *A. flavus/parasiticus* species on maize (Milani, 2013). Maize products were analyzed in Nigeria, a tropical country, finding that *Aspergillus* was the most predominant genus isolated (62% samples). *A. flavus*, *A. niger*, and *A. tamari* were identified (Sule Enyisi et al., 2015). Maize is the main material used to prepare *arepas*, a basic component of the diet among rural and urban people alike (ICBF, 2010).

Many *arepas* producers face the challenges of ensuring that the product can be purchased and consumed without suffering deterioration due to fungal growth, which appears macroscopically in the product (Yousef and Carlstrom, 2001), and whose main source is cross-contamination during the production process. The Colombian NTC 5372 norm sets a maximum allowable limit of 100 CFU/g to identify a good microbiological quality for the *arepa* loaves (ICONTEC, 2007).

In the attempt to protect the *arepa* against fungal contamination, sorbic acid, potassium sorbate and sodium propionate among others, have been used as preservatives (MSPSC, 1991). However, recent research has demonstrated that approved chemical preservatives in *arepa* loaves exert different antifungal effects, showing

that fungal counts in presence of potassium sorbate exceed the maximum allowed by the law between days 6 and 9 of storage (Corpas and Tapasco, 2012a). Consumer demand for products preserved through organic means has increased since the end of the twentieth century, however, there are no studies on the application of biopreservatives on *arepa* loaves.

One alternative that has been widely explored is the application of lactic acid bacteria, which are capable of binding mycotoxins, and which produce organic acids, exhibit antibacterial and antifungal activities (Cortés-Zavaleta et al., 2014; Gerez el al., 2009; 2013; Haskard et al., 2001; Hernández-Mendoza et al., 2009; Londero et al., 2014; Gamba et al., 2015, 2016). Kefir grains contain a symbiotic consortium of lactic acid bacteria (LAB) and yeasts that effects the dual fermentation of saccharide precursors in milk to lactic acid and to alcohol to produce a beverage of characteristic organoleptic properties (Zourari and Anifantakis, 1988). Kefir-fermented milk supernatants at a concentration of 10% (v/v) in a broth completely inhibited the growth of *A. flavus* and *Fusarium graminearum* (Ismaiel et al., 2011). Cell free supernatants obtained from whey permeate fermented with kefir grains inhibited *A. parasiticus* and *F. graminearum* growth and the aflatoxin B_1 and zearalenone production (Gamba et al., 2015, 2016). The antimicrobial properties of kefir-fermented milk have been associated mainly with the presence of lactic and acetic acids (Garrote et al., 2000). Studies conducted with lactic and propionic acid bacteria indicated that the main metabolites inhibiting *A. fumigatus* and *A. nidulans* were acetic and propionic acids, with the lactic acid present having a lesser effect. Moreover, the necessary concentration for fungal inhibition increased with a pH elevation (Lind et al., 2005). The antifungal activity of 91 isolates of LAB was attributed to the presence of lactic, acetic, and phenyllactic acids along with a peptide produced by *Lactobacillus fermentum* (Gerez et al., 2013). Other authors attributed the antifungal activity to a synergistic effect among all the acids present within the fermentation products (Cortés-Zavaleta et al., 2014).

Different types of kefir grains were obtained from two families that traditionally consumed kefir. Such grains had no common `history' previous to their arrival at CIDCA (Centro de Investigación y Desarrollo en Criotecnología de Alimentos - Universidad Nacional de La Plata). They were named CIDCA AGK1 and CIDCA AGK2, characterized and stored in the CIDCA collection. Kefir grains CIDCA AGK1 and CIDCA AGK2 present similar chemical composition (protein, polysaccharide and water concentration), similar acidification kinetics, lactic and acetic acid production and microbial counts (10^8 CFU/mL, 10^7 CFU/mL and

10^5 CFU/mL LAB, yeasts and acetic acid bacteria, respectively). These kefir grains comprised some common species (*Lactobacillus plantarum*, *L. kefir*, *Lactococcus lactis* subsp. *Lactis*, *Saccharomyces* and *Acetobacter*). However, *Leoconostoc mesenteroides* was isolated from CIDCA AGK1 grains and *Lb. parakefir*, *Lc. lactis* subsp. *lactis* biovar *diacetylactis* and *K. marxianus* from CIDCA AGK2 grains (Garrote et al., 2000; 2001). As the kefir microbiota produces organic acids, in the present study we determined the antifungal activity of the cell free supernatants (CFS) of the milk fermented by kefir grains at different conditions and compared the inhibition with that of milk artificially acidified either with pure organic acids or with strong acid (hydrochloric acid). We also incorporated fermented milk in the preparation of the *arepa* in order to study the subsequent resistance to either natural fungal contamination or artificial contamination produced by a deliberate exposure to *A. flavus*. Additionally, kefir fermented milk was added to *arepa* and its organoleptic characteristics were tested.

