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INHIBITION OF SOME FOODBORNE PATHOGENS BY PURE AND MIXED LAB CULTURES DURING FERMENTATION AND STORAGE OF *Ergo*, A TRADITIONAL ETHIOPIAN FERMENTED MILK

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ABSTRACT

The antagonistic effect of nine lactic acid bacterial strains, either as pure or defined mixed-cultures, was assessed against foodborne pathogens (*E. coli* ATCC 25922, *Salmonella* Typhimurium DT104 and *Staphylococcus aureus* ATCC 25923) during fermentation and storage (at ambient and refrigeration) of *ergo*. At the end of fermentation at 72 h, the pure LAB cultures reduced the mean count of the target enteropathogens by 3 log units. The count of all target pathogens was also reduced almost by 2, 3 and 5 log units at 24, 48 and 72 h, respectively during fermentation by mixed LAB cultures. During storage of ready-to-consume *ergo* at ambient condition, the count of test organisms decreased by 3-4 log units at 24 h; and the test strains were totally eliminated within 30-48 h. In contrast, during storage of *ergo* at refrigeration condition, the average count of the test pathogens was reduced by 3-4 log units at 72 h. The LAB strains survived at counts of log 8.0 cfu/ml or higher up to 72 h during ambient and refrigeration conditions. These findings suggest that the isolates are possible candidates for the formulation of bioprotective starter cultures that can be employed for production of safe and potentially probiotic *ergo*.

Keywords: fermentation, *ergo*, lactic acid bacteria, foodborne pathogens, antagonism, probiotic starter, safety.

INTRODUCTION

Fermentation technology is one of the oldest known methods of food preservation. Fermentation processes promote the development of essential and safe microflora, which play a vital role in preventing the outgrowth of spoilage bacteria and food borne pathogens (Gibbs, 1987). Lactic acid bacteria (LAB) are important in much fermentation and the antagonistic effects of LAB are attributed to some of their biochemical features. They can utilize carbohydrates and produce organic acids as lactic acid or acetic acid. The majority of foods borne contaminants, either pathogenic or nonpathogenic, are sensitive to these acids and the resulting low pH. They also produce antibacterial substances such as bacteriocines, hydrogen peroxide, diacetyl, and CO_2 which may also play part in the antagonism of LAB on other microorganisms (Magnusson and Schnurer, 2001). LAB also produces different types of compounds that offer fermented foods their characteristic flavor, color, aroma, and test (Rodriguez *et al*., 2002).

The killing effect of human origin *Lab. acidophilus* strain against *Salmonella enterica* serovar *Typhimurium* as non-lactic acid associated mechanisms was reported by Coconnier et al., (2005). Mufandaedza *et al*., (2006) reported 2 log cycle reductions in the count of *E. coli* 3339 and *S. enteritidis* 949575 during fermentation of milk. The use of living culture of *Lueconostoc carnosum* in preventing the growth of *Listeria monocytogenes* than partially purified leucosins 4010 or bacteriocin produced during fermentation before heat treatment of meat products was indicated by Jacobsen *et al*., (2003). Considerable decrease in the count of coliforms was observed to be consistent with increase in the number of lactic acid bacteria and drop in pH after 12 hours during *ergo* fermentation (Ashenafi, 1995).

Ergo is a naturally processed indigenous Ethiopian fermented dairy product, which is commonly prepared at household level. The fermentation of this product is dominated by LAB (Ashenafi, 2006). Although fermented food products are usually considered safe because of the antagonistic effect of LAB, some foodborne pathogens have been reported to survive and grow in fermented milks (Feresu and Nyathi, 1990). De Buyser *et al*., (2001) indicated the involvement of milk and milk products in staphylococcal food poisoning. Ashenafi (1992, 1994) indicated the survival of *L. monocytogenes, Salmonella spp*. *Staphylococcus aureus* and *Bacillus cereus* for 24-48 h during *ergo* fermentation. Since *ergo* is commonly consumed soon after 24 h of fermentation, the survival of pathogen beyond 24 h would be undesirable (Tsegaye and Ashenafi, 2005).

