MICROBIAL DYNAMICS DURING THE FERMENTATION OF *WAKALIM*, A TRADITIONAL ETHIOPIAN FERMENTED SAUSAGE

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ABSTRACT

Wakalim *is a spiced traditional Ethiopian fermented beef sausage. Early stages (0–12 h) of* wakalim *fermentation were dominated by lactic acid bacteria and aerobic mesophilic bacteria including* staphylococci *and members of Enterobacteriaceae. Gram-negative bacteria were below detectable level after day 4 of fermentation.* Staphylococci *were detected at low levels (around 4 log cfu/g) until the end of fermentation. Lactic acid bacteria grew and dominated the flora at the end of fermentation. Various species of* Lactobacillus *and* Pediococcus *initiated the fermentation and the lactic flora was finally dominated by* Lb. plantarum1 *and* Ped. pentosaceus1. *The pH of the fermenting* wakalim *dropped from 5.5 ± 0.22 to 4.1 ± 0.19 while the titratable acidity increased from 0.09 to 0.6% in the course of fermentation. Moreover, moisture content of the fermenting* wakalim *dropped from 66.5% ± 2.12 to 22.0% ± 0.71 during the 6 days of fermentation. Molecular characterization, using 16S rDNA partial sequence analysis and repetitive sequence-based polymerase chain reaction, of the isolates confirmed some of the earlier phenotypic identification made based on API carbohydrate fermentation profile. Some of the strains were, however, identified as different species of the same genus or entirely different genus. The findings of this study are the first*

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of their kind from traditional Ethiopian fermented sausage and could have paramount importance as a baseline data for large-scale production of the traditional product using defined starter cultures. Moreover, the strains characterized in this study could be exploited as potential starter cultures for the commercialization of wakalim.

PRACTICAL APPLICATIONS

Traditional fermented foods and beverages are products of spontaneous fermentation. This type of process results in microbiological and chemical variability in the products, as it depends on the microflora naturally present in the substrates, on utensils and equipment used. Isolation and characterization of the microorganisms involved in the fermentation, and identification of the most important starter species may help to undertake controlled fermentation with selected mixed culture starters to optimize the process conditions. This would result in products which are consistent and definable in their flavor and other biochemical parameters, have good keeping quality and are, in general, wholesome. This may pave the way for large-scale commercial production, which may also improve the shelf life of the products, and reduce wastage during processing, which is significant at household level. The strains characterized in this study could be exploited as potential starter cultures for the commercialization of *wakalim*.

INTRODUCTION

Sausage fermentation is one of the oldest technologies used to preserve food for long periods, and it is a process in which microbes, meat and technology converge. Fermented sausages are essential parts of diets in many regions of the world. *Wakalim* is a spiced traditional Ethiopian fermented beef sausage. Traditionally, its preparation relies on spontaneous fermentation with ingredients as the chief source of inocula. Lean meat, fat, salt, garlic, onion and other spices account for a proportion of 70, 5, 2, 1, 17 and 5%, respectively. Its preparation is a four-step process that includes the preparation of a casing (locally called *merechi*), mincing of meat, stuffing and fermentation.

Sausage fermentations are characterized by the succession of microbial groups in the course of fermentation. Although several groups are involved in the initiation of fermentation, only those tolerant to acids and metabolites generated during fermentation survive and dominate the final microflora. In naturally fermenting beef sausage, raw meat yields lactic acid bacteria (LAB)

in low numbers. However, the lactic flora rapidly dominates the fermentation because of the anaerobic environment generated during fermentation (Hammes and Knauf 1994).

Many of the LAB isolates from other fermented sausages have been identified as *Lb. plantarum*, *Lb. sakei* and *Lb. curvatus* (Hugas *et al*. 1993; Lucke 1998). There is no scientific report on microbial dynamics and characterization of strains of LAB isolated during *wakalim* fermentation. The aim of this study was therefore to assess the microbial dynamics and the associated chemical changes during *wakalim* fermentation under laboratory conditions.

MATERIALS AND METHODS

Laboratory Preparation of *Wakalim*

Wakalim was prepared using the following ingredients (g/kg) following traditional techniques: lean meat (700 g), mixed with fat (50 g), salt (20 g), onion (*Allium ascalonicum*) (160 g), red pepper (*Capsicum annuum*) (20 g) and 10 g each of Ethiopian cardamom (*Aframomum corrorima*), black cumin (*Nigella sativa*), Kemun (*Trachyspermum capticum*), Ethiopian mustard (*Brassica nigra*) and garlic (*Allium sativum*). The ingredients were mixed in a container of 5-kg capacity. About 200–250 g of the meat–spice mix was stuffed manually into a prewashed and dried animal casing cut in to 20-cm length, and allowed to ferment at ambient temperature (20–25C) for 6 days. Meat was obtained from butchers in Addis Ababa. Spices were purchased from open market in the town of Harar and Addis Ababa. Intestine for the making of casing was obtained from Abattoir in Addis Ababa.

Microbiological Analysis

Fermenting *wakalim* was surface sterilized by swabbing casing with cotton dipped in 70% ethyl alcohol. Samples (25 g) were removed aseptically and homogenized in 225 mL of sterile peptone-water (0.1%) for 2 min using a Stomacher lab blender (Stomacher 400, Seward, London, UK). Samples were drawn at 12-h intervals for the first 48 h and 24-h intervals for the remaining 5 days. A total of three batches of laboratory-prepared *wakalim* processed on different days were analyzed. Ingredients (10 g) were separately mixed with 90-mL of sterile peptone water (0.1%) and homogenized for microbiological analysis. Appropriate dilution (0.1 mL) of homogenized samples were surface plated in duplicates on predried surfaces of the following media (all from Oxoid, Basingstoke, Hampshire, U.K.) for microbial enumeration: (PC) agar for aerobic mesophilic bacteria (AMB), violet red bile (VRB) agar for coliforms, VRB glucose agar for members of Enterobacteriaceae and Mannitol

Salt agar for *staphylococci*. The plates were incubated under aerobic conditions for 1–2 days at 30–32C. Similarly, MRS agar was plated and incubated under anaerobic condition, using anaerobic jar (BBL, GasPak Anaerobic Systems, Cockeysville, MD) for 2–3 days at 30–32C for counting of LAB. Chloramphenicol bromophenol blue (CBB) agar was similarly plated and incubated at 25–28C for 3–4 days for yeasts and mold count (CBB consisted of: yeast extract, 6.0 g; glucose, 20.0 g; chloramphenicol, 0.1 g; bromophenol blue, 0.01 g; agar, 15 g; distilled water, 1,000 mL; pH, 6.0–6.4). Aerobic spore counting was done on (PC) agar after appropriate dilutions were heat-treated at 80C for 10 min in water bath.

