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Effect of temperature and pH on the antimicrobial activity of inhibitory substances produced by lactic acid bacteria isolated from Ergo, an Ethiopian traditional fermented milk

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Lactic acid bacteria (LAB) predominates the microflora of milk and milk products. They produce antimicrobial metabolites that inhibit growth of food-borne pathogenic and spoilage microorganisms, thereby enhancing the shelf-life of the food. To evaluate the antimicrobial activity and the effect of temperature and pH on the culture supernatant of LAB, 112 strains of LAB, belonging to *Lactococcus, Lactobacillus, Entorococcus, Streptococcus, Leuconostoc* and *Pediococcus* species, were isolated from Ergo. The culture supernatants of all isolates were examined for antimicrobial activity on indicator strains, that is, *Salmonella typhi, Shigella flexneri*, *Staphylococcus aureus* (ATCC-25923) and *Escherchia coli* (ATCC-25922), using the disc diffusion assay. Twelve strains of LAB that produced antimicrobial substances were subsequently selected and their culture supernatants were evaluated for stability at different temperatures and pH values. The antimicrobial activity of the culture supernatants of the LAB was completely inactivated when treated at 121^{O}C for 15 min, whereas at 30, 60 and 80^{O}C, there was no significant (*P*>0.05) difference in the diameter of the inhibition zones compared to the control. The culture supernatants was stable over a wide pH range (pH 2 - 10) and no significant (*P*>0.05) difference was observed between the treated and untreated (control) supernatants. However, the diameter of the inhibition zones was significantly (*P* <0.05) decreased at pH 12. Thus, Ergo may be a promising source of antimicrobial substance-producing LAB that may in future be applied to food.

Key words: Lactic acid bacteria, antimicrobial substances, fermentation, ergo

INTRODUCTION

Microbial fermentation has played an important role in food processing and preservation for thousands of years. Lactic acid bacteria (LAB) are widely utilized to produce fermented foods, contributing to flavor development as well as safe metabolic activities. During growth in foods they utilize available sugar for the production of organic acids and other metabolites (Ayele, 1998). In common fermented products, such as yogurt, lactic acid is produced by the starter cultures to prevent the growth of undesirable microorganisms (Daeschel, 1993). LAB have an important role in the inhibition of food-borne pathogenic and spoilage microorganisms, since they produce antimicrobial metabolites that include lactic acid, acetic acid and other organic acids, hydrogen peroxide, bacteriocins and bacteriocin-like substances (Cadirci and Citak, 2005).

The rural communities in Ethiopia produce fermented milk by traditional methods. The major fermented milk products produced by small-holder farmers include “Ergo” (fermented sour milk), “Ititu” (Fermented milk curd), “Kibe” (traditional butter), “Neter kibe” (spiced butter), “Ayib” (cottage cheese), “Arerra” (Sour defatted milk),

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and “Aguat” (whey). Ergo is a traditional naturally fermented milk product, which has some resemblance to yogurt. It is thick, smooth, of uniform appearance and usually has a white milky color when prepared carefully. The product is semi-solid and has a pleasant odor and taste. It constitutes a primary sour milk product from which other products may be derived. Depending on the storage temperature, it can be stored for 15 - 20 days (Almaz et al., 2001) and it has been reported that *Lactococcus garvieae* and *Lactococcus lactis* subsp. *lactis* were dominant (Almaz et al., 1999).