2. Materials and methods

2.1 Fungal strains and preparation of conidial inoculum

A toxigenic strain *A. flavus* AFUNL5 isolated from maize samples was obtained from Laboratorio de Micología, Universidad Nacional del Litoral, Argentina. *A. flavus* was maintained at 4 °C in aqueous agar (0.2% w/v). The inoculum was prepared by growing the fungus on Potato Dextrose Agar (Merck, Darmstadt, Germany) slants for 7 days at 30 °C. After incubation, 10 mL of 0.01% (w/v) sodium lauryl sulfate (Merck, Darmstadt, Germany) in 1% (w/v) sodium chloride solution were added to the tubes and conidia were loosened by gently scraping with a spatula (Molina and Giannuzzi, 1999). The number of conidia was determined by counting in a Neubauer chamber. The number of conidia was adjusted to 10^4 conidia/mL to conduct fungal inhibition assays in solid media and in *arepa* loaves.

2.2 Preparation of cell-free supernatants (CFS)

CIDCA AGK1 and AGK2 kefir grains were characterized at CIDCA, UNLP (Garrote et al., 2000; Garrote et al., 2001) and stored in whole milk at –20 ºC. The kefir grains were activated through two consecutive fermentation passages in commercial ultra high temperature processed (UHT) milk (Sancor®, Santa Fe,

Argentina). The grains were then transferred to fresh milk at a concentration of 10% w/v and incubated at 20, 30 and 37 ºC until reaching the respective pHs of 4.5, 3.5 and 3.3. The pH readings were made with an Altronix TPX-III™ (Altronix, Taiwan) instrument. The fermented products were passed through a strainer of mesh size 1 mm^2 to remove the grains. The remaining microorganisms in the fermented filtrate were precipitated by centrifuging for 15 min at 14,000 *g* in an Eppendorf 5415D™ centrifuge (Eppendorf, Hamburg, Germany). The resulting supernatant was filter-sterilized by passage through a nitrocellulose membrane of 0.22-µm pore size (Sigma-Aldrich, St. Louis, USA) before storage at –20 ºC until use in the antifungal activity assays.

2.3 Determination of organic acids content in the kefir fermented CFS

CFS from kefir fermented milk were obtained as described previously (Section 2.2). A volume of 10 µL CFS was injected in the chromatograph.

The organic acid contents in kefir fermented milk CFS were quantified by high performance liquid chromatography (HPLC) in a Series 1200™ chromatograph (Agilent Technologies, Santa Clara, CA, USA), equipped with an ultraviolet detector set at a wave length of 214 nm and containing an Aminex HPX-87H™ ion-exchange column (Bio-Rad, Hercules, CA, USA). The mobile phase was 45 mM sulfuric acid (Merck, Darmstadt, Germany), the temperature 60 ºC, and the flow rate 0.7 mL/min. Curves of pure organic acids were made with 0.34, 0.68, 1.35, 2.71, 9.48, 20.32, 40.00, 50.00 and 100 mM of acetic acid (Merck, Darmstadt, Germany) and with 8.66, 17.32, 69.28, 138.55 173.00, 200.00, 250.00, 300, 400 and 500 mM of lactic acid (Carlo Erba, Milan, Italy). Additionally, 10 g of the *arepas* were homogenized with 90 mL double distilled water in a Stomacher 400™ laboratory blender (Stomacher, England). The pH of the resulting homogenate was measured in a pH meter Altronix TPX-III (Altronix, Taiwan).

2.4 Preparation of cell free supernatants from artificially acidified milk

The lactic and acetic acids were dissolved in UHT milk at the same concentrations previously determined by HPLC in milk fermented with CIDCA AGK1 and AGK2 kefir grains. In addition, UHT milk was acidified with hydrochloric acid 3 M (Merck, Darmstadt, Germany), as representative of a strong acid, to final pHs of 3.5 and 3.3. After acidification, the solutions were centrifuged and filtered through nitrocellulose membrane

in order to obtain the respective CFS with lactic and acetic acids (named CFS L+A) and with hydrochloride acid 3 M (named CFS HCl).

2.5 Fungal inhibition assays

Fungal inhibition assays were performed in Petri dishes with sterile basal medium, containing malt extract (10 g/L) (Biokar, Beauvais, France), yeast extract (20 g/L) (Biokar, Beauvais, France) and agar (20 g/L) (Merck, Darmstadt, Germany) as described previously (Leon et al., 2012; Gamba et al., 2016). The media were autoclaved at 121 ºC for 15 min and different treatments were performed. The basal medium at 45 °C was mixed with a given CFS obtained from kefir fermented milk in the range of 50% and 10% (v/v). Other media were mixed with CFS obtained from artificially acidified milk with lactic and acetic acids (CFS L+A). Finally, other media were amended with CFS obtained from artificially acidified milk with HCl (CFS HCl). The final pH of the medium was measured. Each treatment was inoculated with 10 µL of the suspension at 10^4 conidia/mL dispensed by micropipetting in the center of the solidified growth medium. The diameter of the circular inocula obtained was assumed as the colony initial diameter. Inoculated plates were incubated at 30 ºC inside plastic boxes containing dishes of water to prevent dehydration. Growth was allowed until the maximum 80 mm diameter, corresponding to mycelial growth across the total Petri dish; all cultures were incubated for up to 30 days in order to determine the total fungal inhibition (López-Malo et al., 2007). Colony diameters were measured each day by placing the Petri dishes on a millimeter scale illuminated from beneath by a light box. Four diameter measures were taken from the center of each colony and the results were calculated from the mean diameter of the replicate colonies (Horner and Anagnostopoulos, 1973). The control medium was the basal medium without any addition. All treatments were performed in triplicate. Two independent assays were performed.

