The role of spices and lactic acid bacteria as antimicrobial agent to extend the shelf life of *metata ayib* (traditional Ethiopian spiced fermented cottage cheese)

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Abstract Spices and lactic acid bacteria have natural antimicrobial substances and organic compounds having antagonistic activity against microorganisms. The objective of this study was to investigate the role of spices and lactic acid bacteria as antimicrobial agent to extend the shelf life of *metata ayib*. Antimicrobial activities of spices and lactic acid bacteria (LAB) filtrates were determined by agar well diffusion method against *E. coli*, *S. aureus*, *S. flexneri* and *S. pneumoniae*. Antimicrobial activity of garlic was found to be the most effective against all the tested pathogens. Inhibition zones of garlic extract against all pathogens was significantly ($P \leq 0.05$) greater than the remaining spice extracts. Inhibition zones (12.50 ± 1.00 to 15.50 ± 1.00 mm) of ginger and *R. graveolens* ethanol extracts against all tested pathogens were significantly ($P \leq 0.05$) greater than the remaining solvent extracts. Inhibition zone of *O. basilicum* ethanol extract against all pathogenic bacteria was significantly ($p \leq 0.05$) greater than hexane and acetone extracts. *Lactobacillus* isolates were shown the highest antimicrobial activity than the other LAB isolates against all pathogens. The synergistic effect of spices together with LAB might be contributed a lot to preserve and extend shelf life of *metata ayib*. Their antimicrobial activity can reduce the risk of spoilage and pathogenesis. The possible reason of LAB isolates was may be due to production of lactic acid, acetic acid and secondary metabolites like bacteriocins. Aseptic processing of traditional cottage cheese (*ayib*) is by far needed to minimize risks associated during consumption of *metata ayib*.

Keywords Antimicrobial activity · Cottage cheese · Inhibition zone · Lactic acid bacteria · *Metata ayib* · Spices

Introduction

Spices have been used for many centuries across different regions of the world to improve aroma and flavour of our foods substances. Our ancestors have been used spices for a long period of times to preserve food and for treatment of diseases. Plant derived products have been used for medicinal purposes for many centuries. At present it has been estimated that about 80 % of the world population rely on botanical preparations as remedy to treat different diseases without any side effects (Joe et al. 2009). Spices have innate antimicrobial substances that widely used to extend shelf life and preserve foods for a long period of time (Eyassu 2013). Additives are well known harmful for human health, especially aspartame, monosodium, sodium cyclamate, glutamatesaccharin, sulphites, nitrites and nitrates. It causes nausea, headache, weakness, cancer, anorexia and mental retarding (Keskin et al. 2010). As a result, consumer's interest in natural products, especially plant extracts like spices increased from time to time. The spices have a special flavour and aroma which are already derived from substances known as secondary metabolites or phytochemicals (Avato et al. 2002). Essential oils and their derivatives are well known inherent antimicrobial compounds widely applied for different activities, natural therapeutics including complementary drug and food preservation (Cosentino et al. 2003).

Apart from spices, lactic acid bacteria play significant role to preserve and extend shelf life of fermented food products. There are a number of bacteria that can preserve

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and increase shelf life of dairy foods by generating different organic compounds with significant potential to inhibit the growth of food spoilage and pathogenic microorganisms. Of those significant compounds, proteinaceous-bacteriocins play great role to inhibit the growth of pathogenic organisms in dairy foods (Hemashenpagam and Saranya 2011). Lactic acid bacteria (LAB) are the most well-known non-pathogenic bacteria that play a significant role in our everyday life, such as preservation of food, fermentation and production of vitamins and nitrous foods. They also play great role for prevention of certain cancer and diseases due to their antimicrobial action. Lactic acid fermentation process is generally economical often involving modest environment with full-efficient production capacity (Keith 1991). A number of *Lactobacilli* have been shown to have anti-cholesterol, anti-carcinogenic activities, have nutritional benefits, improved lactose utilization and protection against other different types of diseases. In addition to the above mentioned medicinal significance of *Lactobacilli*, it also plays important role in the prevention of intestinal pathogens. For example, *Lactobacillus acidophilus* (*L. acidophilus*) has been applied for treatment of different type of diarrhoea in human (Noordiana et al. 2013). The antimicrobial activity of LAB isolated from fermented products on various food borne pathogens is well documented. In Ethiopia, different spices are traditionally used to enhance flavor, color and keeping quality of milk and milk products. Use of spices in the preparation of the traditional cottage cheese *ayib* has been reported from different parts of the country (Eyassu 2013). The roles of antibiotic activity of such types of spices are not yet determined using test pathogenic bacteria. Therefore, there is a need to determine their antimicrobial activity against pathogenic bacteria. In addition to this, the role of LAB to discriminate and suppress food spoilage microorganism and thereby help to preserve and extend shelf life of *metata ayib* was not documented. The present study objective was to investigate the role of spices and lactic acid bacteria as antimicrobial agent to extend the shelf life of *metata ayib*.

Materials and methods

Description of the study area

Gondar town is located in Amhara Regional state, North West of Ethiopia, at about 723 kms from Addis Ababa. It is located at an altitude of around 2,225 m above sea level, 12° 35' 60, 000" of latitude and 37° 28' 120" of longitude. The study was conducted from February–March 2012 at Biotechnology laboratory in University of Gondar.

