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Production of reuterin in a fermented milk product by *Lactobacillus reuteri*: Inhibition of pathogens, spoilage microorganisms, and lactic acid bacteria

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ABSTRACT

We assessed the antimicrobial activity of reuterin produced in vitro in glycerol aqueous solutions in situ by *Lactobacillus reuteri* ATCC 53608 as part of a fermented milk product against starter (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*), spoilage (*Penicillium expansum*), pathogenic (*Staphylococcus aureus*, *Salmonella enterica* ssp. *enterica*, and *Listeria monocytogenes*), and pathogen surrogate (*Escherichia coli* DH5α) microorganisms. We also assayed the influence of cold storage (28 d at 4°C) and reuterin on the color and rheology of the fermented milk product. We obtained maximum reuterin concentrations of 107.5 and 33.97 m*M* in glycerol aqueous solution and fermented milk product, respectively. Reuterin was stable throughout its refrigerated shelf life. Gram-positive microorganisms were more resistant to reuterin than gram-negative microorganisms. *Penicillium expansum* and *Lactobacillus reuteri* ATCC 53608 survived at concentrations up to 10 and 8.5 m*M*, respectively. *Escherichia coli* DH5α was the most sensitive to reuterin (0.9 m*M*). The presence of reuterin did not cause relevant changes in the quality parameters of the fermented milk product, including pH, acidity, soluble solids, color, and rheological aspects (storage and loss moduli and viscosity). This study demonstrated the viability of using *Lactobacillus reuteri* ATCC 53608 as a biopreservative in a fermented milk product through reuterin synthesis, without drastically modifying its quality parameters.

Key words: reuterin, *Lactobacillus reuteri*, fermented milk product, biopreservation

INTRODUCTION

The most common method used to extend the shelf life of yogurt and fermented milks is refrigeration. The refrigerated shelf life of these products vary greatly depending on many factors, such as storage temperature (Birollo et al., 2000) and the presence of spoilage (Mataragas et al., 2011) or pathogenic microorganisms (O'Mahony et al., 1990; El-Sharoud, 2009). Important outbreaks of foodborne diseases caused by *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., and *Listeria* spp. in dairy products have been reported in recent years (Morgan et al., 1993; Gulmez and Guven, 2003; Braden and Tauxe, 2013; Bianchi et al., 2014; Kemal, 2014). Benzoic and sorbic acids, as well as their salts, are typically employed as preservatives in dairy products to avoid the growth of unwanted microorganisms (Mroueh et al., 2008). However, consumers are increasingly demanding a reduction in the use of synthetic chemical agents for food preservation (Mills et al., 2011), and alternative strategies to extend the shelf life of foods have been proposed. Of special interest are those based on the use of natural substances (Gyawali and Ibrahim, 2014). Lactic acid bacteria may be successfully used as a biopreservation strategy and a substitute for artificial preservatives. The use of lactic acid bacteria takes advantage of the capacity of some strains to synthetize chemical compounds such as alcohols, organic acids, carbon dioxide, diacetyl, hydrogen peroxide, and other substances that are capable of inhibiting the growth of unwanted microorganisms (Helander et al., 1997).

An example of this strategy is the use of *Lactobacillus reuteri*. Some strains of *Lb. reuteri* can synthesize reuterin (3-hydroxypropionaldehyde), a broad-spectrum antimicrobial agent produced during the anaerobic metabolism of glycerol (Talarico et al., 1988). Reuterin has potent antimicrobial effects against bacteria, yeast, fungi, and protozoa (Talarico et al., 1988; Axelsson et al., 1989; Chung et al., 1989; Talarico and Dobrogosz,

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1989). The use of reuterin to prevent the growth of spoilage and pathogenic microorganisms in dairy products has been evaluated (el-Ziney and Debevere, 1998; Langa et al., 2013). Arqués et al. (2004) reported that 8 AU/mL of reuterin in milk caused bacteriostatic activity against *Listeria monocytogenes* and bactericidal effects against *Staph. aureus, E. coli* O157:H7, *Salmonella choleraesuis* ssp. *choleraesuis*, *Yersinia enterocolitica*, *Aeromonas hydrophila* ssp. *hydrophila*, and *Campylobacter jejuni.* Several other studies have demonstrated the effectiveness of reuterin as an antimicrobial agent (Axelsson et al., 1989; Chung et al., 1989; Rasch, 2002; Cleusix et al., 2007; Spinler et al., 2008; Garde et al., 2014). However, the synthesis and accumulation of reuterin in situ through the activity of *Lb. reuteri* in preserved food has not been explored as fully, probably due to the technical difficulties involved in achieving the conditions required for reuterin synthesis in food products without drastically modifying their quality traits. Still, the use of *Lb. reuteri* as a biopreservative is very relevant from a commercial point of view, because in situ production of reuterin allows for clean labeling. The ability of *Lb. reuteri* to synthesize reuterin is strongly dependent on temperature, pH, oxygen concentration, cell age, and biomass (Lüthi-Peng et al., 2002b); developing a food manufacture process in which *Lb. reuteri* survives and synthesizes reuterin is a challenge.