Growth rate K_D (mm/h), was calculated from the regression slope of colony diameter versus time during the linear growth phase, using the Sigma Plot 9.0™ software. The lag phase is the time in hours required for the colony to grow beyond the inoculation zone (typically 5-7 mm). This value corresponded to the point on the *abscissa* where the regression line intersected the horizontal line representing the initial inoculation-zone diameter:

$$\text{Lag (h)} = (D_0 - Y_0)/K_D \quad (1)$$

Where: D_0= diameter of the inoculation zone, Y_0= intersection of the regression line with the *ordinate* and K_D= slope of the regression line (*i. e.*, growth rate) (Cuppers et al., 1999; Molina and Giannuzzi, 1999).

2.6 Effect of kefir fermented milk on the resistance to fungal contamination, and on the organoleptic properties of *arepa*

Corn was boiled in water for two hours in order to prepare the *arepas*. After draining off the water, corn was ground manually to produce the dough, which was then shaped into disks of about 8 cm in diameter and 8 mm thickness before baking in a gas oven (Segesa, Argentina) for 7 min on each side at 140 ºC. After baking, *arepas* were left for 90 min to cool down to 20 °C. Two treatments were assessed. Milk kefir obtained with kefir grains CIDCA AGK1 was added to the crude dough before shaping into disks (10% v/w), named kefir baked (KB) *arepas*. Alternatively the fermented milk was brushed onto the surface of the baked *arepas* (10% w/v) and left to dry for one hour, named kefir sprayed (KS) *arepas*. The control (CA) *arepas* received no addition of fermented milk at either step. All *arepas* were packaged into polyethylene bags, heat-sealed and stored at 14 ºC for 10 days.

2.6.1 Microbial counts in kefir and in *arepa*s

A total of 10 mL of kefir fermented milk or 10 g of the *arepas* were homogenized with 90 mL of 1 g/L aqueous peptone solution and necessary serial dilutions were performed. A volume of 100 µL were inoculated and homogenized on the agar by sterile beads. The number of filamentous fungi (FF), LAB and yeasts in the *arepas* (control and treated) was assessed by counts on Dichloran Rose Bengal Chloramphenicol agar (BIOKAR, Beauvais, France), De Man-Rogosa-Sharpe (MRS) agar (Difco, Beauvais, France) and Yeast Glucose Chloramphenicol (YGC) agar (Merck, Darmstadt, Germany), respectively, at 1, 5 and 10 days of storage. Plates were incubated at 30 ºC for 48 h for LAB, and at 30 ºC for 120 h for yeasts and FF. After these treatments, the colony forming units (CFU/mL or CFU/g) were quantified (Magalhães et al., 2010).

2.6.2 Determination of the resistance to artificial fungal contamination of *arepa* loaf prepared with kefir fermented milk

A fungal suspension of A. *flavus* AFUNL5 at 10^4 conidia/mL was prepared. Then, 1 mL of this suspension was pulverized per 100 g *arepa*, whereas the uncontaminated *arepa* control was treated in the same way but with sterile distilled water. The samples were then left to dry before storage at 14 ºC in sealed polyethylene bags. Thereafter the bags were inspected daily for determination of visible signs of the fungal presence (Gerez et al., 2009). The intervening time was considered the *arepa* resistance to the fungal contamination.

2.6.3 Organoleptic profile

All sensory analyses were conducted at the Laboratory of Sensory Analysis at the University of Antioquia, Medellin, Colombia. Test room design and preparation area complies with the Colombian Technical Guide 226. The environmental conditions were monitored throughout the test (50 ± 5% relative humidity and 24 ± 2 °C) (ICONTEC, 2012).

Flavor profile was performed according to the Colombian Technical Standard NTC 3929 - ISO (International Standards) 6564 (ICONTEC, 2009; ISO, 1985) with a sensory panel of eight trained panelists according to the Colombian Technical Guide 245 and 246 (ICONTEC, 2013). Sensory analysis was carried out with KB *arepas* (fermented milk was added to the crude dough before shaping into disks 10% v/w), KS *arepas* (fermented milk was brushed onto the surface of the baked *arepas*) and CA *arepas* (received no addition of fermented milk at either step). Before sensory analysis, panelists were trained in order to improve their ability to identify and assess the attributes of the product in three sessions. *Arepas* added with kefir fermented milk and *arepas* without addition of kefir fermented milk were used during the training sessions.

During the evaluation, each panelist was situated in an individual booth under incandescent light of intensity approximately 350 lx. At the preparation area, the different *arepa* samples were heated and given to the panelists at a temperature of 50 °C. White dishes with 20 g of *arepas* at 50 °C were provided. Samples were coded by three-digit numbers. The order of sample evaluation was randomized for each panelist and was presented in such a manner that the panelist could not identify the sample. Tap water was provided between samples to rinse the palate. All assays were performed in triplicate.

To carry out flavor profile test, panelists described the flavors found in the sample, ordered perception, rated each flavor descriptor, described residual or persistent flavors and rated the general quality flavor by a scale of 0 to 5, where five it is the maximum intensity.