Experimental design

Antimicrobial activities of six spices (Table 1) were evaluated by agar well diffusion method against four bacterial pathogens, namely *E. coli* (ATCC25922), *S. aureus* (ATCC25923), *S. flexneri* (ATCC 12022) and *S. pneumoniae* (ATCC49619). The role of LAB isolates as antimicrobial agent in fermentation process of *metata ayib* was also evaluated against the above pathogenic bacteria using the well diffusion assay method (Hemashenpagam and Saranya 2011).

Sample collection

The fresh spices (Table 1) used for traditional *metata ayib* preparation were purchased from the local market in Gondar town. Cheese samples were also separately collected from four households producing cheese following traditional practice. These samples were collected in four sterilized flasks. Samples were kept at 4 °C temperature with the help of an icebox and brought to the laboratory for further investigation. The samples were kept for 2–4 h in the refrigerator until further analysis was conducted.

Metata ayib preparation using traditional method

The preparation steps and ingredients used during preparation of *metata ayib* were identified by interviewing the four vendors from whom cheese samples were purchased. Additional information was also obtained from Eyassu (2013) and used

Table 1 Spices used in the preparation of *metata ayib*

Scientific name	Vernacular name		
	English	Amharic	Part of the plant used
<i>Brassica nigra</i>	Mustard	<i>Senafitch</i>	Seed
<i>Coriandrum sativum</i>	Coriander	<i>Dimbillael</i>	Seed
<i>Zingiber officinale</i>	Ginger	<i>Zingebil</i>	Rhizomes
<i>Allium sativum</i>	Garlic	<i>Netchishinkurt</i>	Bulbs
<i>Ocimum basilium</i>	Basil	<i>Zekakibe (Basobila)</i>	Seed
<i>Ruta graveolence</i>	Rue	<i>Tenadam</i>	Seed

to verify the processing steps and ingredients of *metata ayib*. The raw material used for the preparation of *metata ayib* was a traditional cottage cheese called *ayib* and different spices. *Metata ayib* was prepared according to the following procedure. The different batches of *ayib* were mixed with 2–9 g of NaCl per 500 g of *ayib* and spices prepared from finely ground seeds of *Coriandrum sativum* and *Brassica nigra* 2 g. Each of them was added into the mixture after which whey was drained for three consecutive days. Then the curd was mixed with four additional spices of 2 g each listed in Table 1. Then the mixture was allowed to ferment normally at room temperature (25 °C) in a firmly closed container for 20 days. Finally, the vessel was opened and *metata ayib* was ready for consumption and further analysis.

Preparation of spice crude extracts for antimicrobial activity

The fresh spices used for traditional *metata ayib* preparation were purchased from the local market (Table 1), oven-dried at 45 °C for 72 h and the dried materials were powdered using blender. Ethanol, hexane and acetone were used as solvents for the extraction of antimicrobial substances. Commercial antibiotic disc (vancomycine) and sterilized distill water were used as positive and negative controls, respectively. Twenty grams of each spice was immersed and extracted with 100 ml of 95 % solvents separately for a period of about 120 h, then homogenized by using a Heidolph Homogenizer (DIAX 900, Germany). The homogenous substances were mixed using a rotary shaker at 120 rpm for 24 h. The preparations were then centrifuged at 12,000 rpm for 20 min at room temperature and then the supernatant was collected and filtered using What-man No. 2 filter paper. Then, extracts were evaporated using vacuum rotary evaporator to near dryness at 37 °C for 48 h and stored in glass vials in dark at 4 °C until the determination of antimicrobial activity (Das et al. 2012) was carried out. The final concentration of each extract was adjusted to 50 %.

Sources of test organisms and inoculum preparation

In this study, the standard Gram positive and Gram negative bacterial species commonly implicated in causing infectious disease such as *S. aureus* (ATCC25923), *E. coli* (ATCC25922), *S. flexneri* (ATCC 12022) and *S. pneumoniae* (ATCC49619) were used for in vitro antibacterial activity. The microorganisms were selected because they were of great public health importance. The organisms were obtained from the Department of Medical Microbiology, University of Gondar. The antibacterial activity was tested at Molecular Biology and Microbial Laboratory Department of Biotechnology, University of Gondar. The standard pathogenic bacteria were inoculated and spread over on Muller-Hinton (MH) agar (Oxoid) and incubated for 24 h at 37 °C. Prior to inoculation, from 2 to 3 colonies were picked up by wire loop aseptically

into sterile normal saline solution and the turbidity of the resulting suspension was adjusted to 0.5 McFarland's standard solution (a concentration of 1.5×10^8 CFU/ml) (Alsaied et al. 2010).