After colony counting, 10–15 colonies were randomly picked from countable plates of PC and MRS agar plates for further identification. Colonies of LAB were transferred into 5-mL MRS broth (Oxoid) and purified by repeated streaking on MRS agar. AMB were similarly transferred into Nutrient broth (Oxoid) for repeated purification on Nutrient agar (Oxoid). The pure cultures of LAB and AMB were stored at 4C on agar slants for further characterization. Part of the pure cultures of LAB were preserved in 30% glycerol and maintained in deep freeze at −70C for further use.

Biochemical Characterization of Isolates. Isolates were microscopically characterized by cell shape, cell grouping, motility, and presence or absence of endospores. Gram reaction of isolates was tested by the KOH test (Gregerson 1978). Production of the enzyme oxidase was tested according to Kovacs (1956) and formation of catalase was determined by flooding young colonies with 3% solution of H_2O_2. Oxidative or fermentative utilization of glucose by each isolate was assessed by the O/*F*-test (Hugh and Leifson 1953). The testing medium consisted of (g/L): peptone, 2 g; yeast extract, 1 g; NaCl, 5 g; K_2HPO_4, 0.2 g; glucose, 10 g; bromophenol blue, 0.08 g; agar, 2.5 g; distilled water, 1,000 mL, pH, 7.1.

Purified isolates from MRS agar plates which were Gram-positive, nonsporing cocci or rods, and which did not produce the enzyme catalase were considered as LAB and were subjected to biochemical tests using API 50CH (Biomeriuex, Marcy I'Etoile, France). Gram-negative, nonsporing rods, and did not produce an enzyme oxidase were considered as members of the family Enterobacteriaceae and further characterized biochemically using API 20E (Biomeriuex, Marcy I'Etoile, France). The biochemical profiles of isolates were analyzed to species and subspecies level using identification software (API WEB, V1.1.0, Biomeriuex, Marcy I'Etoile, France).

Chemical Analysis

The pH of samples was measured using digital pH meter (Nig 333, Naina Solaris LTD, New Delhi, India) after homogenizing 10 g of *wakalim* in 90 mL

of distilled water. Titratable acidity (TA) was measured according to Antony and Chanrda (1997) and was expressed as g lactic acid/100 g of *wakalim*. Moisture content was determined by drying 10 g of sample to constant weight at 30–32C. The difference in weight between the initial and final reading was considered as moisture content of the sample.

Numerical Analysis and Molecular Characterization of Isolates

The degree of similarities among strains of LAB isolated during *wakalim* fermentation was evaluated by cluster analysis using unweighted pair group method with arithmetic mean based on their carbohydrate fermentation profiles. A dendrogram was produced using NTSYSpc statistical software package (NTSYSpc version 2.1, Exeter Software, Setauket, NY).

Bacterial strains were subtyped using genomic fingerprinting methods. Repetitive sequence-based polymerase chain reaction (Rep-PCR) was performed using a 15-mer oligonucleotide (5′-GTGGTGGTGGTGGTG-3′) according to a program described by Versalovic *et al*. (1994). PCR amplifications (PCR [30 cycles of 94C for 30 s, 54C for 30 s, and 72C for 80 s], for denaturation of DNA strands, annealing of oligonucleotide primers and extension with polymerase, respectively) were performed with a DNA thermal cycler (Perkin Elmer 9600, Applied Biosystems, Foster City, CA). The PCR products were separated by electrophoresis at 2.5 V/cm in a 1% agarose gel in 1 × Tris-acetate-EDTA buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The gels were stained with ethidium bromide (0.2 μg/mL) for 5 min and digitized images were captured under ultraviolet transillumination. Band patterns were evaluated visually.

Species identification of strains was confirmed by 16S rDNA sequence analysis. The isolates were grown separately overnight on MRS agar at 37C under anaerobic condition (AneroGen, Oxoid). A sterile toothpick was used to transfer a small part of a single colony from the plates to the PCR reaction. A part of the 16S rDNA gene (1,411 bp) was amplified by PCR (30 cycles of 94C for 30 s, 54C for 30 s, and 72C for 80 s, for denaturation of DNA strands, annealing of oligonucleotide primers, and extension with polymerase, respectively) using the primers 16S.S (5′-AGAGTTTGATCCTGGCTC-3′) and 16S.R (5′-CGGGAACGTATTCACCG-3′). The resulting PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The purified fragments were partially sequenced (approximately 500 bp of the 5′-end of the gene comprising the variable regions V1-V3) according to standard methods using the 16S.S primer, Big Dye Terminator v3.1 sequence analyzer and the Cycle Sequencing Kit (Applied Biosystems). The sequences obtained were subjected to a BLAST search in the ribosomal database project (RDP-II) (Cole *et al*. 2007).

Data Analysis

The microbial contribution of meat and each spice ingredient was evaluated using mean separation tests (Tukey, LSD) of microbial counts of each ingredient at $P = 0.05$. Furthermore, the patterns of succession of the different microbial groups was presented by plotting mean counts during the course of fermentation. Data was analyzed using SPSS for Windows (version 10.0, SPSS Inc, Chicago, IL). Coefficient of variation was calculated to evaluate the significances of differences within samples of the same item.