As a natural fermentation, different groups of microorganisms may participate in and contribute to the final characteristics of *ergo*. The safety and wholesomeness of a fermented product may depend on the types of LAB that are involved in the fermentation process. The aim of this study was, therefore, to evaluate the antagonistic effect of pure and mixed LAB cultures against some foodborne pathogens during the fermentation of *ergo* and its storage at ambient and refrigeration temperatures.

MATERIAL AND METHODS

Bacterial strains

Salmonella Typhimurium DT104, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as target test strains in this study. All LAB were recovered from traditional fermented dairy (*ayib* and *ergo*) and low-alcoholic beverages (*borde* and *shamita*). The recovery and enumeration of LAB were done following the protocols indicated in Tsegaye and Ashenafi (2005) for fermented dairy (*ayib* and *ergo*) and fermented beverages (borde and shamita) as indicated in Bacha *et al*., (1998; 1999). They were identified to species and subspecies level using API 50CHL kit, and selected based on their *in vitro* and *in vivo* probiotic qualities (data not included). Nine pure and four mixed LAB cultures were employed as starter cultures for preparation of *ergo*. Each mixed culture was formulated based on fermentation characteristics of LAB strains as homofermentative and heterofermentative (Table-1).

Table-1. Pure and mixed LAB cultures used as starter cultures during *ergo* fermentation.

Starter LAB cultures		
Pure LAB cultures	**Mixed LAB cultures**	
Lab. acidophilus 1*, *Lab. brevis* 1**, Lab. *cellobiosus***, *Lab. delbrueckii ssp delbrueckii**, *Lab. paracasei ssp paracasei* 3**, *Lab. plantarum 1***, *Lab. plantarum 2***, *Lac. lactis ssp lactis* 1* *Ped. pentosaceus 1***	MLC 1	*Lac. lactis ssp lactis* 1*, *Lab. paracasei ssp paracasei* 3**, *Lab. brevis* 1**
	MLC 2	*Lab. acidophilus* 1*, Lab. *cellobiosus*** *Lab. plantarum 1***
	MLC 3	*Lab. delbrueckii ssp delbrueckii**, *Lab. plantarum 2*** *Ped. pentosaceus 1***
	MLC 4	MLC 1 + *Lab. delbrueckii ssp delbrueckii**

* homofermentative, **heterofermentative

Detection of LAB antagonism during souring and storage of *ergo*

A volume of 200 ml of pasteurized milk was co-inoculated in duplicates with a pure LAB culture and a culture of a target pathogen to get a final inoculum level of log 6cfu/ml and log 3cfu/ml, respectively following the protocol given by Tsegaye and Ashenafi (2005). An equal amount of pasteurized milk was inoculated with the target organism to a final level of log 3 cfu/ml and served as control. The enumeration of *E. coli*, *S. Typhimurium* and *Staph. aureus* from each co-culture and respective control bottles was conducted at 12 h intervals for 72 h by plating an appropriate dilution on duplicate PC (Plate Count) plates to allow metabolic recovery of injured cells. Plates were overlaid after 30 minutes with Violet Red Bile (VRB) Agar for *E. coli*, Xylose Lysine Desoxycholate (XLD) Agar for *S. Typhimurium* and Mannitol Salt Agar (MSA) for *Staph. aureus*. Plates were incubated at 32^0C for 24/48 h. LAB isolates were enumerated using MRS agar plates incubated at 32^0C for 24/48 h in anaerobic jar. Similarly, the effect of each of four mixed LAB cultures was determined against each of three test pathogens during *ergo* fermentation following the same procedure.

To assess the survival of each test pathogen in the fermented and ready-to-consume *ergo,* pasteurized milk was separately fermented with the various mixed LAB cultures. The test pathogens were separately inoculated in duplicates into *ergo* to give a final inoculum level of log 6cfu/ml and incubated at ambient condition (20-25^0C). Fermented *ergo* was also prepared with each mixed LAB culture, separately inoculated with the test pathogens to give a final inoculum level of log 6cfu/ml and stored at refrigeration condition (4^0C). Phosphate Buffered Saline (200 ml) separately inoculated with the test pathogens served as control at both storage temperatures. Enumeration of test pathogens, LAB and measurement of pH during storage of ready-to-consume *ergo* was done at 0, 6, 12, 24, 30, 36 and 48 h as indicated previously. When counts of target pathogens dropped beyond the detection level (<log 1cfu/ml), samples were enriched in Tryptic Soya broth and incubated at 32^0C overnight. Any growth of *E. coli*, *S. Typhimurium* DT104, and *Staph. aureus* was checked by streak plating on VRB, XLD and MSA plates, respectively.