Preparation of 0.5 McFarland turbidity standards

The McFarland 0.5 turbidity standard was prepared by adding 50 µl of a 1.175 % (w/v) barium chloride dehydrate ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) solution to 9.95 ml of 1 % (v/v) sulfuric acid. The standard tube containing the solution was then sealed with paraffin to prevent evaporation and stored in dark at room temperature. The accuracy of the density of a prepared McFarland standard was checked using a spectrophotometer with a 1-cm light path length. For the 0.5 McFarland standards, the absorbance was adjusted at a wavelength of 625 nm and water was used as a blank. The 0.5 McFarland standards were vigorously agitated to turbidity on a vortex mixer before use. As with the barium sulfate standards, a 0.5 McFarland standard is equivalent to a bacterial suspension of 1.5×10^8 colony forming units (CFU)/ml (NCCLS 2000).

Antimicrobial susceptibility test

The agar-well diffusion method prescribed by NCCLS (2000) was employed in the susceptibility testing (Patel et al. 2011). Suspensions of the standard test bacteria were made in sterile normal saline solution and adjusted to the 0.5 McFarland's standard. Each Mueller Hinton (MHA) agar plate was uniformly seeded with 0.1 ml of the respective test organism and spread with sterile cotton swab. The seeded plates were left on the bench to evaporate and absorb excess fluid. Wells of 6 mm in diameter, about 2 cm apart and 5 mm deep were punched in the MHA with the help of a sterile cork-borer. Then, 100 µl of the extracts were placed into each well. The setup was then allowed to stabilize for 3 h before being incubated as described previously by Patel et al. (2011). Inoculated plates were incubated at 37 °C over night. After a 24-h incubation period, the mean inhibition zones were thereafter measured in mm, for the entire individual test organism. Distilled water and vancomycine (30 µg) were used as negative and positive control, respectively (Patel et al. 2011). Each test experiment was carried out in triplicates and the interpretation of antibacterial characteristics was conducted according to Alsaied et al. (2010). Inhibition zones less than 10 mm was considered as weak activity, from 10 to 15 mm as moderate activity and greater than 15 mm as strong activity following the methods of (Alsaied et al. 2010).

Determination of antimicrobial activity of LAB in broth culture during fermentation and storage of *metata ayib*

Metata ayib associated LAB isolates were separately grown in test tubes containing 10 ml MRS broth (OXOID) without

agitation. Sterilized MRS broth without inoculation of any organism was used as a control. The antimicrobial activities of the isolated LAB (cell free filtrate) against *E. coli* (ATCC25922), *S. aureus* (ATCC 25923), *S. flexneri* (ATCC 12022), and *S. pneumoniae* (ATCC49619) were evaluated using the well diffusion assay method (Hemashenpagam and Saranya 2011). In brief, pathogenic test bacteria were incubated in Nutrient broth at room temperature for 24 h. Plates consisting of 20 ml of MHA were prepared and inoculated with 0.1 ml of 24 h broth culture of test pathogenic bacteria. Once MHA solidified, plates were placed for 2 h in a refrigerator. Then wells were made using the same procedure as mentioned in the text above and filled with 100 μ l of cell-free supernatant or filtrate. The inoculated plates were subsequently incubated for 24 at 37 °C h. The antimicrobial activity of filtrate was evaluated by measuring the clear zone formed around the wells in plate.

Risk assessment of pathogenic bacteria in metata ayib

Fifty gram of *metata ayib* was added with 100 ml distilled H₂O and mixed with a vortex mixer. Ten ml of the mixture was taken to the test tubes and autoclaved. On the autoclaved sample, refreshed loop full of standard bacteria (*S. flexneri* (ATCC 12022), *E. coli* (ATCC25922), *S. pneumoniae* (ATCC49619) and *S. aureus* (ATCC25923)) were inoculated and incubated at 37 °C and stayed for 48 h. After 48 h incubation, a loop full was taken and stricken on MHA and incubated at 37 °C for 24 h.

Statistical analysis

All statistical analyses were conducted using SPSS software version16.0. All the experiments were conducted in triplicate and the data were analyzed and compared statistically using ANOVA at 95 % level of significance. A probability value at $p \leq 0.05$ was considered statistically significant. Data are presented as mean values $\pm$ standard deviation calculated from triplicate determinations.

Results and discussion

The antimicrobial activities of spices in *metata ayib* fermentation

In Ethiopia, different spices are traditionally used to enhance flavor, color and keeping quality of milk and milk products Eyassu (2013). Spices were widely used in different parts of the country during the production of traditional cottage cheese *ayib* (Eyassu 2013). Spices have innate antimicrobial substances and can be applied for preservation of foods (Eyassu 2013).

Ethanol, acetone and n-hexane extracts of six spices have been evaluated for their antimicrobial activities against four commonly prevalent pathogenic gram negative and gram positive bacteria such as *E. coli* (ATCC25922), *S. aureus* (ATCC25923), *S. flexneri* (ATCC 12022) and *S. pneumoniae* (ATCC49619).