RESULTS

Microbial Development during *Wakalim* Fermentation

The count of total AMB of the various ingredients ranged between 3.65 and 5.78 log cfu/g (Table 1). The aerobic mesophilic bacterial flora was dominated by aerobic spore formers (ASF). In meat and garlic, the aerobic flora also consisted of *staphylococci* and some members of the Enterobacteriaceae. However, the counts of most of the various microbial groups varied significantly (CV > 10%) within meat samples. Meat, onion *(A. ascalonicum)*, Ethiopian caraway (*Aframomum corrorima*) and, to a certain extent, garlic (*A. sativum*) were the major sources of LAB with counts >5 log cfu/g (Table 2). The LAB flora of meat consisted of *Lb. acidophilus*3, *Lactococcus lactis ssp. lactis*, *Leuconostoc lactis, Lb. delbruecki subsp delbrueckii, Lb. curvatus, Lb. cellobiosus* and *Pediococcus* species.

A total of 76 isolates, belonging to the family Enterobacteriaceae, were obtained mainly from meat and were grouped in to 12 genera and 20 species. The dominant strains belonged to *Klebsiella* species (data not given).

During fermentation, the counts of all the microbial groups increased until 12 h. Thereafter, the counts of LAB increased by more than 3 log units and reached numbers as high as 9.5 log cfu/g at the end of fermentation. Yeast count also increased after 48 h. Counts of AM**B** and ASF were around 7 log cfu/g and that of *staphylococci* were around 4 cfu/g at completion of fermentation. Molds were eliminated at 96 h and Enterobacteriaceae and coliforms were eliminated at 120 h (Fig. 1).

Although the counts of LAB, in general, increased only gradually after 12 h of *wakalim* fermentation, a certain succession within specific species of LAB was observed in the course of the fermentation. Many species of the genera *Lactobacillus* and *Pediococcus* took part in the initiation of *wakalim* fermentation along with *Lb. plantarum1* and *Ped. pentosaceus1* (Fig. 2). They all increased by at least 1 log unit until 12 h. Thereafter, only *Ped. pentosaceus1* and *Lb. plantarum1* among the identified isolates were able to increase

TABLE 1.
MICROBIAL LOAD (LOG CFU/G) OF INGREDIENTS USED FOR *WAKALIM* FERMENTATION

Ingredients	Microbial groups											
	Aerobic mesophilic bacteria			Aerobic spore formers			Enterobacteriacea			Coliforms		
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
Meat	5.69	0.79	14.00	1.70	0.40	24.00	3.11	0.81	26.00	2.89	0.83	27.00
Allium ascalonicum	5.78	1.29	22.00	2.23	1.15	2.00	3.98	0.83	21.00	3.45	0.21	6.00
Allium sativum	3.65	0.07	2.00	0.00	0.00	0.00	3.50	0.14	4.00	3.35	0.07	2.00
Trachyspermum capticum	4.18	0.67	16.00	3.56	0.13	4.0	3.51	1.46	42.00	3.35	1.48	44.00
Nigella sativa	5.13	0.07	2.00	4.48	0.07	2.00	4.55	0.49	10.00	4.23	0.33	8.00
Aframomum corrorima	4.84	0.74	15.00	4.29	0.18	4.00	1.74	1.46	84.00	1.74	1.16	66.67
Capsicum annuum	5.25	0.19	4.00	5.46	0.07	2.00	4.27	0.38	9.00	4.34	0.198	5.00

Ingredients	Microbial groups											
	Staphylococci			Lactic acid bacteria			Yeasts			Molds		
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
Meat	5.16	1.24	24.00	4.42	0.05	1.00	4.08	0.34	8.00	1.08	0.54	50.00
Allium ascalonicum	4.41	1.01	23.00	4.71	1.12	2.40	4.73	1.31	24.00	3.49	1.00	28.00
Allium sativum	3.74	0.07	2.00	3.86	0.09	2.00	3.87	0.10	3.00	2.69	0.13	5.00
Trachyspermum capticum	3.64	0.51	14.00	2.39	1.96	82.00	0.00	0.00	0.00	1.45	1.05	72.41
Nigella sativa	4.02	0.03	0.70	2.38	0.11	5.00	0.00	0.00	0.00	3.55	0.64	18.00
Aframomum corrorima	3.61	0.18	5.10	4.08	0.25	6.00	1.09	0.54	49.00	1.15	0.63	54.78
Capsicum annuum	3.31	0.45	9.00	1.5	0.71	47.33	3.49	0.69	19.00	3.76	0.76	20.00

TABLE 2.
FREQUENCY OF ISOLATION OF SPECIES OF LAB FROM DIFFERENT INGREDIENTS USED FOR *WAKALIM* FERMENTATION

Ingredients	No. of isolates of LAB	Strains of LAB isolated from the given ingredient	No. of a given strain of LAB	Proportion (%) of the strain
Lean meat	70	*Lb. acidophilus 3*	6	8.6
		Lact. Lactis spp lactis	14	20.0
		Leuc. lactis	4	5.7
		Lb. delbrueckii spp. delbrueckii	8	11.4
		Lb. curvatus	6	8.6
		Lb. cellobiosus	4	5.7
		Lactobacillus species	12	17.1
		Pediococcus species	16	22.9
Onion (*Allium ascalonicum*)	74	*Ped. pentosaceus 1*	30	41
		Pediococcus species	44	59
"Kemun" (*Trachyspermum capticum)*	81	*Lb. cellobiosus*	12	15
		Lactobacillus species	24	30
		Pediococcus species	45	55
"Ethiopian caraway" (*Aframomum korerima)*	68	*Lactobacillus species*	68	100

LAB, lactic acid bacteria; *Lb*, *Lactobacillus*; *Lact*, *Lactococcus*; *Leuc*, *Leuconostoc*; *Ped*, *Pediococcus*.

in count steadily, and they dominated the lactic flora after 24 h. Of the total of 190 LAB isolates obtained from fermenting *wakalim*, the dominant species were *Ped. pentosaceus1* (36%) and *Lb. plantarum1* (29%).