In Ethiopia, some research has been performed on the antimicrobial activity of LAB isolated from traditional Ethiopian fermented foods and beverages. Idris (1999) did some microbial and biochemical studies on the fermentation of two traditional condiments, Awaze and Datta, and reported that entrobacteriaceae and coliforms, which were isolated at the initial stage of Datta fermentation, were inhibited at 32 h by the antimicrobial activity of dominating LAB. The antimicrobial activity of LAB isolated from two traditional fermented beverages (Borde and Shameta) on *S. aureus, S. flexneri, Salmonella* spp. and *E. coli* O157:H7 has also been examined. All isolates, except *E. coli* O157:H7, showed an additional 3 to 4 mm zone of inhibition over the control. *Lactobacillus* isolates had the maximum inhibitory effect against the test strains, followed by *Pedicoccus, Streptococcus* and *Leuconostoc* isolates (Girum et al., 2005). From the studies of Ayele (1998), regarding the microbiological safety of two widely consumed LAB-fermented Ethiopian foods, Kocho and Tef, the dough and freshly baked products were found capable of inactivating asporgenous pathogenic and spoilage bacteria. In their study on the fate of *E. coli* O157:H7 during the processing and storage of Ergo and Ayib, Mekonen and Mogessie (2005) reported that the decrease in the population of *E. coli* O157:H7 in fermenting Ergo could have been due to the antagonistic activities of the LAB through their metabolites and resulting low pH.

Ergo plays an important role in the diet of low income people and the majority of people in the rural areas of Ethiopia. If Ergo is to be produced on large scale, some tasks have to be undertaken according to Mogessie (2002). Thus, it is logical to start with isolating and characterizing as many lactic acid bacteria as possible from Ergo produced in the various ecological zones of the country. Characterization of promising strains of lactic acid bacteria, particularly those which have potential to produce inhibitory substances that are effective over a wide range of pH and temperature may result in improved safety of fermented and processed products (Fekadu et al., 1998). Thus, this research was aimed at isolating antimicrobial substance-producing lactic acid bacteria and to evaluate the inhibitory substances at different temperatures and pHs.

MATERIALS AND METHODS

The study areas

The study area was located in three districts of the East Shewa Zone (Adami Tulu-Jidocombolcha, Lumme and Fentale), Oromiya Regional State, Ethiopia. The altitude of these areas ranges from 910 - 2300 m above sea level and have an arid and semi- arid climate with rainfall averaging between 500 and 900 mm per annum (Lemma, 2004). The predominant production systems are crop and livestock farming, except for Fentale where pastoral and agro-pastoral production systems predominate.

Test (indicator) strains

S. typhi (clinical isolate), *S. flexneri* (clinical isolate), *S. aureus* (ATCC-25923) and *E. coli* (ATCC-25922) were obtained from the Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia, and were used as test microorganisms.

Ergo sample collection

Samples of Ergo (250 ml) were collected from 10 farms from each of the three districts, and 30 samples in total were collected using sterilized flasks. The samples were kept under refrigeration temperature using an icebox and brought to the dairy laboratory. Samples were then kept in a refrigerator until analysis.

Plating and isolation of LAB

Plating was done within 3- 6 h of arrival of the samples at the laboratory. Enumeration of lactic acid bacteria was done after plating 0.1 ml of the 10^{-5} dilution of the samples onto de Mann Rogosa Sharp agar (MRS) (HiMedia). The agar plates were incubated anaerobically in an anaerobic jar (BBL, GasPak Plus) at 35^{o}C for 48 h with replications. Ten colonies were randomly selected from the MRS agar plates. The colonies were purified by successive streaking onto MRS agar, before being subjected to characterization. Five colonies from each plate, with differing colony morphology, were inoculated into MRS broth, incubated for 12 h and then maintained in the refrigerator at 4^{o}C. The isolates were grouped as lactic acid bacteria after examining their Gram reaction, cell and colony morphology, catalase reaction and gas production from glucose fermentation. Isolates that were characterized as lactic acid bacteria, were kept as glycerol stock cultures in the refrigerator at -20^{o}C.