2.7 Statistical analyses

The statistical significance of differences between the data obtained on fungal growth and microbial growth was determined by analysis of variance (ANOVA), and the multiple-range (Fisher) test at the level of 5% (p<0.05) by means of the Statgraphics Centurion XV.II™ program. All experiments were performed in triplicate.

3. Results

3.1 Antifungal action of CFS from milk fermented with kefir grains CIDCA AGK1 and AGK2 at 20, 30, and 37 °C.

Fig. 1 presents some growth curves of *A. flavus* AFUNL5 in solid medium added with CFS obtained from milk fermented by CIDCA AGK1 (a) and CIDCA AGK2 (b) kefir grains to different final pH values at 30 ºC. The time required for *A. flavus* AFUNL5 to reach the plate edge became greater with increased final acidity and with the percent concentration of the CFS supplementing the growth medium, indicating an antifungal effect. CFS obtained from CIDCA AGK1 grains to a final pH of 3.3 and 50% (v/v) exerted the greatest fungal inhibition (240 h to reach the plate edge), whereas CFS obtained from CIDCA AGK2 grains at pH 3.3 and 50% (v/v) caused a less inhibition (198 h to reach the plate edge).

Dilutions of these supernatants and the use of others with less acidic pH (CFS at pH 4.5 and 3.5) caused lower fungal inhibition than those mentioned above. However, the inhibition obtained with these dilutions was higher than the controls.

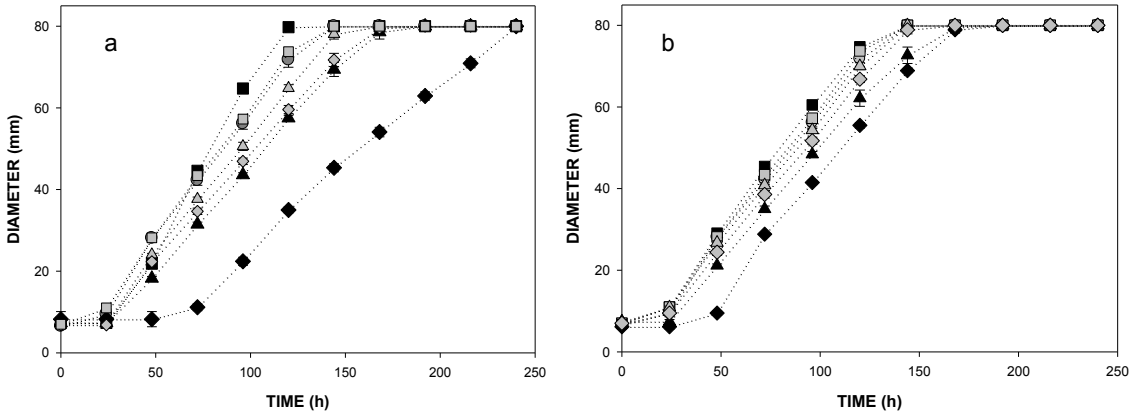

Fig. 1. *A. flavus* AFUNL5 growth curves in the presence of 10% and 50% (v/v) cell-free supernatants (CFS) from milk fermented by kefir grains CIDCA AGK1 (a) and CIDCA AGK2 (b) at 30 ºC to final pHs of 4.5, 3.5, and 3.3. Control:●; CFS at pH 4.5: 50%v/v:■,10%v/v:■; CFS at pH 3.5:50%v/v:▲, 10%v/v:▲; CFS at pH 3.3: 50%v/v:♦, 10%:♦.

Table 1 shows that the fermented milk CFS at three fermentation temperatures (20°C, 30°C and 37°C) inhibited the fungal growth according to the pH decrease and the organic acids concentration increase. Additionally, the fungal inhibition was higher with CFS concentration of 50% v/v than with 10% v/v added in the medium. The fungal inhibition was observed through the fungal lag phase increase and the growth rate decrease. The lag phase was the fungal growth parameter more affected by the CFS. Moreover, the CFS obtained at different fermentation temperatures showed different antifungal effects. The highest inhibition was observed with CIDCA AGK1fermented milk CFS obtained at 30°C, followed by those from fermentations conducted at 20°C. CIDCA AGK2 fermented milk CFS were less inhibitory than CIDCA AGK1-CFS. CFS from CIDCA AGK1 and CIDCA AGK2 kefir grains fermented at 37°C showed the lowest antifungal effect. Moreover, CFS from CIDCA AGK1 and CIDCA AGK2 kefir grains fermented until pH 4.5 (at three temperatures) showed the highest growth rates (between 0.59 and 0.78 mm/h) and the lowest lag phases (between 13.23 and 25.57 h). These CFS presented the lowest concentrations of lactic and acetic acids. These findings suggest that CFS with high pH present a low antifungal effect due to the low concentrations of lactic and acetic acids and possibly to the presence of milk compounds that protect the fungus.

The most inhibitory CFS (AGK1, 30°C, pH 3.3) contained the highest concentration of lactic acid (324.58±4.25 mM) but not the highest concentration of acetic acid (20.76±2.89 mM) among the various preparations. These findings indicate that the growth inhibition depends on the combination of the acetic acid with the highest concentration of lactic acid.

Table 1.