The pH of the fermenting mass in the casing dropped from 5.5 ± 0.2 to 4.1 ± 0.19 at completion of fermentation (Fig. 3). The drop in pH was sharp until 24 h. The TA of fermenting *wakalim* increased from 0.09 to 0.6% (expressed as lactic acid) at the end of fermentation (Fig. 3).Moisture content of the fermenting *wakalim* dropped from 66.5% ± 2.12 to 22% ± 0.70 during the 6 days of fermentation. Rate of loss of moisture was markedly high until 72 h (Fig. 4).

Numerical Analysis of LAB Isolated from Fermenting *Wakalim*

The degree of similarities among the LAB isolated during *wakalim* fermentation, based on their carbohydrate fermentation profiles showed five clusters with two major clusters at 35% similarity (Fig. 5). Cluster 1 contained 69 isolates mainly dominated by strains of *Lb. plantarum1*, *Ped. pentosaceus1* and *Lb. pentosus*. The three species tended to form subclusters of their own.

Cluster 2 contained 15 heterogenous strains of the genus *Lactobacillus*, including strains of *Lb. brevis, Lb. fermentum, Lb. delbrueckii ssp. delbrueckii,*

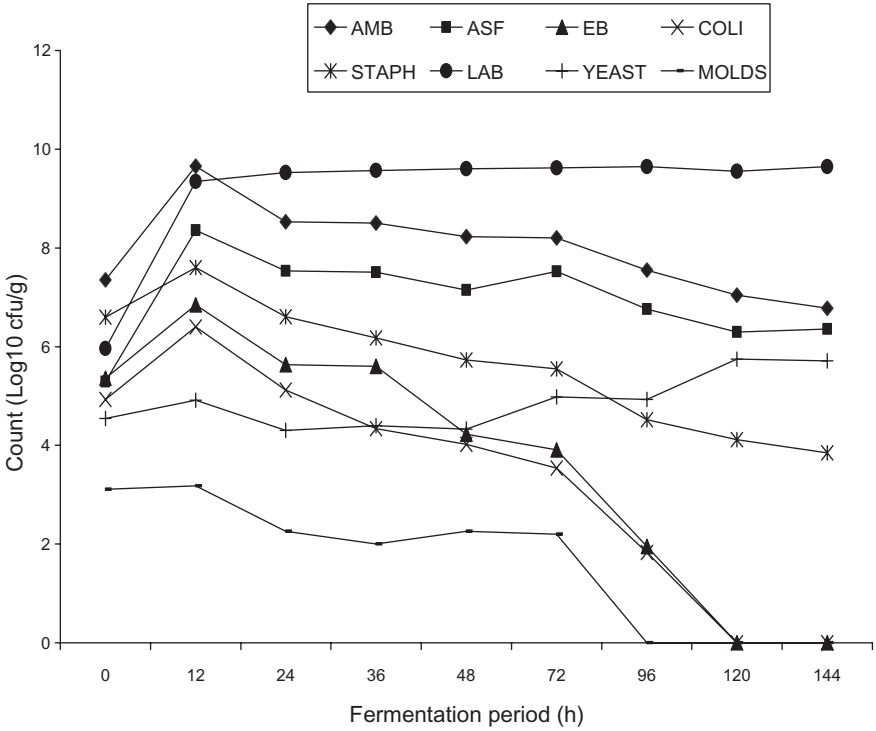

FIG. 1. MICROBIAL DYNAMICS DURING *WAKALIM* FERMENTATION
AMB, aerobic mesophilic bacteria; ASF, aerobic spore former; EB, enterobacteriaceae; COLI, coliforms; STAPH, *staphylococci*; LAB, lactic acid bacteria.

Leuconostoc mesenteroides ssp. mesenteroides and *Leuc. mesenteroides ssp. cremoris*. Two subclusters were recognized within this major cluster. One of the clusters was dominated by strains of *Lb. brevis* and the other cluster by strains of *Lb. fermentum* and *Lb. delbrueckii ssp. delbrueckii*.

All the *Lb. plantarum* 1 were very much related in their carbohydrate fermentation profile. So were the *pediococci*. The *Leuconostoc* isolates were far apart in their profile. The other *lactobacilli* were distant from the *Lb. plantarum1*.

Based on their physiological properties, our LAB in cluster 1 were dominated by both facultative heterofermenters and obligate heterofermenters. Thus, two subclusters of facultative heterofermenters (e.g., *Lb. plantarum*) and obligate heterofermenters (e.g., *Ped. pentosaceus*) were recognized. Likewise, cluster 2 was composed of both obligate heterofermentative and obligate homofermentative *lactobacilli*. All members of the group were *lactobacilli*.