Among the six spices evaluated for antimicrobial activity, garlic was found to be the most effective one against all the tested strains except *S. flexneri*. Contrary to the findings of some previous studies (Silva et al. 2011; Sofia et al. 2007), in this study, it has been observed that the two spice extracts, namely *C. sativum* and *B. nigra*, had no antimicrobial effect against test pathogens. Nonetheless, they might still be useful as flavor enhancers, anti-oxidant and coloring agents. The data also revealed that garlic extracts obtained using different solvents show difference in their antimicrobial activities against the selected bacterial species. Among different solvent extractions used, garlic acetone extract exhibited highest antibacterial activity (ranged from 14.00 ± 2.64 to 20.33 ± 0.58 mm) against all tested bacteria followed by hexane extract of garlic showing its highest antibacterial activity against *S. aureus* with an inhibition zone of 19.33 ± 2.08 mm (Table 2). In this study, the acetone extract of garlic bulb inhibition zone against *E. coli* (ATCC25922) was similar to the result reported by Irorere and Igeleke (2012). In contrast, the ethanol extract garlic bulb inhibition zone against *E. coli* (ATCC25922) was lower than the work reported by Irorere and Igeleke (2012). Garlic ethanol extract was least effective against all tested bacteria. The maximum antibacterial activity was observed in garlic acetone extract against *E. coli* (20.33 ± 0.58 mm) and minimum activity against *S. pneumoniae* (8.67 ± 0.58 mm) using ethanol as a solvent. It is clear that in our study, *E. coli* (ATCC25922) was more sensitive than other bacterial pathogens. The results were in agreement with observations of previous researchers (Joe et al. 2009). The inhibition zones of garlic acetone extract against *E. coli* (ATCC25922) was presented in Fig. 1. Inhibition zones of garlic extract against all tested pathogens was significantly ($P \leq 0.05$) greater than the remaining plant extract. The diameters of inhibition zone against all pathogens ranged from 8.67 ± 0.58 to 20.33 ± 0.58 mm for garlic extract as compared with 16.00 ± 1.00 to 17.00 ± 1.00 mm for vancomycin (Table 2). There was a significant ($P \leq 0.05$) difference in antimicrobial activity of garlic extracts among solvents. In this study, there was no significant ($p \geq 0.05$) difference between solvent extracts against *S. flexneri* (ATCC 12022). There was no significant ($p \geq 0.05$) difference in antimicrobial activity of garlic hexane extract (19.33 ± 2.08 mm) and vancomycin (17.00 ± 1.00 mm) against *S. aureus* (ATCC25923) (Table 2).

The antibacterial activity of garlic is accounted for the action of diallyl thiosulphinic acid or diallyl disulphate or allicin (Avato et al. 2002). Allicin was recognized as the oxygenated sulfur compound, thio-2-propene-1-sulfinic

Table 2 Determination of antibacterial activity of garlic against pathogens using an agar well diffusion method

Test micro organisms	Solvents used for extraction	Mean inhibition zone (mm) $\pm$ standard deviation		
		Crude extracts	Positive control VA	Negative control Pure water
<i>S. aureus</i> (ATCC25923)	A	14.67 $\pm$ 2.52 ^a	17.00 $\pm$ 1.00 ^b	0
	H	19.33 $\pm$ 2.08 ^b	17.00 $\pm$ 1.00 ^b	0
	E	13.66 $\pm$ 1.53 ^a	17.00 $\pm$ 1.00 ^b	0
<i>S. pneumoniae</i> (ATCC49619)	A	19.33 $\pm$ 3.21 ^c	16.00 $\pm$ 1.00 ^{bc}	0
	H	13.00 $\pm$ 1.00 ^b	16.00 $\pm$ 1.00 ^{bc}	0
	E	8.67 $\pm$ 0.58 ^a	16.00 $\pm$ 1.00 ^{bc}	0
<i>S. flexneri</i> (ATCC 12022)	A	14.00 $\pm$ 2.64 ^a	17.00 $\pm$ 1.00 ^b	0
	H	15.33 $\pm$ 3.01 ^a	17.00 $\pm$ 1.00 ^b	0
	E	13.07 $\pm$ 1.01 ^a	17.00 $\pm$ 1.00 ^b	0
<i>E. coli</i> (ATCC25922)	A	20.33 $\pm$ 0.58 ^c	17.00 $\pm$ 1.00 ^{bc}	0
	H	15.33 $\pm$ 3.01 ^{ab}	17.00 $\pm$ 1.00 ^{bc}	0
	E	12.06 $\pm$ 1.10 ^a	17.00 $\pm$ 1.00 ^{bc}	0

Where: mean $\pm$ standard deviation in triplicate

VA Vancomycine, A Acetone, E Ethanol, H Hexane. Values are means of triplicate determination values within the same row and column followed by different superscripts are statistically different ($p \leq 0.05$) against each tested microorganisms

acid S-allyl ester, which is commonly considered as allicin (Berhanu 2012). In brief, allicin hinders with lipid synthesis and RNA production. If RNA might not be produced at all or produced insufficient amount, then protein synthesis might be severely affected. In short, there is no protein synthesis takes place without the action of rRNA, mRNA and tRNA. Moreover, allicin affects lipid production and as the result phospholipids layer of the cell wall cannot be synthesized correctly in both Gram negative and Gram positive bacteria. The cumulative effects of all these things contribute great role to impose on the growth of bacteria in the presence of allicin (Berhanu 2012). According to Khan et al. (2012), it has been found that garlic can be used as a potential inhibitor of food spoilages and pathogens. As the result, it would increase the shelf life of processed foods. In this finding, garlic might be equally contributed to preserve and extend the shelf life *metata ayib* along with LAB.