Antimicrobial activity and bioassay

The antimicrobial activities of the isolates were quantified by modifying the disc diffusion procedure of Savadogo et al. (2004) and Girum et al. (2005). A well-isolated colony was selected from the MRS agar plate. The top of the colony was touched with a loop and the growth was transferred into a tube containing 5 ml of sterile MRS broth. The broth culture was incubated at 35^{o}C for 24 h. After incubation, the culture was centrifuged (10 000 rpm for 20 min at 4^{o}C) to obtain the culture supernatant. The pH of the culture supernatant was adjusted to pH 7 with 1 M NaOH to exclude antimicrobial effects of organic acids (Savadogo et al., 2004). Two controls were prepared using un-inoculated MRS broth. A sterile cotton swab was dipped into culture of the test (indicator) microorganism, which had been grown in Tryptone Soya Broth (Oxoid) for 24 h at 35^{o}C. The swab was rotated several times and pressed firmly on

Table 1. Lactic acid bacteria isolated from Ergo that were collected from three districts (Lumme, Adami Tulu, Fentale)

Site	LAB isolated and identified from the different districts						
	Lactobacillus	***Lactococcus***	***Luconostoc***	***Entrococcus***	***Streptococcus***	***Pediococcus***	**None LAB**
Lumme	10	9	0	5	4	2	20
Adami Tulu	16	20	3	3	2	4	2
Fentale	11	0	5	8	5	5	16
Total.	37	29	8	16	11	11	**38**

firmly on the inside wall of the tube above the fluid level to remove excess inoculum. The dried surface of Mueller-Hinton agar (HiMedia) was inoculated by streaking the swab over the entire agar surface. This procedure was repeated by streaking two more times, rotating the plate each time to ensure an even distribution of inoculum. Sterile filter discs of 6 mm in diameter were prepared from Whatman filter paper no. 1. Using a wire loop of 20 gauge (2 mm in diameter), 5 µl of the culture supernatant was added to each disc (Lalitha, 2005). Four discs (with culture supernatant) were placed on a 100- mm plate, within 5 to 15 min of streaking the test organisms. After 12 and 24 h of anaerobic incubation, each plate was examined. The diameter of the inhibition zones was measured, including the diameter of the disc. Zones were measured to the nearest whole number in millimeter with a transparent ruler.

Characterization and identification of inhibitory substance producing LAB strains

Overnight cultures of the LAB isolates, grown in 10 ml of MRS broth at 30^{o}C, were washed twice with sterile peptone water, centrifuged and the pellets were resuspended in API 50 CHL medium. The carbohydrate fermentation profiles of the selected 12 LAB isolates were then investigated using API 50 CH strips according to manufacturer's instructions (API system, bio-Merieux, France).

Effect of heat treatment on inhibitory substances produced by LAB isolates from Ergo

The culture supernatant of inhibitory substance-producing strains, which were grown in MRS broth for 24 h, was exposed to various heat treatments. The culture supernatants were incubated for 30 min at 30, 60 and 80^{o}C, as well as at 121^{o}C for 15 min.

Effect of pH on inhibitory substance produced by LAB isolates from Ergo

To determine the pH sensitivity of the culture supernatant, recovered during stationary growth phase of the isolates, pH values were adjusted ranging from 2 – 12 by using 1 M NaOH or 1 M HCl (Hernadez et al., 2005). After incubation for 4 h, the pH was readjusted to 6.5 and the antimicrobial activity was then determined, as described above.

Statistical analysis

All laboratory experiments were repeated three times, except the disc diffusion assay, which was repeated twice. The different sample treatments were compared by performing one-way analysis of variance on the replicates at 95% level of significance, using the SPSS 12.0 statistical program. Significant results refer to $P<0.05$.

RESULTS AND DISCUSSION

Isolation of lactic acid bacteria

Gram-positive, catalase- negative, cocci, cocco-bacilli or rod-shaped isolates with characteristic cell arrangements when grown on MRS medium were considered as lactic acid bacteria (Savadogo et al., 2004). From a total of 150 bacteria isolated from the 30 samples (10 from each district), 112 were tentatively considered as lactic acid bacteria (Table 1). Of these, 30 were isolated from Lumme, 48 from Adami Tulu and 34 from Fentale. The LAB isolated comprised five genera, that is, *Lacto-bacillus, Lactococcus, Leuconostoc, Entrococcus* and *Streptococcus.* These results showed the heterogeneity of Ergo at different locations or agro-climatic zones either in its chemical or microbial composition and quality attributes. These findings are consistent with observations of other reports regarding fermented milk (Almaz et al., 1999; Savadogo et al., 2004; Ayad et al., 2004).