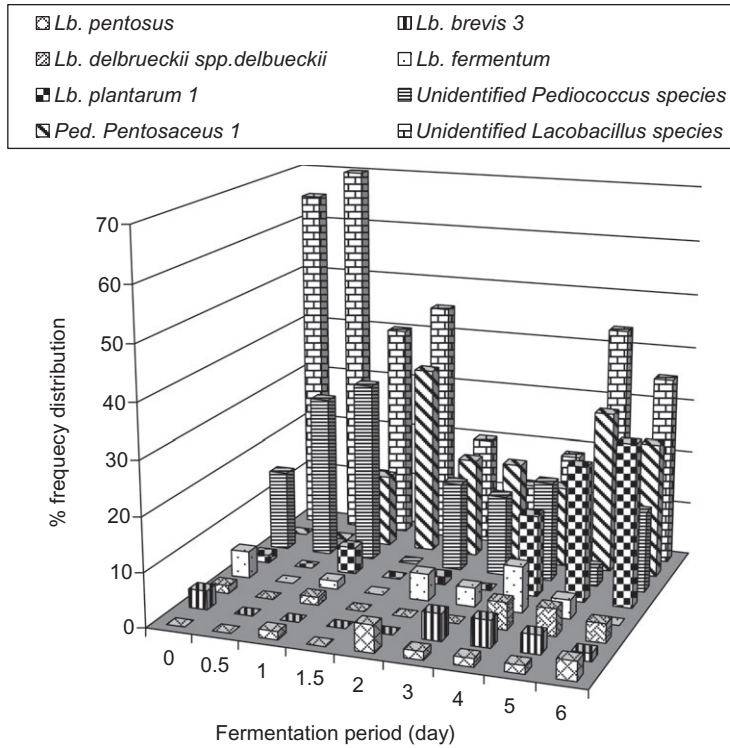

FIG. 2. DYNAMICS OF SOME SPECIES OF LAB DURING *WAKALIM* FERMENTATION

In light of the three phylogenetic group of LAB, namely *Lb. casei* – *Pediococcus* group, *Lb. delbrueckii*, and *Leuconostoc* group, our isolates fell in all the three groups. Members of cluster 1 belonged largely to the *Lb. casei* – *Pediococcus* group. Those of cluster 2 belonged to the *Lb. delbrueckii* and *Lb. casei* – *Pediococcus* group.

Molecular Characterization of LAB

The rep-PCR processing of the 52 representative isolates of LAB produced clearly visible electrophoretic bands for 48 isolates (Fig. 6). Analysis of the banding patterns of these isolates affiliated them to strains of *Lb. plantarum* (24), *Ped. pentosaceus* (13) and *Lb. sakei ssp. carnosus* (11). API, however, assigned these isolates to 10 different species. Differences between the two methods were seen on two of the 12 *Pediococcus* isolates and 17 of the 29 *Lactobacillus* isolates. This could be because of the difference in the degree