RESULTS AND DISCUSSIONS

In fermenting *ergo*, pure or mixed LAB strains grew to more than log 8cfu/ml within 24 h and the count stabilized around log 9cfu/ml after 48 h (Figure-1). The proliferation of LAB in a fermenting product is a necessary condition to lower the pH and possibly to produce and accumulate antimicrobial metabolites against food borne pathogens. The pure LAB cultures had relatively higher counts between 36 h and 72 h. Both types of starter cultures reduced the pH markedly. However, at 72 h, average final pH of *ergo* fermented with pure LAB cultures was 4.74 where as mixed LAB cultures resulted in *ergo* with a final average pH of 4.36 (Figure-1).

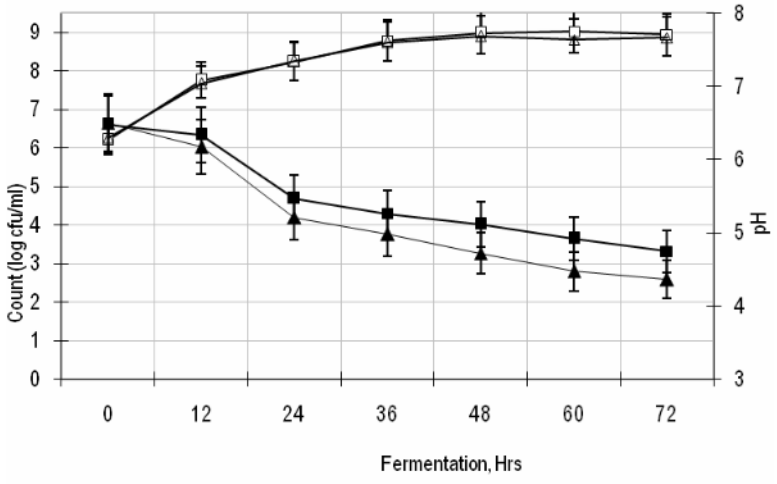

Figure-1. Changes in pH (closed symbols) and counts (open symbols) of various pure and mixed LAB cultures during ergo fermentation. □ Average count of pure cultures, Δ average count of mixed cultures, ■average pH with pure cultures, and ▲average pH with mixed cultures.

In the absence of LAB cultures, *E. coli*, *S.* Typhimurium DT104 and *Staph. aureus* grew to around log 9cfu/ml at 48 h with a slight decrease (to log 8.1cfu/ml) at 72 h (Figure-2a). The pH in the control cultures gradually decreased from an initial value of 6.5 to 4.9 at 72 h (Figure-2b). In the presence of the various pure LAB cultures, mean count of the test organisms in fermenting *ergo* initially rose to over log 6.5cfu/ml at 48 h; and finally dropped to about log 5cfu/ml at 72 h. The corresponding pH declined to about 4.21 at 72 h (Figure-2b).

