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Survival of the functional yeast *Kluyveromyces marxianus* B0399 in fermented milk added with sorbic acid

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ABSTRACT

In this study, the survival of the functional yeast *Kluyveromyces marxianus* B0399 in an industrially produced fermented milk was evaluated. In particular, the yeast viability was assessed throughout the entire shelf-life of the product (30 d) to ensure the presence of the effective yeast dose (20 million viable cells for each serving of 125 g) while avoiding, by sorbic acid addition, yeast growth, which could affect product quality and stability. To find the best combination of yeast and sorbic acid concentration, 13 different combinations were tested, and then 2 of them were chosen for industrial production. In production at lower concentrations (30 million viable cells, 150 mg/kg of sorbic acid) the effective dose was maintained only at 4 and 6°C, whereas at higher dosages (70 million viable cells, 250 mg/kg of sorbic acid) the effect of temperature was less evident. In all the trials, the concentration of sorbic acid was not affected by microbial metabolism and remained stable throughout the entire shelf-life.
Key words: functional yeast, fermented milk, sorbic acid, *Kluyveromyces marxianus*

INTRODUCTION

Probiotics are defined as viable microorganisms that, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002). After ingestion, they must overcome biological barriers (such as gastric acidity and bile toxicity) to reach the gastrointestinal tract and exert their health-promoting effects (Sánchez et al., 2009). Moreover, before their addition in functional foods, their safety and efficacy have to be demonstrated. Foods used as probiotic sources are mainly dairy products, such as yogurt, fermented milk, and cheese, are regarded as ideal vehicles for delivering probiotic microorganisms to the human gastrointestinal tract (Ross et al., 2002). Most of the microorganisms employed as probiotics are bacteria belonging to the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Bifidobacterium*, *Enterococcus*, and *Bacillus* (Shah, 2004). Recently, an increased interest in probiotic fungi has been observed. *Saccharomyces boulardii* has, for a long time, been the only yeast commercialized as a probiotic for humans (Martins et al., 2005) and its beneficial effects have been demonstrated (Surawicz et al., 1989; Mombelli and Gismondo, 2000). In recent years much research has been focused on the isolation and characterization of yeasts from a technological and functional point of view (Pennacchia et al., 2008; Binetti et al., 2013). Among yeasts, *Kluyveromyces marxianus* (formerly *Kluyveromyces fragilis*), isolated from different dairy products (Farnworth, 2005; Bolla et al., 2011; Tofalo et al., 2014), has gained attention because of its peculiar characteristics (i.e., high thermotolerance and growth rate, broad substrate spectrum, and absence of fermentative metabolism upon sugar excess). Regarding its biotechnological applications, *K. marxianus* is well known for the production of enzymes (Fonseca et al., 2008), of aromatic compounds (Fabre et al., 1995), and ethanol in high-temperature processes (Fonseca et al., 2008). Moreover, it can be used also for the reduction of lactose content (Rajoka et al., 2004), for the recovery of high-value bioingredients (Belem et al., 1997). Finally, many studies reported its use for heterologous gene expression (Dellomonaco et al., 2007; Raimondi et al., 2008, 2013).

On the contrary, little information is available on the putative functional properties of *K. marxianus* (Yoshida et al., 2005; Romanin et al., 2010). Recently, Maccaferri et al. (2012a) investigated the potential of the strain *K. marxianus* B0399, isolated from milk, for its applica-

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tion as a probiotic. This strain was chosen because it is included in the European Food Safety Authority list of qualified presumption of safety biological agents added to food and feed (EFSA Panel on Biological Hazards, 2010). Moreover, it is able to survive the gastrointestinal transit, maintaining its vitality and fermentation capacity (Mustacchi et al., 2010). Studies of this strain have shown that it is able to affect colonic microbiota, increasing the bifidobacterial concentration in the colonic model system, and to induce the formation of higher amounts of the short-chain carboxylic acids acetate and propionate. Moreover, it is highly adhesive to human enterocyte-like Caco-2 cells and can modulate the immune response (Maccaferri et al., 2012a). These findings demonstrated that *K. marxianus* B0399 possesses several beneficial and strain-specific properties that make it suitable for application as a probiotic. Hence, some studies were focused on the effect of consumption of a fermented milk containing *Bifidobacterium animalis* ssp. *lactis* BB12 and *K. marxianus* B0399 on patients with irritable bowel syndrome (Lisotti et al., 2011; Maccaferri et al., 2012b).

