The Inhibition of Some Foodborne Pathogens by Mixed Lab Cultures During Preparation and Storage of Ayib, a Traditional Ethiopian Cottage Cheese

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Abstract: The antagonistic impact of mixed lactic cultures against foodborne pathogens (Escherichia coli, Salmonella Typhimurium DT104 and Staphylococcus aureus) were evaluated during preparation and storage of ayib. Soursing of milk reduced the count of the test pathogens by 4 log units at 48 h. The test pathogens were reduced to log 4 and log 0.8 cfu/g at day 9 while ayib was stored at refrigeration and ambient conditions, respectively. Complete elimination of the test organisms was achieved at day 7 when dipped ayib was kept at ambient conditions. Dipping ayib was observed to improve its safety.

Key words: Ayib · Lactic acid bacteria · Antagonism · Foodborne pathogens

INTRODUCTION

Ayib is a traditional Ethiopian cottage cheese produced by slowly heating naturally soured milk until a distinct curd mass forms and floats over the whey. Ayib is a popular milk product consumed by the various ethnic groups in the country [1]. Temperature of ayib preparation could vary between 40°C to 70°C without any significant impact on composition and yield. However, temperature above 80°C imparts a cooked flavor to the product. Ashenafi [2] suggested that curd preparation at 70°C produced a wholesome product with low microbial count.

During preparation of ayib, the high initial count of microorganism in milk, which increases during the fermentation process, is shown to decrease by the combined action of cooking and low pH [3]. The presence of high microbial load of ready-to-consume ayib is believed to be introduced from plant parts used for packaging and imparting flavor; and from handlers, too [2]. The safety of cheeses with respect to food-borne diseases is of great concern around the world and especially true in developing countries, where production of milk and various dairy products often take place under unsanitary conditions [1].

Generally, fermented food products are usually considered safe because of the low pH and production of antimicrobial substances by the fermenting organisms. However, some enteropathogens have been reported to survive and grow in fermented milks [4]. Isolation of Staph. aureus from ayib collected from open market in Awassa was reported by Ashenafi [2]. Salmonella heidelberg was associated with consumption of contaminated cheese at a restaurant [5].

Although low pH is expected to inactivate bacterial pathogens, acid adaptation was reported to promote the survival of different foodborne pathogens in different types of cheeses [6, 7]. Use of pediocin producing Lactobacillus plantarum (L. plantarum) culture to combat Listeria contamination in red smear cheese was suggested by Loessner et al. [8]. The inhibition of E.coli 0157: H7 during the souring of milk in the course of preparation of ergo and ayib and during storage of the two products was shown by Tsegaye and Ashenafi [9].

Praveez et al. [10] suggested the potential benefits of consuming fermented dairy products containing viable lactic acid bacteria (LAB). Workers have also indicated that cheese can serve as a vehicle for provision of probiotic organisms when compared with the other more acidic dairy products [11]. LAB with probiotic properties can be incorporated into cheese as starters to cheese milk or to the curd before hooping [12].
The aims of this study were to assess the antagonistic effect of defined mixed LAB cultures with probiotic potential against some foodborne pathogens during preparation and storage of ayib; and the possible use of ayib as a carrier of potentially probiotic LAB by dipping ayib preparations into mixed LAB cultures.

**MATERIALS AND METHODS**

**Bacterial Strains:** *E. coli* ATCC 25922, *Staph. aureus* ATCC 25923 and *S. Typhimurium* DT104, were used as test foodborne pathogens in this study. The LAB strains were recovered from locally fermented dairy products, *ayib* and *ergo*; and traditional low-alcoholic beverages, *borde* and *shamita*. LAB isolates 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). Briefly, colonies of pure LAB cultures were transferred into API 50CHL medium (Biomerieux, Marcy l’Etoile, France) and homogenized. Homogenized suspensions were filled into 50 wells of API 50CHL strips and covered with mineral oil and incubated at 30 to 32°C for 48 h. Identification of LAB strains using the biochemical profile was done using software database (V5.0) provided by the company. The nine LAB isolates were arranged into four mixed LAB cultures based on their fermentative characteristics (Table 1).