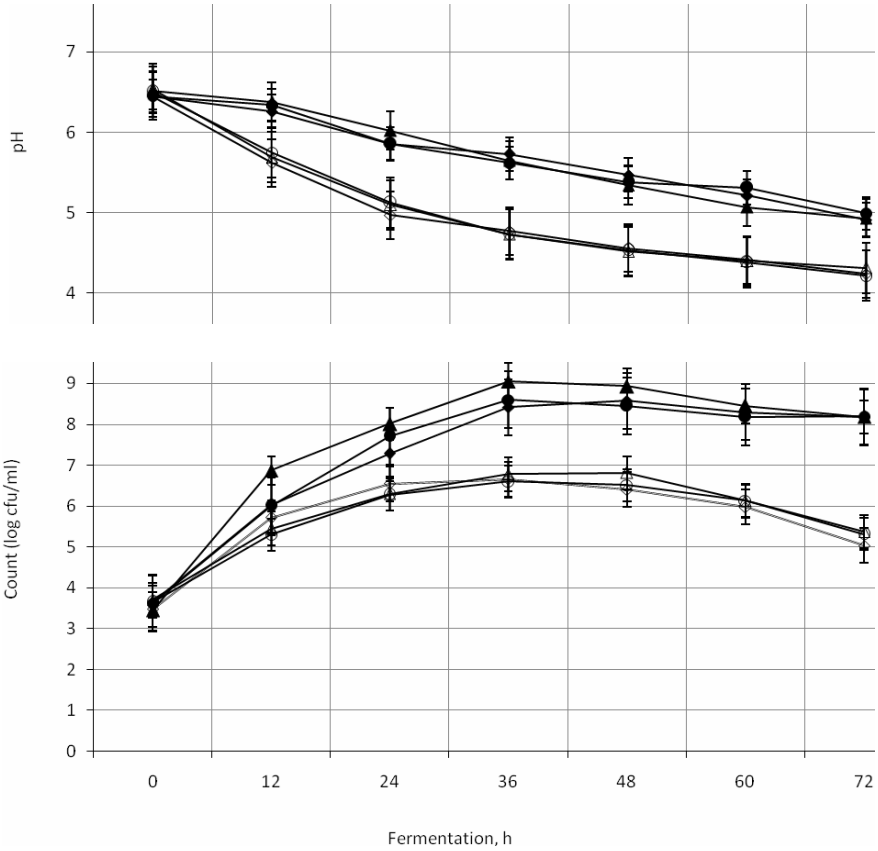

Figure-2. Changes in pH and mean counts of *E. coli* (triangle), *S. Typhimurium* (diamond), and *S. aureus* (circle) in the presence (open symbols) and absence (closed symbols) of pure LAB cultures during fermenting *ergo*.

Mixed LAB cultures showed a stronger inhibition on the test organisms. Initially, increase by 1 to 2 log units was noted in the count of the test organisms until 12 h of fermentation. However, the count decreased markedly thereafter (Figure-3a). The growth rate of *Staph. aureus* was markedly lower than the other test organisms and final counts at 72 h were around log 3.5cfu/ml. The pH of fermenting *ergo* dropped to 3.94 at 72h (Figure-3b).

Various workers reported the isolation of different strains of *E. coli* including pathogenic types from raw milk and fermented milk products like yoghurt (Morgan *et al*., 1993) and cheeses (Rodriguez *et al*., 2005). The count of different strains of enterohaemorrhagic *E. coli* during spontaneous fermentation of *ergo* decreased by 2 log units at 24 h of fermentation (Tsegaye and Ashenafi, 2005). Mufandaedza *et al*., (2006) reported that counts of *E. coli and Salmonella* strains decreased by 2 log units during fermentation of milk with a *Lactococcus* biovar alone or in combination with *Candida kefyr*. Similarly, our result showed the average reduction of the test pathogens by 3 log and 5 log units at 72 h by pure and mixed lactic cultures, respectively. This result indicated the possible synergistic effect of individual members of a mixed lactic culture. Dineen *et al*., (1998) showed that mixed thermophilic LAB cultures showed a stronger inhibition of *E. coli* O157:H7 than pure lactic cultures during fermentation of dairy products.

Longer survival, up to 72 h, of our test pathogens was observed when they were exposed to pH 4.61- 6.36 for up to 36 h, possibly due to development of acid tolerance at this pH range. Different workers reported that exposure and growth of *S. Typhimurium* (Hickey and Hirshfield, 1990), *E. coli* 0157: H7 (Lin *et al*., 1995) and *Staph. aureus* (Bore *et al*., 2007) to mildly acidic pH induced acid-tolerance and longer survival of the organisms at low acidic environment. Other workers, on the other hand, noted increase in number of *E. coli* O157: H7, *S. Typhimurium, and Staph. aureus* at 24 h during fermentation of *kefir* (Karagozlu *et al*., 2007). Similarly, the survival of *Salmonella spp.* in souring *ergo* for up to 60 h was also reported by Ashenafi (1993).

