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Antifungal effect of kefir fermented milk and shelf life improvement of corn *arepas*

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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 °C 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 °C 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.

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

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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 et 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 °C. 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 °C 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 °C 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 °C, 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