A. flavus AFUNL5 growth parameters in the presence of cell-free supernatants (CFS) from milk fermented by kefir grains CIDCA AGK1and CIDCA AGK2 at 20, 30 and 37 °C and lactic and acetic concentration of each CFS.

Fermentation Temperature	*CFS*	*pH*	*LA[a](mM)*	*AA[b](mM)*	*CFS Concentration (%v/v)*	*K_D[c] (mm/h)*	*Lag[d](h)*
20 °C	AGK1	4.5	48.33±4.21	6.24±3.15	50	0.61±0.02	17.15±2.15
					10	0.59±0.05	13.23±054
		3.5	156.09±2.78	15.58±1.54	50	0.49±0.01	20.99±1.11
					10	0.54±0.01	15.77±2.01
		3.3	191.41±3.74	18.57±2.36	50	0.44±0.01	26.66±0.91
					10	0.56±0.02	16.2±1.54
	AGK2	4.5	39.98±5.24	4.43±1.25	50	0.63±0.03	19.24±2.95
					10	0.60±0.01	15.82±0.95
		3.5	119.28±2.62	11.48±1.85	50	0.48±0.02	17.72±3.31
					10	0.56±0.01	12.77±2.12
		3.3	202.36±3.01	20.57±1.14	50	0.40±0.02	52.3±1.23
					10	0.50±0.01	13.18±2.25
	CONTROL[e]	6.5	NC[f]	NC[f]	NC	0.63±0.01	16.94±1.03
30 °C	AGK1	4.5	55.55±1.63	5.20±2.01	50	0.64±0.04	25.57±0.62
					10	0.61±0.01	16.23±0.52
		3.5	174.36±3.27	7.22±1.17	50	0.52±0.01	24.47±1.16
					10	0.58±0.02	21.48±0.28
		3.3	324.58±4.25	20.76±2.89	50	0.40±0.03	59.01±2.54
					10	0.53±0.02	20.67±1.37
	AGK2	4.5	47.42±2.28	4.42±1.12	50	0.67±0.02	15.89±0.23
					10	0.64±0.01	16.55±0.96
		3.5	73.70±1.88	7.87±3.22	50	0.55±0.01	22.76±0.43
					10	0.58±0.04	15.72±0.14
		3.3	203.28±2.14	17.39±1.36	50	0.56±0.03	31.98±0.62
					10	0.58±0.02	18.52±0.46
	CONTROL[e]	6.5	NC[f]	NC[f]	NC	0.63±0.01	14.38±2.14
37°C	AGK1	4.5	33.52±2.83	5.36±1.24	50	0.78±0.04	20.44±2.41
					10	0.66±0.01	15.84±3.15
		3.5	122.06±4.21	13.45±3.87	50	0.64±0.02	23.41±0.31
					10	0.62±0.02	18.72±1.11
		3.3	121.40±1.37	54.36±0.87	50	0.54±0.01	24.34±1.21
					10	0.61±0.01	16.14±1.30
	AGK2	4.5	28.36±2.74	4.12±1.41	50	0.71±0.02	18.52±1.28
					10	0.69±0.05	14.35±1.19
		3.5	109.52±2.78	12.29±1.13	50	0.60±0.02	22.11±1.21
					10	0.56±0.03	15.89±1.01
		3.3	139.10±3.72	46.50±2.25	50	0.57±0.02	22.81±0.81
					10	0.61±0.04	16.23±1.32
	CONTROL[e]	6.5	NC[f]	NC[f]	NC	0.61±0.11	13.04±1.01

[a]Lactic acid (LA) and [b]acetic acid (AA) concentrations in the CFS determined by HPLC. [c]K_D: growth rate (mm/h); [d]Lag: lag phase (h). [e]Control medium without CFS. [f]NC: Not quantified.

3.2 Antifungal effect of milk artificially acidified with pure lactic and acetic acids and with hydrochloric acid

Fig. 2 shows that in the presence of the CFS L+A AGK1 and CFS L+A AGK2, lag phases were higher than the controls, but lower than those obtained with the kefir fermented milk CFS. The fungal lag phase in the medium added with CFS HCl was lower than those obtained with the treatments and the control. This indicates that there is no antifungal effect due to the mere presence of the strong acid.

Growth rates obtained with artificially acidified milk were higher or did not have statistical difference with respect to the control values. These results indicate that the antifungal effect of kefir fermented CFS can be attributed exclusively neither to the presence of the organic acids nor to the acidity of the medium *per se*, but rather to a combination of those two factors.