Table 3 represents the results of inhibition zone of antibacterial activity of the ginger extracts. The study shows

Fig. 1 Inhibition zones of garlic acetone extract against *E. coli* (ATCC25922)

that the capacity of inhibition differed with the type of solvent used for extraction and the tested pathogens. The results showed that the extracts have antibacterial activity against all tested pathogens. Inhibition zones (12.50 $\pm$ 1.00 to 15.50 $\pm$ 1.00 mm) of ginger ethanol extract against all tested pathogens was significantly ($P \leq 0.05$) greater than the remaining solvent extracts. This might be due to that the bioactive compounds were polar and readily extracted by organic solvents such as ethanol. This observation clearly indicates that the polarity of antibacterial compounds make them more readily extracted by polar solvents, and using such polar solvents does not negatively affect their bioactivity against bacterial species. The diameter zone of inhibition of ginger extract prepared in ethanol was in the range of 12.50 $\pm$ 1.00 and 15.50 $\pm$ 1.00 mm. Ginger has a high antimicrobial activity (15.50 $\pm$ 1.00 mm) against *S. aureus* (ATCC25923) extracted with ethanol (Fig. 2). In this study, Gram-negative bacteria were found to be the least sensitive as compared to Gram-positive bacteria against ginger extract. *S. aureus* (ATCC25923) was the most susceptible among all the bacterial species in ginger ethanol extract. Ginger ethanol extracts had a very little effect on Gram-negative bacteria specifically against *E. coli* (ATCC25922). This might be due to the differences in chemical composition and structure of cell wall of both types of microorganisms. This result was in line with other studies which have reported about ginger for its comparatively broad antibacterial activity against test bacterial pathogens (Supreetha et al. 2011).

The diameter zone of inhibition of vancomycine against all tested pathogens was significantly ($p \leq 0.05$) greater than ginger extract using all solvents. The diameter of zone of inhibition for vancomycine was ranging from 18.66 $\pm$ 0.57 to 19.33

Table 3 Determination of antibacterial activity of ginger against bacterial pathogens using an agar well diffusion method

Test micro organisms	Solvents used for extraction	Mean inhibition zone (mm) $\pm$ standard deviation		
		Crude extracts	Positive control VA	Negative control Pure water
<i>S. aureus</i> (ATCC25923)	A	9.33 $\pm$ 1.25 ^a	19.33 $\pm$ 1.25 ^c	0
	H	9.00 $\pm$ 1.80 ^a	19.33 $\pm$ 1.25 ^c	0
	E	15.50 $\pm$ 1.00 ^b	19.33 $\pm$ 1.25 ^c	0
<i>S. pneumoniae</i> (ATCC49619)	A	10.83 $\pm$ 2.08 ^a	18.83 $\pm$.76 ^c	0
	H	9.50 $\pm$ 1.00 ^a	18.83 $\pm$.76 ^c	0
	E	14.50 $\pm$ 1.00 ^b	18.83 $\pm$ 0.76 ^c	0
<i>S. flexneri</i> (ATCC 12022)	A	8.66 $\pm$ 1.25 ^a	18.66 $\pm$ 0.57 ^c	0
	H	9.00 $\pm$ 1.00 ^a	18.66 $\pm$ 0.57 ^c	0
	E	13.33 $\pm$ 0.76 ^b	18.66 $\pm$ 0.57 ^c	0
<i>E. coli</i> (ATCC25922)	A	10.00 $\pm$ 1.00 ^a	19.00 $\pm$ 1.32 ^c	0
	H	9.00 $\pm$ 1.00 ^a	19.00 $\pm$ 1.32 ^c	0
	E	12.50 $\pm$ 1.00 ^{bc}	19.00 $\pm$ 1.32 ^c	0

Where: mean $\pm$ Standard deviation in triplicate

VA Vancomycine, A Acetone, E Ethanol, H Hexane. Values are means of triplicate determination values within the same row and column followed by different superscripts are statistically different ($p \leq 0.05$) against each tested microorganisms

± 1.25 mm), as compared with 8.66 ± 1.25 to 15.50 ± 1.00 mm for ginger extract. This result was in line with the findings of Joe et al. (2009) (Table 3).

The antibacterial activity of ginger extracts could be attributed to the chemical properties of ginger, the fact that it consists of antimicrobial agents such as zingiberine, bisabolene and zingiberol (Michael derrida 1999). The rhizome of ginger composes of pungent vanillyl ketones comprise of paradole and, gingerol etc. (Joe et al. 2009). Gingerol is a mixture of crystal gingerone that mainly cause the of ginger to be acidic naturally and plays a great role in inhibiting bacteria such as *Trichomonas vaginalis*, *S. aureus* and help to cure bacterial vaginosis and skin diseases (Derrida 1999). The terpenoids are one of significant substances in pharmacy due to their connection with such compounds as vitamin A and have wide range of application in medicen. The gingerols could make ginger available for treatment of stomach acidity and may have analgesic and sedative properties (Malu et al. 2009).