Antimicrobial activity of the isolates

The antimicrobial activity of isolated LAB and degree of inhibition is shown in Table 2.From a total of 112 lactic acid bacteria, the culture supernatants of 12 LAB strains yielded zones of inhibition when tested against the indicator strains. The diameters of the inhibition zones ranged from 7 mm to 12 mm. The biggest diameters (12 mm) were obtained from the culture supernatants of strains Mj-622 on *S. flexneri,* Ad- 211 on *S. typhi*, and Ad-855 on both *S. typhi* and *S. flexneri* (Figure 1). In contrast, *E. coli* (ATCC-25922) was the most resistant strain; it was only inhibited by two strains (Ad-855 and Fn-133) and the diameter of inhibition zones was 10 mm and 9 mm, respectively. *S. aureus* (ATCC-25923) was inhibited by four LAB strains with low diameter inhibition zones, i.e. Ad-211 (9 mm), Ad-233 (10 mm), Ad-411 (9 mm) and Ad-522 (9 mm). Similarly, Girum et al. (2005) and Cadirici and Citak (2005) observed varying degrees of inhibition using various indicator microorganisms, although inhibitory substances produced by the lactic acid bacteria strains act differently on the reference indi-cator strains (Savadoge et al., 2004). Inhibitory sub-stances produced by the lactic acid bacteria can be gene-

Table 2. Average diameter of inhibition zones and antimicrobial activity of the inhibitory culture supernatant, on some indicator strains, using the disc diffusion assay

No	LAB strain	Test bacteria	Diameter of inhibition zone (mm) (Mean ± SD)
1	Mj-622	*Salmonella typhi*	10 ± 0.00
		Shigella flexneri	11.8 ± 0.35
2	Ad-211	*Salmonella typhi*	11.8 ± 0.35
		Shigella flexneri	10 ± 0.00
		Staphylococcus aureus (ATCC-25923)	9 ± 0.00
3	Ad-233	*Salmonella typhi*	9 ± 0.00
		Shigella flexneri	10 ± 0.00
		Staphylococcus aureus (ATCC-25923)	10 ± 0.35
4	Ad-355	*Salmonella typhi*	9.5 ± 0.70
		Shigella flexneri	9 ± 0.00
		Staphylococcus aureus (ATCC-25923)	9.25 ± 0.35
5	Ad-411	*Salmonella typhi*	10 ± 0.00
6	Ad-522	*Shigella flexneri*	10.5 ± 0.00
		Staphylococcus aureus (ATCC-25923)	9.25 ± 0.35
7	Ad-855	*Salmonella typhi*	11.8 ± 0.35
		Shigella flexneri	11.3 ± 0.35
		Escherichia coli (ATCC-25922)	10 ± 0.00
8	Fn-133	*Salmonella typhi*	10 ± 0.00
		Escherichia coli (ATCC-25922)	9 ± 0.00
9	Fn-144	*Salmonella typhi*	9.25 ± 0.35
10	Fn-233	*Salmonella typhi*	9.25 ± 0.35
11	Fn-533	*Shigella flexneri*	9 ± 0.00
12	Fn-555	*Shigella flexneri*	9 ± 0.00

Figure 1. Inhibition zone on test strains formed by lactic acid bacteria isolated from Ergo.

rally proteins (Vandenberg, 1993). Inhibition caused by hydrogen peroxide and organic acids was ruled, out as the producer strains were cultured anaerobically and the culture supernatant was neutralized before assaying the antimicrobial activity.