**Fate of Test Pathogens During Ayib Processing:** The effect of each mixed culture against each test strain during souring of milk was done following the protocol given by Tsegaye and Ashenafi [9]. Pasteurized milk samples (800 ml each) were separately soured by introducing into them initial inoculum level of log 6 cfu/g of each mixed LAB culture and log 3 cfu/g of each test pathogen. Pasteurized milk, separately inoculated with log 3 cfu/g of test strain in duplicates served as control. The enumeration of test strains was conducted by plating 0.1 ml of appropriate dilutions on PC agar. Plates for counting *E. coli*, *S. typhimurium* DT104 and *Staph. aureus* were overlayed after 30 minutes with Violet Red Bile (VRB) agar, Xylose Lysine Desoxycholate (XLD) agar and Mannitol Salt agar (MSA), respectively.

<table>
<thead>
<tr>
<th>Pure LAB cultures</th>
<th>Mixed LAB cultures (MLC)</th>
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<tbody>
<tr>
<td><em>L. acidophilus</em> 1*, <em>L. brevis</em> 1*, <em>L. cellobiosus</em>†, <em>L. delbrueckii</em> ssp <em>delbrueckii</em>††, <em>L. paracasei</em> ssp <em>paracasei</em> 3*, <em>L. plantarum</em> 1*, <em>L. plantarum</em> 2*, <em>L. acidophilus</em> ssp <em>lactis</em> 1* and <em>Ped. pentosaceus</em> 1††</td>
<td><em>L. lactis</em> ssp <em>lactis</em> 1, <em>L. paracasei</em> ssp <em>paracasei</em> 3 and <em>L. brevis</em> 1</td>
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<td></td>
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<td>*- homofermentative, †- heterofermentative</td>
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Counting was done at 0, 12, 24, 36, 48, 60 and 72 h. Plates were incubated at 32°C for 24 to 48 h. The pH was measured during sampling.

Soured milk was defatted by churning with a shaker at 100 RPM for 1 h. The floating fat was removed with a sterile spoon. Defatted soured milk samples were separately cooked at 50°C and 70°C in a water bath for 55 min. The curd (ayib) was separated from the whey after cooling cooked solid mass to room temperature and by filtering through sterile cheese cloth. Counting of pathogens and pH determination of cooked curd was done at 24 h intervals [9].

**Survival of the Test Pathogens During Storage of Ready-to-consume Ayib:** Ayib was prepared by cooking pasteurized milk fermented with the various mixed starter cultures. The test pathogens were separately inoculated into duplicates into 200 g of cooled ayib in sterile stomacher bags to give initial inoculum level of log 6 cfu/g mixed thoroughly and incubated at ambient condition. Separately, ayib samples was similarly processed and stored at refrigeration condition. Phosphate buffered saline (PBS) inoculated with the test pathogens served as control. Enumeration of pathogens was done at 24 h intervals for 9 days. When counts were <log1 cfu/g, enrichment followed by streaking on the nutrient agar plates were done to determine complete inhibition [9].

**Survival of the Test Pathogens During Storage of Dipped Ayib:** This experiment was carried out to examine the potential use of ayib as a vehicle of probiotic cultures as the starter LAB cultures would be inactivated by the curd cooking temperature. Ayib (200 g) prepared with the various mixed starter cultures was wrapped in sterile cheese cloth and dipped into pasteurized whey inoculated with log 8 cfu/g of respective mixed starter LAB cultures for 30 minutes. Dipped ayib samples were re-drained for 1 h. Test pathogens were inoculated into 200 g of drained-dipped ayib in sterile stomacher bag to give final inoculum level of log 6 cfu/g. Enumeration of LAB isolates was done on MRS agar plates after anaerobic incubation at 32°C for 24/48 h. Test pathogens and LAB were enumerated initially and at 24 h interval for 9 days as described earlier [9].

Table 1: Composition of the four mixed LAB cultures prepared for antagonism against target foodborne pathogens during preparation of and storage of ayib with probiotic potential against some foodborne pathogens measured during sampling.
Statistical Analysis: Experiments were conducted in triplicates. Results indicated are averages values and were described by descriptive statistics.