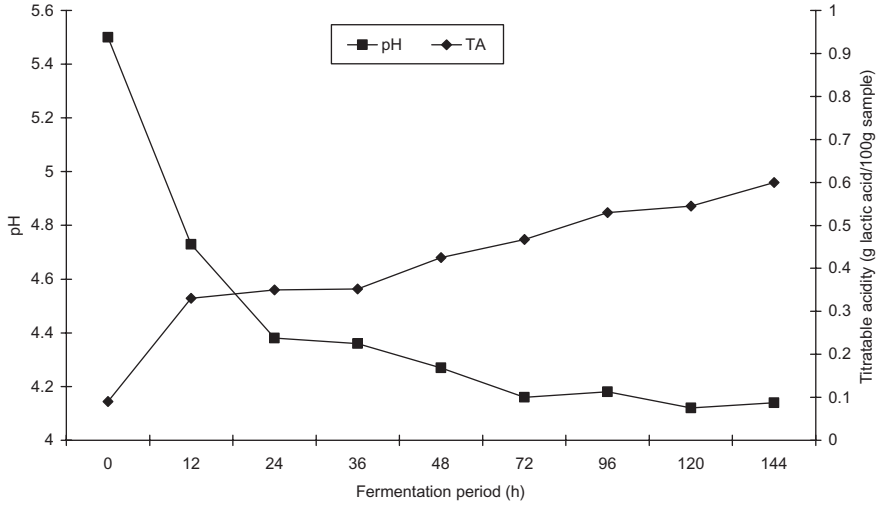

FIG. 3. PATTERNS OF CHANGE IN pH AND TITRATABLE ACIDITY (TA) DURING *WAKALIM* FERMENTATION

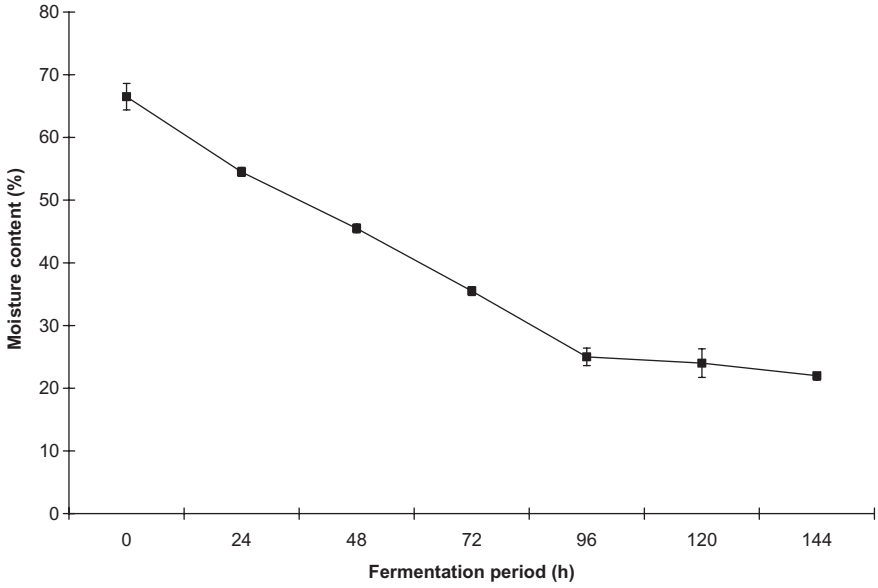

FIG. 4. CHANGES IN MOISTURE CONTENT DURING THE SPONTANEOUS FERMENTATION OF *WAKALIM*

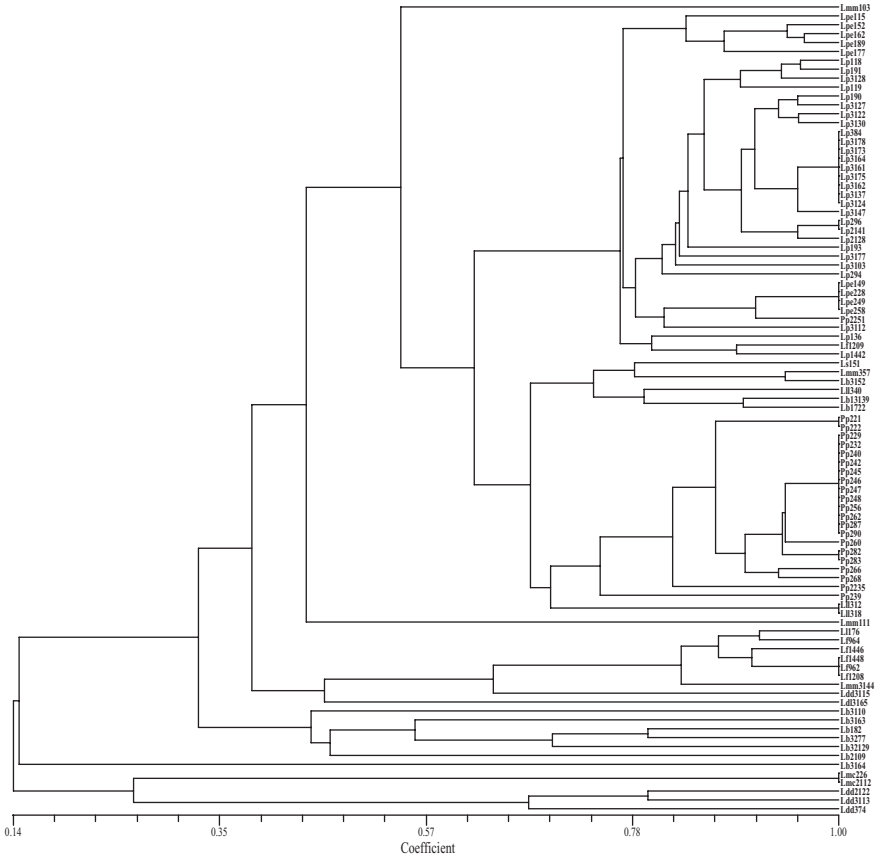

FIG. 5. DENDROGRAM OF SPECIES OF LACTIC ACID BACTERIA CONSTRUCTED BASED ON CARBOHYDRATE FERMENTATION PROFILE (API 50CH) OF THE ISOLATES
Lmm, *Leuc. mesenteroides* spp. *mesenteroides*; Ls, *Lb. salivarius*; Lp, *Lb. plantarum1*; Lpe, *Lb. pentosus*; Pp2, *Ped. pentosaceus2*; Lf, *Lb. fermentum*; Pp, *Ped. pentosaceus1*; Lb1, *Lb. brevis1*; Lb3, *Lb. brevis3*; Lb2, *Lb. brevis2*; Lb, *Lb. brevis*; Ll, *Leuc. Lactis*; Ldd, *Lb. delbrueckii* spp *delbrueckii*; Lmc, *Leuc. mesenteroides* spp. *cremoris*.

of sensitivity of the phenotypic and genetic methods of analysis. Both methods, however, agreed completely on the assignment of all the12 *Lb. plantarum* isolates (Table 3).

DISCUSSION

Availability of fermentable carbon sources, mainly in meat, encouraged the growth of diverse microbial community in the fermenting mash during the

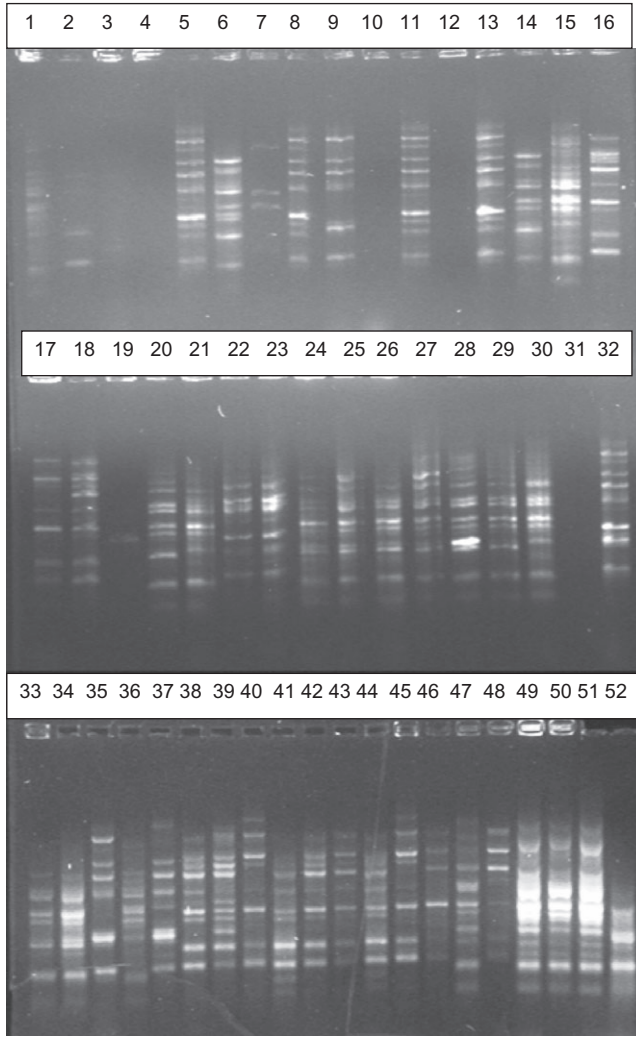

FIG. 6. REPETITIVE SEQUENCE-BASED POLYMERASE CHAIN REACTION AGAROSE GEL ELECTROPHORESIS BAND PROFILES OF SOME REPRESENTATIVE LAB ISOLATED FROM FERMENTING *WAKALIM*
The isolates were *Lactobacillus plantarum* 1 (Lanes 1,6,14,15,19–30, 33,34,36,47, 49–52); *Lb. sakei subsp. carnosus* (Lanes 2,5,7–9,11,13,35,37); and *Ped. pentosaceus* 1 (Lanes 16–18, 38–46, 48).