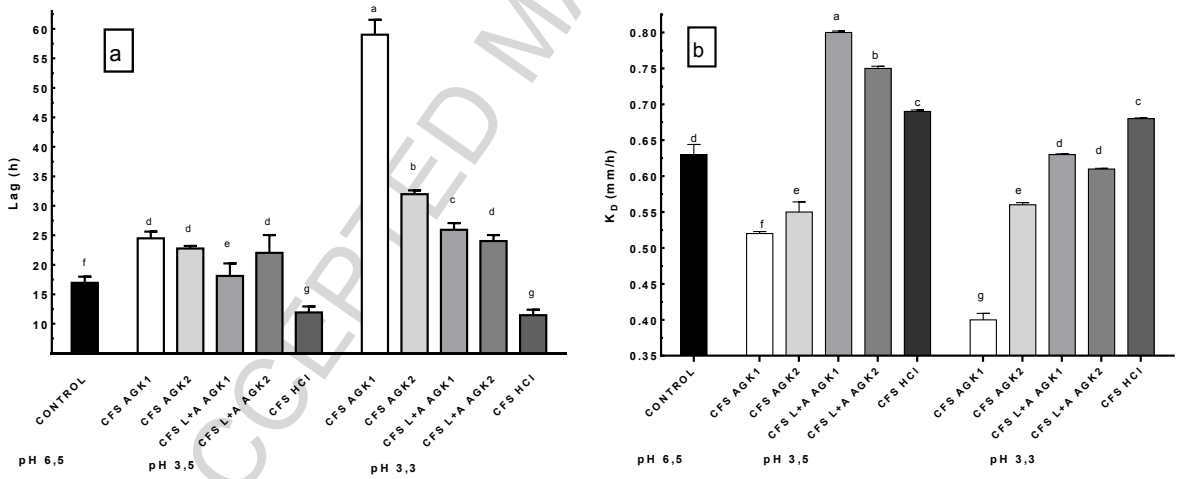

Fig. 2. *A. flavus* AFUNL5 growth parameters K_D (mm/h) (a) and Lag (h) (b) in the presence of kefir fermented milk supernatants CIDCA AGK1 and CIDCA AGK2 grains (CFS AGK1 and CFS AGK2), artificially acidified with lactic and acetic acids milk supernatants (CFS L+A AGK1 and CFS L+A AGK2) and artificially acidified with HCl milk supernatants (CFS HCl). Acid mixtures simulated the respective compositions of the AGK1 and AGK2 fermented supernatants. Control was milk supernatant at pH 6.5 (CONTROL). Different letters indicate statistically significant differences in values.

3.3 Effect of kefir fermented milk on the resistance to fungal contamination.

In the first experiments, the resistance to fungal contamination on *arepas* was studied, as described in Materials and Methods. When the *arepas* were contaminated artificially with *A. flavus* AFUNL5, fungal growth first appeared at 4.2±0.3, 9.1±0.1, and 11.1±0.5 days for the CA, KS, and KB *arepas*, respectively (P<0.05). The greatest resistance to fungal growth relative to control values occurred with the KB *arepas*.

In order to determine the shelf life improvement of *arepas* added with kefir fermented milk, the microbial composition of treated or non-treated arepas at different storage times was assayed. Table 2 shows that kefir baked *arepas* (KB-*arepas*) exhibited the lowest microbial counts (LAB, yeasts and FF) at different storage days. Kefir sprayed *arepas* (KS-*arepas*) showed the highest counts of LAB and yeasts at Day 1, whereas control *arepas* (CA-*arepas*) showed low counts of LAB and yeasts at Day 1. These results suggest that LAB and yeasts present in KS-*arepas* derived from the kefir fermented milk. Consequently, CA*arepas* showed an increase of yeast and FF counts at Day 5 and 10. These results suggest that yeasts in CA-*arepas* came from the environment.

Table 2.

Microorganism counts in *arepa* loaf preparations during storage

Treatment	*Arepas pH*[d]	*Storage day*[e]	*LAB*[f] *(CFU/g)*	*YEASTS (CFU/g)*	*FF*[g] *(CFU/g)*
KB-*arepas*[a]	4.0	Day 1	<30	2.00±0.10 x10^2	1.07±0.10 x10^2
		Day 5	<30	3.00±0.23 x10^2	1.11±0.20 x10^2
		Day 10	<30	3.36±0.04 x10^2	1.51±0.23 x10^2
KS-*arepas*[b]	4.6	Day 1	2.14±0.42x10^7	1.05±0.06 x10^6	<30
		Day 5	5.54±0.59 x10^7	1.95±0.42 x10^6	<30
		Day 10	5.98±0.36 x10^7	5.75±0.07 x10^5	<30
CA-*arepas*[c]	4.8	Day 1	<30	3.52±0.06 x10^2	3.66±0.12 x10^2
		Day 5	<30	3.65±0.58 x10^4	2.81±0.13 x10^3
		Day 10	<30	1.53±0.59 x10^6	4.71±0.31 x10^4

[a]*Arepas* baked with kefir included as an ingredient. [b]*Arepas* sprayed with kefir. [c]Control *arepas*. [d]*Arepas* pH was determined at day 1 of storage. [e]Days of storage at 14°C. [f]LAB: lactic acid bacteria, [g]FF: filamentous fungi.

3.4 Organoleptic profile of the kefir-containing *arepa*s

Fig. 3 summarizes the tastes and trigeminal sensations perceived with the different *arepas* on a *subjective* and semiquantitative scale from 0 (weak) to 5 (intense). All the *arepas* shared most of the tastes such as boiled corn, salty, roasted, alkaline, earthy, burnt, toasted, milky, bran, butter, acid, sweet and bitter. Nevertheless, KS and KB *arepas* showed significantly higher milky taste than those obtained with the CA *arepas*. The KS and KB *arepas* shared some tastes such as fermented milk and fat. KB *arepas* showed exclusively tastes such as alcoholic, chemical, soap and mouldy. On the other hand, all the *arepas* shared trigeminal sensations such as metallic, astringent and spicy. Additionally, KB *arepas* showed exclusively numbness sensation.