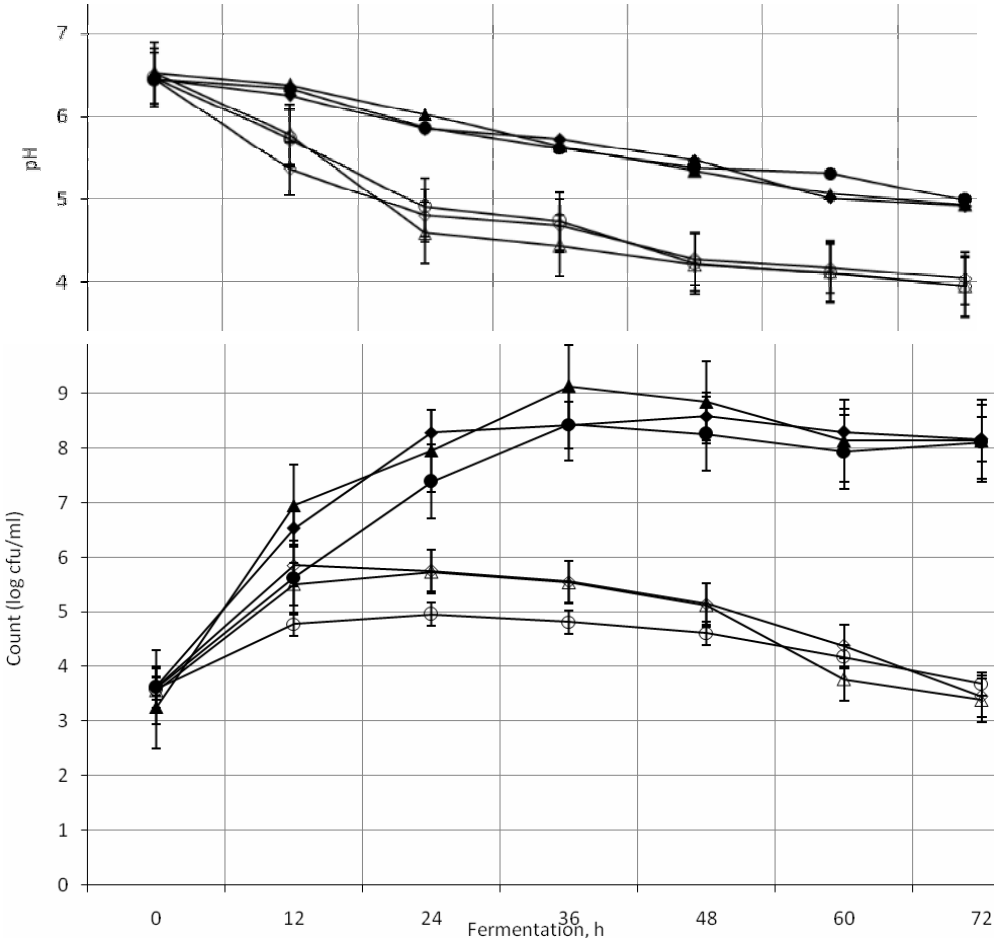

Figure-3. Changes in pH and mean counts of *E. coli* (triangle), *S. Typhimurium* (diamond), and *S. aureus* (circle) in the presence (open symbols) and absence (closed symbols) of mixed LAB cultures during fermenting *ergo*.

During refrigeration storage of ready-to-consume *ergo*, the average counts of *E. coli, S. Typhimurium* DT104 and *Staph. aureus* fell markedly at 48 h and further declined at 72 h (Figure-4). Elimination of the test pathogens was distinctly higher at ambient temperature storage of *ergo*. *E. coli* was totally eliminated at 36 h, whereas it took about 48 h to eliminate *S. Typhimurium* DT104 and *Staph. aureus* (Figure-4). The pH of *ergo* remained around 4.2 during refrigeration storage and slightly fell to around 3.8 during ambient storage. Similar to our findings, Tsegaye and Ashenafi (2005) reported the complete inactivation of different strains of *E. coli* 0157:H7 at 36 h of *ergo* storage at ambient condition. The same test strains survived longer at refrigeration temperature.