In the various studies, the therapeutic capacity of ginger has been reported which includes antipyretic, anti-

ulcer, antiplatelet, anti-emetic activity, antioxidant and anti-inflammatory activity. The antibacterial and antifungal activity of ginger has been associated with shagelol and gingerol obtained from ginger extracts using ethanol as solvent (Supreetha et al. 2011).

The antimicrobial activities of *Osmium basilicum* (ethanol, acetone, and hexane) extracts against the test pathogens examined in this study and their antimicrobial potency were determined by the evaluation and measurement of inhibition zones diameter (Table 4). The ethanol extract has strongest and broadest spectrum of antimicrobial activities in comparison with acetone and hexane extracts. In this study, the inhibition zone of *O. basilicum* ethanol extract against to all pathogenic bacteria was significantly ($p \leq 0.05$) greater than inhibition zone of hexane and acetone extracts. Inhibition zone of vancomycine against all tested pathogenic bacteria was significantly ($p \leq 0.05$) greater than to inhibition zone of basil extracts. The diameter zone of inhibition for *O. basilicum* extracts ranges from 7.33 ± 0.57 to 16.33 ± 1.15 mm, as compared with 17.66 ± 0.57 to 18.66 ± 1.25 mm for vancomycine. It has been documented that the extract demonstrated maximum inhibitory activity against *E. coli* (ATCC25922) with inhibition zone of 16.33 ± 1.15 mm, while it showed moderate activity against *S. aureus* (ATCC25923), *S. pneumoniae* (ATCC49619) and *S. flexneri* (ATCC 12022).

Methanol, acetone, and chloroform extracts from *O. basilicum* were examined for their in vitro antimicrobial properties by Kaya et al. (2008). They reported that that the methanol extracts of *O. basilicum* showed the antimicrobial activity against all tested pathogens. Though the acetone and chloroform extracts had no effect,

Fig. 2 Inhibition zones of ginger ethanol extract against *S. aureus* (ATCC25923)

Table 4 Inhibition zone of bacterial growth by *O. basilicum* using an agar well diffusion method

Test micro organisms	Solvents used for extraction	Mean inhibition zone (mm) $\pm$ standard deviation		
		Crude extracts	Positive control VA	Negative control Pure water
<i>S. aureus</i> (ATCC25923)	A	9.16 $\pm$ 1.25 ^a	17.66 $\pm$ 0.57 ^c	0
	H	7.50 $\pm$ 0.50 ^a	17.66 $\pm$ 0.57 ^c	0
	E	13.00 $\pm$ 1.00 ^b	17.66 $\pm$ 0.57 ^c	0
<i>S. pneumoniae</i> (ATCC49619)	A	8.83 $\pm$ 1.04 ^a	18.33 $\pm$ 1.04 ^c	0
	H	7.33 $\pm$ 0.57 ^a	18.33 $\pm$ 1.04 ^c	0
	E	11.50 $\pm$ 1.00 ^b	18.33 $\pm$ 1.04 ^c	0
<i>S. flexneri</i> (ATCC 12022)	A	9.33 $\pm$ 1.52 ^a	18.66 $\pm$ 1.25 ^c	0
	H	8.16 $\pm$ 0.76 ^a	18.66 $\pm$ 1.25 ^c	0
	E	12.33 $\pm$ 1.04 ^b	18.66 $\pm$ 1.25 ^c	0
<i>E. coli</i> (ATCC25922)	A	14.00 $\pm$ 1.00 ^a	18.16 $\pm$ 0.28 ^c	0
	H	13.00 $\pm$ 1.00 ^a	18.16 $\pm$ 0.28 ^c	0
	E	16.33 $\pm$ 1.15 ^b	18.16 $\pm$ 0.28 ^c	0

Where: mean $\pm$ Standard deviation in triplicate

VA Vancomycine, A Acetone, E Ethanol, H Hexane. Values are means of triplicate determination values within the same row and column followed by different superscripts are statistically different ($p \leq 0.05$) against each tested microorganisms

the methanol crude extracts was shown inhibition zones to strains of *Shigella* sp., *P. aeruginosa*, *S. aureus*, *L. monocytogenes*, and two different strains of *E. coli*. In this study, ethanol extracts have shown the most effective effect in comparison with other extracts tested to test pathogenic bacteria. This investigation was in line with the report of Kaya et al. (2008).

Table 5 presents the results of inhibition zone of antibacterial activity of the *Ruta graveolens* crude seed extracts tested against pathogenic bacteria. Ethanolic extract of *R. graveolens* seed was shown most potent antibacterial activity against *S. aureus* (ATCC25923) and *S. pneumoniae* (ATCC49619) (Fig. 3). Their zones of

inhibition were 21.33 $\pm$ 1.15 and 15.50 $\pm$ 2.00 mm, respectively. While *E. coli* (ATCC25922) zone of inhibition was 11.00 $\pm$ 1.00 mm, which was the least sensitive bacteria strain in this study.