Together with its functional potential, the technological properties of the strain *K. marxianus* B0399 need to be better investigated. In particular, a good viability throughout the entire shelf-life of the product in which it is incorporated is essential to induce the claimed benefits and, therefore, to commercialize the product as a functional food. In fact, a minimum daily intake of probiotics has to be assured to exert its beneficial functions (Vinderola et al., 2000).

The aim of this work was to optimize the formulation of a fermented milk containing *K. marxianus* B0399 to maintain at least 20 million viable yeast cells for each serving (a 125-g cup) throughout 30 d of refrigerated storage. This yeast amount was defined by studies evidencing beneficial effects with dosages between 10 and 20 million viable cells (Mustacchi et al., 2010; Lisotti et al., 2011; Maccaferri et al., 2012a). However, in this specific case, the viability of *K. marxianus* B0399 had to be maintained while avoiding its growth, which could affect product quality and stability (ethanol and CO_2 production, off-flavors, and so on). Therefore, sorbic acid was added to the formulation to prevent yeast multiplication. Thus, the main purpose of this research was to find the best combination of yeast concentration and sorbic acid (added at concentrations far below amounts able to exert a lethal effect on *K. marxianus* B0399), able to ensure a cell load of at least 20 million viable cells without product alteration. Moreover, a fast chromatographic method for the determination of sorbic acid in the samples was set up modifying the method proposed by other authors (Kamankesh et al., 2013).

MATERIALS AND METHODS

Kluyveromyces marxianus B0399 Strain

The probiotic strain *K. marxianus* B0399 was provided by Turval Laboratories (Udine, Italy) as a concentrated cell suspension in a liquid medium. The functional properties of this strain were reported by Lisotti et al. (2011), Maccaferri et al. (2012a,b), and Mustacchi et al. (2010).

To evaluate the effect of sorbic acid on *K. marxianus* B0399, MIC and minimum fungicidal concentrations (**MFC**) of this compound were assessed on yeast extract peptone-dextrose agar (**YPD**; Oxoid, Basingstoke, UK) at pH 6.5 (normal pH of the medium) and 4.2 (YPD added with proper amounts of HCl, 1 *N*), to simulate the pH of the tested product. For this determination, 198 μL of YPD inoculated with the strain at a level of approximately 5 log cfu/mL were placed into 200-μL microtiter wells (Corning Inc., Corning, NY). Because of the scarce solubility in water, an alcoholic stock solution (100 g/L) of sorbic acid (Fluka, Milan, Italy) was prepared and further diluted in ethanol. Two microliters of the proper dilutions were added to each well to convey different amounts of sorbic acid, obtaining the same final concentration of ethanol (1% vol/vol).

The sorbic acid concentrations tested ranged from 0 to 1,000 μg/mL for trial at pH 6.5 and from 0 to 500 μg/mL for trial at pH 4.2. A control with ethanol alone was also included. For each sorbic acid concentration, 8 repetitions were considered. Microtiter plates were incubated at 37°C and, for each condition, the presence of a visible growth after 72 h of incubation was recorded. The MIC was defined as the lowest concentration of the compound preventing visible growth of the inoculated cells after 72 h in all the 8 repetitions; MFC was defined as the lowest concentration of sorbic acid that caused the death of the inoculated cells, as no growth was observed (for all the 8 repetitions) after 72 h of incubation at 37°C of a 10-μL spot plated onto YPD agar.

Fermented Milk Production

Fermented milk used in our study was industrially produced from raw cow milk (4,000 L) that was concentrated, homogenized, and pasteurized. Then, milk was cooled, inoculated with *Streptococcus thermophilus* and