RESULTS AND DISCUSSION

In milks soured in the presence of mixed starter LAB cultures, the test organisms were reduced by 2 and 4 log factors at 24 and 48 h, respectively. The pH of souring milk was about 4.0 at 48 h (Table 2). In the control milks (milks inoculated with test pathogens in the absence of LAB), the test pathogens grew to log 8.4 cfu/g at 48 h. The mean pH value of control milks dropped from initial 6.44 to 5.43 (Table 2). Similar results were reported by Tsegaye and Ashenafi [9]. Vernozy-Rozand et al. [7] also indicated that E. coli 0157:H7 survived the lactic cheese manufacturing process, when milk was inoculated with less than log 3 cfu/g. The increase in the count of S. Typhimurium during manufacturing of cheese until the curd was salted and its subsequent reduction was showed by Goepfert et al. [13].

Curd-cooking at different temperature (50°C and 70°C) reduced the count of the test enteropathogens both in the control and the experimental milk. In the control (which did not sour to coagulation), curd-cooking at 50°C reduced the mean count of the test pathogens from the initial load of log 8.48 cfu/g to around log 2 cfu/g. Further maintaining the control at ambient temperature, however, resulted in the growth of the test organisms to log 4.83 cfu/g at 48 h (Table 3). Curd-cooking at 70°C resulted in total inactivation of the test pathogens.

In milks soured in the presence of mixed starter cultures, cooking curds at 50°C and 70°C reduced the counts of the test enteropathogens to log 1.28 cfu/g and to undetectable levels, respectively. In related study, Tsegaye and Ashenafi [9] reported total inactivation of strains of E. coli 0157:H7 after curd-cooking at 70°C. Abdella et al. [14] also reported the absence of Salmonella spp. after curd cooking during the process of ayib making. Ercolini et al. [15] also reported curd cooking at 55°C for 20 min reduced the viable cell load of E. coli 0157:H7 and S. Typhimurium F01, but Staph. aureus and L. monocytogenes persisted at a higher level.

Similar to our result, Arocha et al. [16] reported that the count of E. coli 0157:H7 increased during the manufacturing of cottage cheese, but was eliminated during heating the curd at 57°C for 90 minutes. During mozzarella cheese manufacturing, Spano et al. [17].

Table 2: The inhibitory effect of mixed LAB cultures on the test organisms during souring of milk

<table>
<thead>
<tr>
<th>TOs-MLCs</th>
<th>Average values</th>
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<tbody>
<tr>
<td></td>
<td>0 Hour</td>
</tr>
<tr>
<td></td>
<td>pH</td>
</tr>
<tr>
<td>E. coli-MLCs</td>
<td>6.42</td>
</tr>
<tr>
<td>S. Typhimurium-MLCs</td>
<td>6.47</td>
</tr>
<tr>
<td>Staph. aureus-MLCs</td>
<td>6.47</td>
</tr>
<tr>
<td>Stdeva¹</td>
<td>0.01</td>
</tr>
<tr>
<td>TOs alone (control)</td>
<td>6.44</td>
</tr>
<tr>
<td>Stdeva²</td>
<td>0.04</td>
</tr>
</tbody>
</table>

(Tos: E. coli, S. Typhimurium and Staph. aureus)

Stdeva¹ = Standard deviation among counts of the TOs in the presence of MLCs; Stdeva² = Standard deviation among values of the control

Table 3: The average effect of curd cooking against test organisms (TO) during preparation of ayib with mixed LAB cultures

<table>
<thead>
<tr>
<th>Test organisms</th>
<th>Average values</th>
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<tbody>
<tr>
<td></td>
<td>Defatted milk</td>
</tr>
<tr>
<td></td>
<td>0 Hr</td>
</tr>
<tr>
<td></td>
<td>Count (log/ml)</td>
</tr>
<tr>
<td>E. coli-MLCs</td>
<td>4.21</td>
</tr>
<tr>
<td>S. Typhimurium-MLCs</td>
<td>4.21</td>
</tr>
<tr>
<td>Staph. aureus-MLCs</td>
<td>4.19</td>
</tr>
<tr>
<td>TOs alone (control)</td>
<td>ND</td>
</tr>
</tbody>
</table>