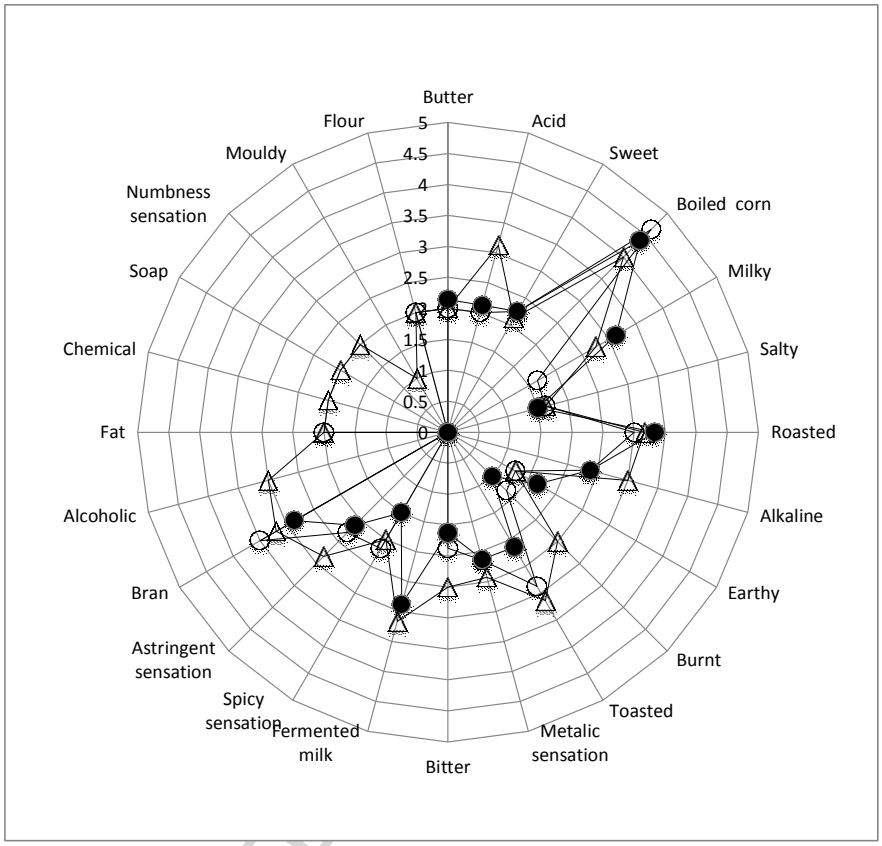

Fig. 3. Organoleptic profile of *arepas* with and without the addition of kefir-fermented milk. Control arepas-CA (O), kefir sprayed *arepas*-KS (●), kefir baked arepas-KB (Δ). The concentric circles mark the semiquantitative degrees—from 1 (the slightest) to 5 (the most pronounced)—of the taste quality indicated at the intersection of the each radius with the outer circle.

4. Discussion

Previous studies have demonstrated that different kefir fermented products inhibit both bacteria and fungi (Caro and León, 2014; Cevikbas et al., 1994; Gamba et al., 2015; Garrote et al., 2000; Golowczyc et al., 2008; Londero et al., 2014). In this work we have demonstrated that kefir fermented products and their CFS exert an antifungal effect against *A. flavus* AFUNL5. Our results agree with those obtained with CFS from whey fermented with CIDCA AGK10 grains, which reduced the germination of conidia of *Rhizopus* sp., *A. fumigatus*, *A. terreus*, *A. flavus* and *A. parasiticus* (Londero et al., 2014). Additionally, comparable CFS from kefir products showed an antifungal effect against *A. flavus* AH3 and *Fusarium graminearum* CZ (Ismaiel et

al., 2011). In addition, CFS from 'panela' (product obtained from unrefined sugarcane) fermented with water kefir grains decreased the growth parameters of *A. ochraceus* AFUNL9 (Caro and León, 2014).

The fermentation products obtained in this study reached pH values lower than the pK_a of the predominant weak acids thus far described in kefir (lactic and acetic acids with pK_a 3.75 and 4.76, respectively) (Garrote et al., 2000). Previous studies have demonstrated the relationship between the final pH and the undissociated forms of acetic and lactic acid on *A. flavus* and *A. parasiticus* inhibition. The mixture of lactic and acetic acid acts synergistically to generate the fungal growth inhibition. Since lactic acid has a lower pKa than acetic acid, at a given pH, the lactic acid present is the one mainly responsible for lowering the pH. Under the minimal pH obtained for kefir (3.5 and 3.3), acetic acid is in a maximally undissociated state and thus more able to penetrate the fungal membranes and exert the antifungal effect (Gamba et al., 2015; León et al., 2012).

The results demonstrating fungal inhibition obtained with the CFS from kefir fermented milk were characterized by two opposite effects on the fungus. The fermentation products in the CFS inhibited fungal growth, but the nutritional components of the milk that remained after fermentation succeeded in offsetting that inhibition producing a marginal stimulation under certain conditions.

The highest concentration in the medium of the CFS at pH 3.3 and 3.5 caused fungal inhibition, but CFS added at the lowest concentration exhibited fungal growth parameters similar to the controls. In contrast fungal growth stimulation was obtained with CFS at pH 4.5 at both concentrations in the medium (Table 1).