The most inhibited indicator strains were Gram-positive bacteria. *S. typhi* was the most sensitive pathogenic the lactic acid bacteria isolates, followed by *S. flexneri* and *S. aureus* (ATCC-25923). Similar results were reported by Savadago et al. (2004) and Girum et al. indicator strain to the inhibitory substances produced by (2005). *E. coli* strains were the least sensitive to inhibitory substance produced by the lactic acid bacteria as compared to the other indicator strains. The resistance of Gram-negative bacteria is attributed to the nature of their cell wall. According to Bhunia et al. (1991), pediocin (bacteriocin produced by *Pediococcus acidilactic*) interacts with lipoteichoic acids that are absent in Gram-negative bacteria. Also, all the *Lactococcus lactis* ssp. *lactis* isolates (Fn-555, Ad-411 and Ad-211) did not show an inhibitory effect on *E. coli* ATCC-25922. This could be due to the fact that these isolates are nisin producers (Soormro et al., 2002), and the primary target of nisin's antimicrobial action is the cell membrane. Nisin has an inhibitory effect against a wide variety of Gram-positive food-borne pathogens and spoilage microorganisms (Rodriguz, 1996).

Identification of the inhibitory substance-producing LAB strains

The morphological and physiological characteristics, and the carbohydrate fermentation profile of the 12 inhibitory substance-producing LAB isolates were used to tenta-

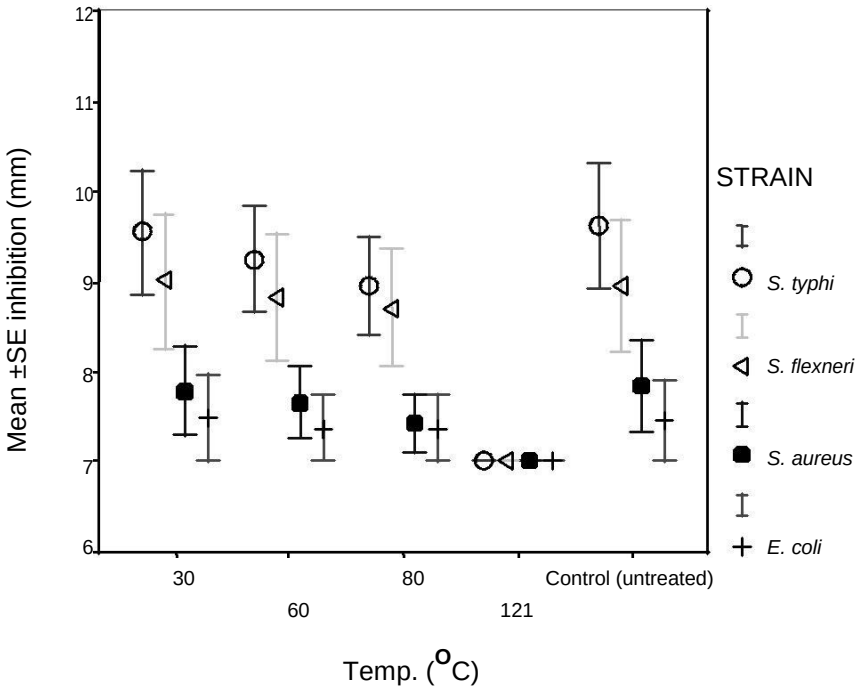

Figure 2. Effect of temperature on the antimicrobial activity of inhibitory substances produced by lactic acid bacteria strains isolated from Ergo.

tively designate them to species according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994), in addition to the identification table in the API50 CH user manual. Two of these isolates were identified as *Lactobacillus plantarum* (Ad-522 and Ad-233), two as *Lactococcus lactis* ssp. *cremoris* (Mj-622 and Ad-855), three as *L. lactis* ssp. *lactis* (Fn-555, Ad-411 and Ad-211), two as *Lactobacillus acidophilus* (Fn-133 and Fn-233), one as *Leuconostoc lactis* (Fn-533), one as *Pediococcus pentosace* (Fn-144) and one as *Pediococcus* sp. *(*Ad-355*)*.