The Gram negative bacterium such as *E. coli* (ATCC25922) was comparatively less sensitive than Gram positive bacteria; this might be due to the structural differences in the outer cell membrane of Gram negative bacteria, which further block the penetration of antibiotics including the extracts of spices making them less sensitive or due to polarity difference between the extract and porine nature of lipopolysaccharide (outer membrane). This result was similar or in line with the findings of Pandey et al. (2012). They reported that,

Table 5 Antibacterial activity of the *R. graveolens* crude seed extracts using the agar well diffusion method

Test micro organisms	Solvents used for extraction	Mean inhibition zone (mm) $\pm$ standard deviation		
		Crude extracts	Positive control VA	Negative control Pure water
<i>S. aureus</i> (ATCC25923)	A	17.66 $\pm$ 0.57 ^b	20.16 $\pm$ 0.76 ^c	0
	H	17.00 $\pm$ 0.86 ^b	20.16 $\pm$ 0.76 ^c	0
	E	21.33 $\pm$ 1.15 ^c	20.16 $\pm$ 0.76 ^c	0
<i>S. pneumoniae</i> (ATCC49619)	A	9.83 $\pm$ 2.08 ^a	19.33 $\pm$ 0.76 ^c	0
	H	11.00 $\pm$ 1.00 ^a	19.33 $\pm$ 0.76 ^c	0
	E	15.50 $\pm$ 2.00 ^b	19.33 $\pm$ 0.76 ^c	0
<i>S. flexneri</i> (ATCC 12022)	A	11.16 $\pm$ 1.25 ^{ab}	16.50 $\pm$ 0.70 ^c	0
	H	8.00 $\pm$ 1.00 ^a	16.50 $\pm$ 0.70 ^c	0
	E	13.00 $\pm$ 1.00 ^b	16.50 $\pm$ 0.70 ^c	0
<i>E. coli</i> (ATCC25922)	A	9.50 $\pm$ 1.32 ^a	16.16 $\pm$ 1.25 ^b	0
	H	9.33 $\pm$ 1.52 ^a	16.16 $\pm$ 1.25 ^b	0
	E	11.00 $\pm$ 1.00 ^a	16.16 $\pm$ 1.25 ^b	0

Where: mean $\pm$ Standard deviation in triplicate

VA Vancomycin, A Acetone, E Ethanol, H Hexane

Values are means of triplicate determination values within the same row and column followed by different superscripts are statistically different ($p \leq 0.05$) against each tested microorganisms

Fig. 3 Inhibition zones of *R. graveolens* ethanol extract against *S. aureus* (ATCC25923)

the ethanolic extract showed most susceptible activity against *S. aureus* (22.0 ± 0.04 mm) and *B. subtilis* (19.4 ± 0.24 mm), where as *P. aeruginosa* zone of inhibition (12.5 ± 0.17 mm) was the least sensitive bacteria at a concentration of 100 µg/ml. In this study there was no significant difference ($p \geq 0.05$) between vancomycine and ethanolic extracts of *R. graveolens* against *S. aureus* (ATCC25923). Inhibition zone of vancomycine against all tested pathogenic bacteria was significantly ($p \leq 0.05$) greater than inhibition zone of crude extracts except *S. aureus* (ATCC25923) (Fig. 3). There was no significant difference ($p \geq 0.05$) between solvent extracts of *R. graveolens* against *E. coli* (ATCC25922). In this study, ethanolic extracts of *R. graveolens* has shown the highest antibacterial activity than the remaining solvents. The reason might be due to the antimicrobial component of

Table 7 Antimicrobial activities of isolates of LAB obtained from *metata ayib* samples against test microorganisms

Pathogens	Cell free filtrates (mean zone of inhibition in mm)			
	<i>Lactobacillus</i>	<i>Pediococcus</i>	<i>Weissella</i>	<i>Lactococcus</i>
<i>S. aureus</i> (ATCC25923)	14	15	12	9
<i>S. pneumoniae</i> (ATCC49619)	13	11	14	7
<i>S. flexneri</i> (ATCC 12022)	18	14	10	6
<i>E. coli</i> (ATCC25922)	8	7	5	5

R. graveolens was more soluble in ethanol as compared to other solvents due to its degree of polarity.

Natural chemical compounds originated from plants are generally believed to be more acceptable and less hazardous than chemically synthesized compounds and have huge potential for the application of food preservation and disease control agent. Understanding of plant biochemistry, physiology and chemistry of natural products have shown that the secondary metabolites may be used to control infectious organisms to overcome the problems associated with synthetic chemicals and chemical preservatives (Ahmad et al. 2011). One of such method involves the use of plant derived products such as plant essential oils and other components that has antimicrobial effect. Many plants have antimicrobial effects because of their secondary metabolites. Such secondary metabolite products are well known active substance, for instance, due to the presences of phenolic compounds which are part of the tannin as well as essential oil (Jansen et al. 1987).