early stage of fermentation. Meat and spices used for the production of *wakalim* were the major sources of inoculum as they were not subjected to any

TABLE 3.
COMPARISON OF RESULTS OF CARBOHYDRATE FERMENTATION (API 50CH) AND 16S rDNA PARTIAL SEQUENCE ANALYSIS

Isolates	API 50CH (carbohydrate fermentation)	16S rDNA sequencing/rep-PCR	% ID (API 50CH)
239	*Ped. pentosaceus*	*Ped. pentosaceus*	99.8
242	*Ped. pentosaceus*	*Ped. pentosaceus*	99.9
245	*Ped. pentosaceus*	*Ped. pentosaceus*	99.9
247	*Ped. pentosaceus*	*Ped. pentosaceus*	99.9
249	*Lb. pentosus*	*Ped. pentosaceus*	97.9
258	*Lb. pentosus*	*Ped. pentosaceus*	97.4
262	*Ped. pentosaceus*	*Ped. pentosaceus*	99.9
266	*Ped. pentosaceus*	*Ped. pentosaceus*	99.9
268	*Ped. pentosaceus*	*Ped. pentosaceus*	99.9
287	*Ped. pentosaceus*	*Ped. pentosaceus*	99.9
290	*Ped. pentosaceus*	*Ped. pentosaceus*	99.9
294	*Lb. plantarum*	*Lb. plantarum*	99.7
312	*Lact. Lactis*	*Lb. sakei* subsp. *carnosus*	98.1
357	*Leuc. mesenteroides spp. mesenteroides*	*Lb. sakei* subsp. *carnosus*	93.6
374	*Lb. delbrueckii*	*Lb. sakei* subsp. *carnosus*	93.7
384	*Lb. Plantarum*	*Lb. plantarum*	99.9
390	*Lb. paracasei*	*Lb. plantarum*	80.9
405	*Lb. plantarum*	*Lb. plantarum*	83.1
408	*Lb. plantarum*	*Lb. plantarum*	99.9
411	*Lb. plantarum*	*Lb. plantarum*	99.9
422	*Weissella viridescens*	*Lb. plantarum*	88.5
425	*Lact. lactis*	*Lb. plantarum*	92.0
427	*Lact. lactis*	*Lb. plantarum*	82.9
430	*Leuc. lactis*	*Lb. plantarum*	91.3
435	*Ped. pentosaceus2*	*Ped. pentosaceus*	95.1
457	*Lb. brevis 1*	*Lb. plantarum*	98.1
483	*Lb. pentosus*	*Ped. pentosaceus*	88.9
484	*Lb. fermentum*	*Lb. sakei* subsp. *carnosus*	88.5
500	*Ped. pentosaceus2*	*Lb. plantarum*	99.9
513	*Ped. pentosaceus2*	*Lb. plantarum*	86.8
722	*Lb. brevis*	*Lb. sakei* subsp. *carnosus*	98.0
964	*Lb. fermentum*	*Lb. sakei* subsp. *carnosus*	88.5
1,446	*Lb. fermentum*	*Lb. sakei* subsp. *carnosus*	97.0
1,448	*Lb. fermentum*	*Lb. sakei* subsp. *carnosus*	93.3
2,122	*Lb. delbrueckii*	*Lb. plantarum*	90.0
2,128	*Lb. plantarum*	*Lb. plantarum*	99.8
2,141	*Lb. plantarum*	*Lb. plantarum*	98.8
3,103	*Lb. plantarum*	*Lb. plantarum*	98.8
3,112	*Lb. plantarum*	*Lb. plantarum*	99.8
3,113	*Lb. delbrueckii*	*Lb. sakei* subsp. *carnosus*	87.3
3,115	*Lb. delbrueckii*	*Lb. sakei* subsp. *carnosus*	85.9
3,124	*Lb. plantarum*	*Lb. plantarum*	99.9
3,127	*Lb. plantarum*	*Lb. plantarum*	99.3
3,128	*Lb. plantarum*	*Lb. plantarum*	99.9
3,137	*Lb. plantarum*	*Lb. plantarum*	99.9
3,139	*Lb. brevis 1*	*Lb. sakei* subsp. *carnosus*	80.6
3,144	*Lb. fermentum*	*Lb. plantarum*	94.0
3,147	*Lb. plantarum*	*Lb. plantarum*	99.9

Lb, Lactobacillus; *Lact, Lactococcus*; *Leuc, Leuconostoc*; *Ped, Pediococcus.*

microbe-inactivating process. Mean comparison, using one-way analysis of variance, of the counts of the different groups of microorganisms associated with the different spices used for the making of *wakalim* revealed significant variations not only between ingredients but also within ingredients ($P < 0.05$). The mean difference (Turkey, HSD) was significant ($P < 0.005$) when comparison was made between mean counts of AMB versus yeasts and molds. Multiple mean comparisons (Turkey, LCD), however, revealed significant mean difference among AMB and mean counts of ASF, coliforms, LAB, yeasts and molds. No significant variation was observed between AMB and *staphylococci*, showing the dominance of the later among the AMB. The variability in microbial count among the different spices could be accounted to difference in their antimicrobial activities, nutrient content and other intrinsic parameters.

The rise in the counts of both AMB and LAB during the fermentation process was marked. LAB and AMB were the fastest-growing microbes during the production of different sausages (Drosinos *et al*. 2005; Rantsiou and Cocolin 2006). Similar to our observation, LAB increased during the first 5 days of Spanish dry sausage fermentation and remained at constant levels thereafter (Sanz *et al*. 1988). Because of the dynamic nature of fermenting sausage (changes in pH and water activity) complex microbiological activities occur in the initial stage of fermentation (Kaban and Kaya 2006).