Effect of temperature treatment on the inhibitory activity of the culture supernatant

The antimicrobial substances produced by the isolates were relatively stable during heat treatments at 30, 60 and 80^{o}C for 30 min (Figure 2). Treating the culture supernatant at 30, 60 and 80^{o}C for 30 min did not show significant difference (P>0.05) from the control. However sterilization (121^{o}C for 15 min) led to complete inactivation of the inhibitory activity of the culture supernatant, and showed a significant difference (P<0.05) from the control. These results are similar to those of Hernadez et al. (2005).

Effect of pH on the inhibitory activity of the culture supernatant

The antimicrobial activity of the culture supernatant after treatment at different pHs (2-10) was not significantly (P>0.05) affected, but the antimicrobial activity of the culture supernatant was significantly (P<0.05) affected at a pH of 12 (Figure 3). However, all the culture supernatant were heat stable up to 80^{o}C and, in addition; they all were stable over a wide pH (2 - 10) range. These are characteristic of a number of bacteriocins (Hernadez et al., 2005; Messi et al., 2001).

Conclusions

The culture supernatants from 12 strains of lactic acid bacteria isolated from Ergo exhibited antimicrobial activity against four indicator strains. The potential application of the antimicrobial substances as consumer -friendly bio-preservatives either in the form of protective culture or as additives is significant besides being less potentially toxic or carcinogenic than current antimicrobial agents. Lactic acid bacteria and their by-products are more effective and flexible in several applications. Most inhibitory substances produced by lactic acid bacteria are safe and effective natural inhibitors of pathogenic and food spoilage bacteria in various foods. Due to their broad spectrum, heat stability and stability over a wide range of pH, all the inhibitory substances produced by the isolated strains can effectively be used as bio-preservative in food with a wide range of pH, even after pasteurisation. However, a simple *in vitro* antimicrobial evaluation of the isolates only cannot lead to a final conclusion regarding

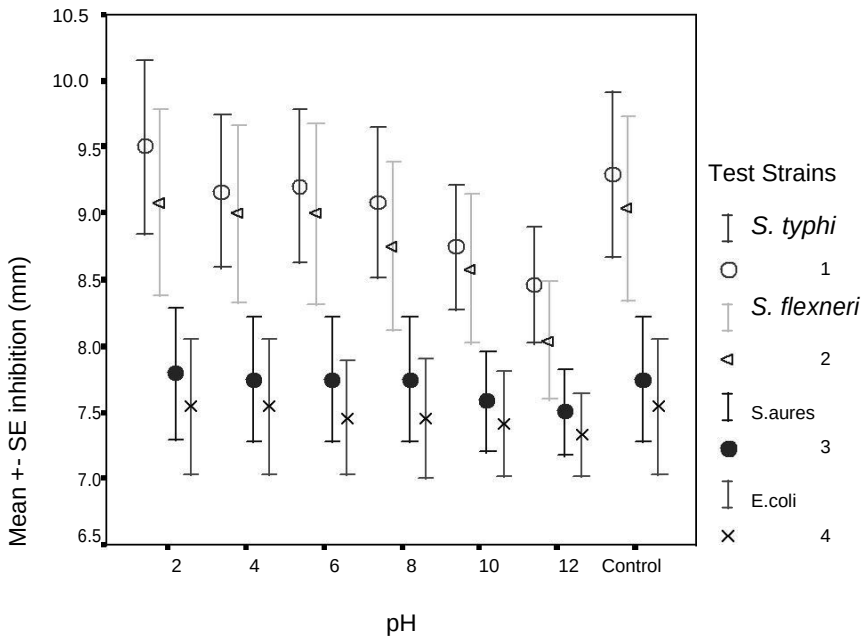

Figure 3. Effect of pH on the antimicrobial activity of inhibitory substances produced by lactic acid bacteria strains isolated from Ergo.

use of the potential strains as bio-preservatives. Additional technological characteristics of the isolates that contribute to the flavour, texture and nutritional attributes of the product, such as acidification activity, proteolytic activity, resistance to bacteriophage and production of exopolysacharides should be studied.

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