The antimicrobial activities of *R. graveolens* crude seed extract have been linked to the presence of bioactive compounds. Some of the phytochemical substances are saponin, glycoside, flavonoids, tannin, alkaloids and terpenoid have different degree of antimicrobial activity (Pandey et al. 2011). The crude extract of this plant also consists of phenolics and alkaloidal chemical substances which also have biological activities (Pandey et al. 2012). The presence of

Table 6 Identification of LAB from *metata ayib* fermentation product

Percentage of isolates with positive reactions				
Isolates of LAB	A	B	C	D
No. of isolates	3	8	4	16
Cell morphology	R	C	1R/3C	R
Gram test	+	+	+	+
Catalase	–	–	–	–
CO ₂ from glucose	75	0	0	0
Growth at 4 °C	100	75	75	50
10 °C	100	100	100	100
15 °C	100	100	100	100
37 °C	100	75	100	100
45 °C	0	100	50	50
Growth at 4 % NaCl	100	100	100	100
6 % NaCl	100	100	100	100
10 % NaCl	0	25	0	0
Growth at pH 3.9	100	100	50	100
Growth at pH 4.4	66	100	25	75
Growth at pH 9.6	0	25	25	25

Genus A = *Lactobacillus*, Genus B = *Lactococcus*, Genus C = *Pediococcus* and Genus D = *Weissella*, R = rod shape, C = cocci (spherical shape)

Table 8 Risk assessment of pathogenic bacteria contamination possibility of *metata ayib*

Standard pathogens	<i>Metata ayib</i>	<i>Ayib</i> (control)
<i>S. pneumoniae</i> (ATCC14619)	–	–
<i>E. coli</i> (ATCC25922)	+	+
<i>S. flexneri</i> (ATCC 12022)	–	–
<i>S. aureus</i> (ATCC 25923)	–	+

+ grow well, – not grow

flavonoids, tannins and alkaloids in the extract might be in charge for activity to pathogenic microorganisms.

Antimicrobial activity of lactic acid bacteria isolated from *Metata ayib*

Identification of LAB from fermented *metata ayib* was carried out using standard methods (Table 6). All colony isolates isolated from *metata ayib* samples were found to be Gram-positive and none motile. Moreover, none of the isolates were shown catalase activity. Morphologically, the cells of isolates were coccous type arranged either in pairs or tetrad and rod shaped. A total of 31 isolates were belonging to four LAB genera namely *Lactobacillus*, *Pediococcus*, *Lactococcus* and *Weissella*.

Antimicrobial activity of isolates of LAB obtained from *metata ayib* sample against test pathogenic bacteria was shown on Table 7. *Lactobacillus* isolates was shown higher antimicrobial activity against all test pathogens than the other LAB isolates. *Shigella flexneri* (ATCC 12022) was the most sensitive (18 mm) to *Lactobacillus* isolates extract followed by *S. aureus* (ATCC25923) (14 mm). *Pediococcus* isolates extract was shown a relatively larger antimicrobial activity on the test strains next to *Lactobacillus* isolates (15 mm). *Staphylococcus aureus* (ATCC25923) was the most sensitive to extracts of *Pediococcus* isolates. Among the test strains, the most sensitive were *S. flexneri* (ATCC 12022) for extract of *Lactobacillus* isolates, *S. pneumoniae* (ATCC49619) for extracts of *Weissella* isolates and *S. aureus* (ATCC25923) for extract of *Pediococcus* isolates. In this study, *Lactococcus* isolates were shown the least antimicrobial activity against all the test strains. *Escherichia coli* was the least sensitive in all cases. This result was in line with the findings of Hemashenpagam and Saranya (2011). The possible reason of LAB isolates, which capable of inhibiting the growth of pathogenic bacteria, was may be due to production of lactic acid, acetic acid and secondary metabolites like bacteriocins.

Risk assessment of pathogenic bacteria in *metata ayib*

The possibility of pathogenic bacteria contamination of *metata ayib* was determined by deliberately inoculating the standard pathogenic bacteria into autoclaved *metata ayib* (Table 8). The results indicate that *E. coli* (ATCC25922) was the only organism that grows in autoclaved *metata ayib*. The remaining test organisms were not grown in *metata ayib*. In contrast, *E. coli* (ATCC25922) and *S. aureus* (ATCC25923) were grown in the control (*ayib*). This result showed that the growth of most of the pathogenic bacteria might be prevented by the spices that were added during *metata ayib* preparation. Although spices in *metata ayib* have such huge significant role to suppress the growth of food spoilage and pathogenic organisms, aseptic processing of traditional cottage cheese

(*ayib*) is needed to minimize risks associated during consumption of *metata ayib*.

Conclusions

In tropical countries like Ethiopia, dairy products are responsible for many outbreaks of gastrointestinal infections. Dairy products produced under unhygienic environment create a great threat to the health of consumers. Despite the fact that *metata ayib* is produced under non- hygienic environment with a high possibility of contamination with desirable, pathogenic, and/or spoilage bacteria, the combined effect of spices which were incorporated during traditional *metata ayib* preparation and the LAB antimicrobial activity can reduce the risk of spoilage and pathogenesis.

In general, the finding of the present study showed that ginger and garlic extracts had exhibited wide spectrum of antimicrobial properties. Therefore, in this study it is found that spices decrease and hinder the growth of food spoilage and food borne pathogens. The use of spices would decrease the chances of food poisoning, food spoilage and increase the food shelf life. Our result also indicated that the culture filtrates of lactic acid bacteria isolated from “*metata ayib*” exhibited antimicrobial activity against four pathogenic test strains. LAB isolates have a strong antimicrobial activity against pathogenic and food spoilage microorganisms and play a vital role in the preservation of *metata ayib*.

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