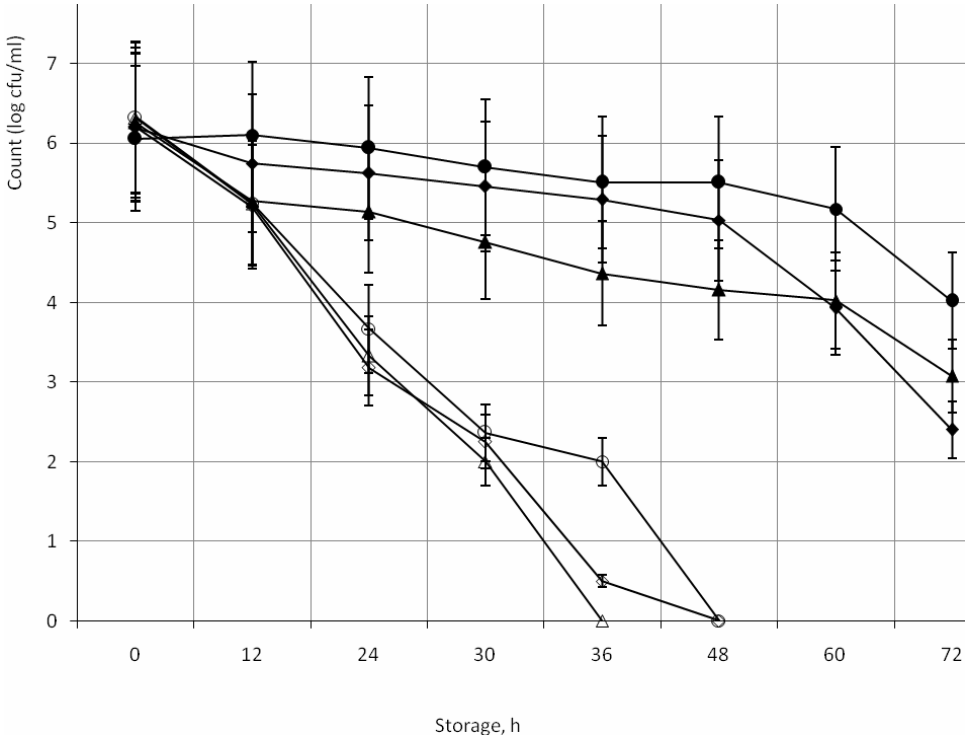

Figure-4. Changes in mean counts of *E. coli* (triangle), *S. Typhimurium* (diamond), and *S. aureus* (circle) in ready-to-consume *ergo* stored at refrigeration (open symbols) and ambient (closed symbols) condition fermented with various mixed LAB cultures.

The inactivation of pathogens when introduced into fermented products is noted by various workers. Mufandaedza *et al*., (2006) reported significant reduction of *E. coli* 3339 after 48 h when inoculated into fermented milk. Ogwaro *et al*., (2002) indicated that *E. coli* 0157:H7 was inactivated after 96 h in traditional African yoghurt at ambient storage, whereas inactivation took about 144 h at refrigeration temperature. According to Massa *et al*., (1997), *E. coli* 0157:H7 was only reduced slightly in number after 7 days of storage of traditional yoghurt at 4^0C. Some surviving strains could have developed acid adaptation to the environment resulting in acid tolerance. At lower pH values, the presence of organic acid metabolites in the fermented product enhanced survival of *E. coli* O157:H7 at 4^0C (Connor and Kotrola, 1995). An acid tolerant state can persist for extended periods if the cells are stored at refrigeration temperatures (Cheville *et al*., 1996).

Our LAB cultures survived in a large numbers without significant variation during refrigeration and ambient storage of ready-to-consume *ergo* (Table-2). Large numbers of viable lactic acid bacteria are required in order to exert a possible probiotic effect in the food product (Gardiner *et. al*., 2004) as observed in vacuum packaged sliced ham (Kotzekidou and Bloukas, 1996) and vacuum-packaged bologna-type sausage (Andersen, 1995).

Table-2. Mean count of the various mixed LAB cultures (MLCs) during ambient and refrigeration storage of *ergo.*

	20-25^{0}C		**4^{0}C**	
Time	**Mean count of MLC** (log cfu/ml)	**S.D**	**Mean count of MLC** (log cfu/ml)	**S.D**
0	9.06	0.04	9.11	0.03
24	8.88	0.05	8.8	0.07
48	8.74	0.88	8.58	0.02
72	8.67	0.11	8.38	0.11

S.D. = Standard deviation

This study has shown that selected mixed LAB cultures may be considered in *ergo* fermentation for a safer product both during fermentation and storage. The study also suggests that the isolates, especially in the form of mixed lactic cultures are possible candidates for the formulation of bioprotective starter cultures that can be employed for production of safe and potentially probiotic *ergo*.

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