¹+ = detected after enrichment, ⁻ = not detected after enrichment, ND = Not done, † = Not enriched
reported that curd stretching at 80°C and 70°C for 5 min resulted in destroying the culturability and reducing culturability of strains of *E. coli* O157:H7 by 10 factors, respectively. Spahr and Schafroth [18] indicated that the most important factors responsible for the death of *M. avium* subsp. *paratuberculosis* in cheese were the temperatures applied during cheese manufacture and the low pH at the early stages of cheese ripening. Since *ayib* is a cottage cheese which is not ripened, the most important factors involved in eliminating pathogens could be the cooking temperature, the low pH and microbial metabolites [3].

In ready-to-consume *ayib* maintained at refrigeration temperature, the mean log counts of our test pathogens decreased by 2 - 3 log units at day 9 (Fig. 1). The pH of *ayib* remained around 4.0 during refrigeration storage and slightly fell to around 3.6 during ambient storage. During ambient storage, the count of test pathogens decreased by 3 log units at day 3 and were only detected with enrichment at day 9. In a similar study, Tsegaye and Ashenafi [9] reported the gradual decrease in the count of *E. coli* O157:H7 by 3 log units at day 3 and complete inactivation at day 9 during storage at ambient condition. Detection of *Salmonella* for up to 7 months in ripened cheddar cheese stored at 7°C was reported by El-Gazzar and Marth [19]. Ercolini et al [15] indicated the reduction of *Listeria monocytogenes*, *Salmonella Typhimurium* and *Staphylococcus aureus* beyond detection level; and the survival of *Escherichia coli* O157:H7 at low levels after curd cooking at 55°C for 4 h.

In dipped *ayib* kept at ambient situation, the mean count of the test pathogens decreased by 3 - 4 log units at day 3 and they were totally eliminated at day 6 or 7 (Fig. 2). Storage of dipped *ayib* at refrigeration condition reduced the count of test pathogens by 2 - 3 log units at day 9 (Fig. 2). These results were comparable to the effect of similar storage conditions on ready-to-consume *ayib* (Fig. 1). The pH of *ayib* dipped into four respective mixed LAB cultures remained around 4.1 during refrigeration storage and slightly fell to around 3.9 during ambient storage. Results showed the enhanced effect of dipped *ayib* on the test enteric pathogens during ambient storage. In fact, reduction of metabolic activity of both...
Fig. 3: Changes in counts of various mixed LAB cultures during refrigeration (closed symbols) and ambient (open symbols) storage of dipped ayib.

LAB and the pathogens at refrigeration temperature could be the reason for low reduction in count of pathogens under refrigeration storage. Bachrouri et al. [20] demonstrated that low temperatures (4°C) enhanced the survival of *E. coli* O157:H7 at low pH in yoghurt. Cheng and Kaspar [21] indicated the storage of low pH foods at low temperatures improved the survival of *E. coli* O157:H7 compared to storage at ambient temperature.

During ambient storage of dipped ayib, the LAB strains survived at around log 7 cfu/g at day 6 and the count decreased slightly at day 9. However, when dipped ayib was kept at refrigeration condition, LAB decreased in count to about log 5.81 cfu/g at day 5 and nearly maintained the count until day 9 (Fig. 3). Haddadin, et al. [22] showed the survival of individual probiotic bacteria at counts >log 10 cfu/g in yoghurt at 4°C for over 15 days. Generally, fermented food products or live microbial food supplements are understood to have probiotic effect [23]. The need to consume at least log 5 cfu/g probiotic organisms daily with food to achieve the beneficial effects was indicated by Lee and Salminen [24]. To this connection, our result indicated the remarkable effect of our mixed lactic cultures in reducing the test foodborne pathogens during souring milk. Inactivation of the test pathogens during curd cooking at 70°C and storage at ambient conditions was noted. Moreover, in addition to the potential use of ayib as a vehicle for provision of probiotic LAB cultures, dipping ayib in lactic cultures demonstrated its significance in further improving its safety.

REFERENCES