Langa et al. (2013) explored in situ reuterin production in a yogurt model system, in which *Lb. reuteri* survived to produce reuterin. Nevertheless, the maximum reuterin concentration achieved in that study reached only 1.5 m*M*, which may not be enough to effectively protect against microbial development over a product's shelf life. The purpose of this research was to produce a fermented milk product in which live *Lb. reuteri* ATCC 53608 synthesized reuterin at higher levels and to evaluate the antimicrobial effect of reuterin on pathogens, starter cultures, spoilage microorganisms, and *Lb. reuteri* itself, throughout the refrigerated shelf life of the fermented milk product.

MATERIALS AND METHODS

Materials and Strains

We purchased tryptophan; acrolein; USP-grade glycerol; ethanol; gentamicin sulfate; de Man, Rogosa and Sharpe (**MRS**) agar; MRS broth; tryptic soy broth; tryptic soy agar; reinforced clostridial agar; and potato glucose agar from Aldrich Chemical Co. (St. Louis, MO). Sodium chloride and dibasic sodium phosphate anhydrous were supplied by Fermont (Productos Químicos Monterrey, S.A. de C.V., NL, México). We purchased M17 agar, sodium hydroxide, and hydrochloric acid from Dibico S.A. de C.V. (México, México), Alfa Aesar (Haverhill, MA), and Mallinckrodt Baker (Austin, TX), respectively.

We obtained food pathogen microorganisms *Staph. aureus, Salmonella enterica* ssp. *enterica* (*S. enterica*), *L. monocytogenes* wild type, and *E. coli* DH5α (studied as a pathogen surrogate) isolated from food, as well as spoilage fungus *Penicillium expansum,* from the Centro de Investigación en Alimentación y Desarrollo, A.C., culture collection (Ciudad Cuauhtémoc, México). We purchased *Lb. reuteri* ATCC 53608 from IECSA (México, México). We purchased starter culture (*Lactobacillus delbrueckii* ssp. *bulgaricus,* hereinafter called *Lb. delbrueckii,* and *Streptococcus thermophilus*) from Chr. Hansen (Hørsholm, Denmark).

Methods

Reuterin was first bio-synthesized in vitro and purified to characterize *Lb. reuteri* ATCC 53608's synthesis capacity, and to test the in vitro susceptibility to reuterin of all involved microorganisms. Once this was accomplished, we developed a fermented milk manufacturing method that allowed for in situ reuterin production. To test the effectiveness of this method as a bio-preservation strategy, a post-process contamination was simulated, inoculating pathogen and spoilage microorganisms before studying the fermented milk product throughout its refrigerated shelf life, focusing on microbial populations and quality attributes (color and consistency).

In Vitro Lb. reuteri Biomass and Reuterin Production. Reuterin production was achieved following a 2-step process described by Doleyres et al. (2005). Stock frozen culture of *Lb. reuteri* strain ATCC 53608 was inoculated at 1% in MRS broth and incubated at 37°C for 15 h. This overnight culture was transferred at 1% into MRS broth with 20 m*M* glycerol and incubated at 37°C under anaerobic conditions for 15 h, maintaining the pH at 5.5 using 5 *M* NaOH. Cells were harvested after incubation (Incubator VWR International, Radnor, PA) by centrifugation at 1,500 × *g* for 10 min at 20°C (centrifuge 5430 R; Eppendorf, Hamburg, Germany) and washed twice with 0.1 *M* potassium phosphate buffer, pH 7. Pellets from the preceding step were suspended in 200 m*M* glycerol solution and incubated at 37°C for 120 min. For reuterin isolation, the cell suspension was centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant was recovered and filtered (0.2 μm; Corning Inc., Corning, NY). The filtrate was considered the reuterin aqueous extract.

Reuterin Quantification. Reuterin quantification in aqueous solutions and in the fermented milk product