In our study we sought to determine whether the antifungal effect of the CFS was attributable to the organic acids alone, the drop in pH *per se*, or possibly both effects in combination. We found that milk CFS acidified by the pure organic acids succeeded in increasing the fungal lag phase, but the effect was less pronounced than that observed with the kefir fermented CFS; whereas acidification with the strong acid caused no inhibition. Comparable results were obtained in previous studies on fungal inhibition with whey or whey permeate solutions artificially acidified with either lactic and acetic acids or hydrochloric acid. The acidified solutions exerted a lower degree of fungal inhibition than that recorded with CFS from the solutions that had been fermented with kefir grains (Londero et al., 2014; Gamba et al. 2015, 2016). These observations led us to the conclusion that the fungal inhibition imparted by kefir fermented milk CFS was caused by the combined action of the organic acids present along with other metabolites produced by the kefir microorganisms.

Bacteriocins can be produced by LAB, and they represent a wide group of compounds with potential application on the fungal inhibition. It was reported that bacteriocin F1 produced by *Lactobacillus paracasei* subsp. *tolerans* isolated from Tibetan kefir grains exhibited antimicrobial activity, strong heat stability (20 min at 121 ºC, or 60 min at 100 ºC) and pH stability (pH 3.0-9.0) (Miao et al., 2014).

In the present study, we evaluated for the first time the effect on the shelf life of *arepas* of incorporating 10% (v/w) of kefir fermented milk into the recipe using two different treatments, KS and KB *arepas* and a control. We found the greatest resistance against natural and artificial fungal contamination with the second treatment. Moreover, only the KB *arepas* presented yeasts and FF counts lower than those required by the law during the entire assay. On the other hand, KS *arepas* showed FF counts lower than those required by the law, whereas yeast counts were higher than those required by the law (ICONTEC, 2005). However, the results suggest that these microorganisms had come from kefir milk (Table 3).

Previous studies have investigated chemical preservation of *arepas*. A study during 12 days of storage at 18 ºC showed that the addition of sorbic acid resulted in the lowest FF followed by sodium propionate and potassium sorbate, respectively (Corpas and Tapasco, 2012b). The protection afforded by the kefir fermented milk was greater than that observed with potassium sorbate. Therefore this product could be considered as a component that would improve the *arepas* shelf life. Other studies showed that the addition of whey fermented with CIDCA AGK10 kefir grains to poultry feed significantly enhanced the resistance to artificial contamination by *Rhizopus* sp., *A. fumigatus*, *A. terreus* and *Penicillium* sp. (Londero et al., 2014).

Our study provides the first report on the effect of kefir fermented milk on the sensory properties of *arepas*. We found that the organoleptic properties of the *arepas* combined the basic characteristics of the taste of the *arepa* with other flavors that would be associated with a fermented milk product. Previous studies have reported that the addition of powdered earthworms at different proportions in *arepas* changes the organoleptic properties (taste, odor, color, crunchiness and firmness). However, the *arepas* incorporating powdered earthworms were not compared to *arepas* without earthworm powder (Cayot et al., 2009); suggesting that the organoleptic changes could be higher than those reported whether they were compared to *arepas* without any addition. Also, other researchers have reported that the replacement of white maize by rice bran at different proportions in *arepas* does not change their organoleptic properties (taste, odour, color, texture and acceptability) when rice bran is used at low proportions (at 5 and 10% w/w) compared to *arepas* without any

addition (Pacheco Delahaye and Peña, 2006). The previous studies have been conducted with the addition of other ingredients to the *arepa*, but there are no studies examining the incorporation of probiotics on the *arepa* recipe in order to be used as preservatives.

5. Conclusions

The kefir fermented milk exhibited an antifungal effect and the most pronounced inhibition was produced by CFS obtained from CIDCA AGK1 kefir grains. The application of mixtures of lactic and acetic acids to unfermented milk CFS exerted a fungal inhibition that was significantly lower than that obtained with the kefir fermented CFS. Moreover, a lowering of the pH *per se* caused no inhibition at all. We conclude that both the presence of the organic acids and a low pH are necessary to achieve inhibition of fungal growth. Nevertheless, the difference between the inhibition exerted by the pure organic acids and that produced by the fermented CFS suggests that the higher efficacy of the latter must involve the presence of additional inhibitory metabolites produced by the kefir microorganisms.

The inclusion of kefir fermented milk in the *arepa* provides a higher resistance against fungal contamination than that of the traditional *arepa*. Finally this product confers novel flavors over the characteristic background of the traditional taste descriptors of the *arepa*. These promising results form the basis of our ongoing study on the antifungal activity of kefir and its application to the control of fungal contamination in the commercial production of foodstuffs of cereal origin.

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Conflict of Interest

There is no conflict of interest with other co-authors for the publication of this manuscript in this journal. All the co-authors have contributed in the preparation of the manuscript up to the submission stage.

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Highlights:

- Kefir products inhibited *Aspergillus flavus* growth.
- Kefir fermented milk caused an increase of the *arepas'* resistance to natural and artificial fungal contamination.
- The application of kefir on/into *arepas* added new flavors and sensations.