In most European fermented sausages, added sugar serves as the fermentable carbohydrate for LAB starter cultures to lower the pH and improve the shelf life of the final product. In *wakalim* fermentation, however, no sugar was added. The lean meat could be the chief source of fermentable sugar. The comminuted sausage meat system contains a number of sugars, which originate from the meat content as well as from the nonmeat ingredients (Mendiolea *et al*. 1995). A limited amount of carbohydrates could also be supplied by the garlic included in fermenting *wakalim*. Some of our LAB isolates may use garlic. Several members of the LAB flora isolated from *som-fak* (a Thai low-salt fermented fish product), including *Lb. pentosus* and *Lb. plantarum*, were capable of fermenting garlic (Paludan-Muller *et al*. 2002).

Despite the sharp increase in count of microorganisms during the earlier stage of fermentation, the counts of AMB, *staphylococci*, coliforms and other members of Enterobacteriaceae dropped during the fermentation period. The counts of LAB increased gradually almost to the end of fermentation. Toward the latter part of *wakalim* fermentation, the stage that resembled more of ripening, the mean count of LAB was as high as 10 log cfu/g. Similarly, during the ripening process of French dry sausages, the number of LAB increased during the first days of the process before it remained almost constant at approximately 8 log cfu/g (Huerta *et al*. 2004). The high population of LAB during fermentation of *wakalim* resulted in a drop in pH down to about 4.2.

The lowered pH and other metabolites, in turn, resulted in reduction in counts of AMB, *staphylococci*, and elimination of Enterobacteriaceae and molds. In fermenting sausages, members of Enterobacteriaceae and *Escherichia coli* are usually counted only in the first few days of fermentation and their number decreases because of the acidification performed by LAB (Rantsiou and Cocolin 2006).

Similar to *wakalim* fermentation, there was significant increase in counts of LAB (from 2 to 9 log cfu/g) and drop in counts of *staphylococci* to undetectable levels in dry Italian sausages (Rebecchi *et al*. 1998). The rate of drop in pH could be a result of higher ambient temperature during fermentation. The change in TA of our product was similar to that of naturally fermented Turkish dry-sausage (Ceylan and Fung 2000). During *sucuk* fermentation, TA (as lactic acid) increased from 0 to 0.6% after completion of fermentation and drying.

There were no significant variations in pH and TA within samples during any sampling time (CV < 10%). High variability in TA within samples (CV > 10%) at initial phases of fermentation could be because of activities of various groups of microorganisms and thus chemical changes as previously noted by Kaban and Kaya (2006).

Growth Dynamics of LAB during *Wakalim* Fermentation

Although *Lactococcus* spp. were among the dominant isolates in the initiation of *wakalim* fermentation and the final product of fermentation was dominated by *Lb. plantarum1* and *Ped. pentosaceus1*, different species of LAB, including other *Lactobacillus* spp., *Lactococcus* spp., other *Pediococcus* spp. and *Leuconostoc* spp. were isolated at least at some stage of *wakalim* fermentation. In a traditional Italian sausage, the indigenous species of *Lactobacillus* (*Lb. sakei*, *Lb. plantarum*, *Lb. curvatus* and *Lb. farciminis*) were the main agents responsible for fermentation (Rebecchi *et al*. 1998).

There are various reports on the dominant species of LAB at different stages of fermentation. *Lb. sakei* and *Lb. curvatus* were important in the early stages of Greek dry-fermented sausage (Papamanoli *et al*. 2003). These species were important during fermentation and ripening of Greek salami (Rantsiou and Cocolin 2006). During the fermentation of "Urutan," a Balinese indigenous fermented sausage, *Lb. plantarum* and *Ped. acidilactici* initiated the fermentation and the lactic flora was finally dominated by *Lb. farciminis* (Antara *et al*. 2002). In our case, *Lb. curvatus* and *Lb. paracasi* spp. *paracasei* were encountered at different stages of fermentation but at levels below dominance (data not shown). The pH of the ready-to-eat *wakalim* product was lower than that of Greek dry-fermented sausage (Papamanoli *et al*. 2003).

Similar to our observation, *Lb. plantarum* dominated the natural fermentation of Greek dry sausages (Drosinos *et al*. 2005), Italian fermented sausage

(Rantsiou and Cocolin 2006) and Spanish fermented sausage (Hugas *et al*. 1993). *Pediococcus* species were also reported to be dominant in some traditional Italian sausages (Rebecchi *et al*. 1998) and French dry sausages (Huerta *et al*. 2004). The species composition of the dominant flora may vary depending on initial lactic flora, temperature and duration of fermentation and other parameters. In sausage fermentations performed at 18–23C, the indigenous microflora was usually dominated by strains of *Lb. sakei* and *Lb. curvatus* (Hugas *et al*. 1993); *Lb. plantarum* predominated at higher ripening temperature (Lucke 1998). Starter cultures of *Ped. pentosaceus* and *Lb. plantarum* were initially developed for products with shorter curing times at higher fermentation temperature (Erkkila 2001). According to some authors, starters composed of *Lb. sakei* and *Lb. curvatus* were more competitive than that of *Lb. plantarum* and *Ped. pentosaceus* at lower fermentation temperature (Hammes and Knauf 1994).

The moisture content of fermented *wakalim* resembled that of other dry sausages and, thus, has lower moisture content than the semi-dry sausage such as the Italian style Mortadella, some of the South African fermented dry sausages, salami and cabanossi (Wolter *et al*. 2000). Traditionally, the combined processes of *wakalim* fermentation and drying proceed simultaneously with no control on humidity and temperature of the fermentation environment. Thus, the final moisture content of *wakalim* and the duration of fermentation could be affected by the existing environmental condition. As opposed to spontaneous fermentation of *wakalim*, controlled lactic fermentation of sausage usually proceeds at about 20C within 2–4 days with subsequent drying at progressively declining relative humidity to ensure homogenous removal of water (Bozoglu and Ray 1996). Under controlled fermentation, the relative humidity is also controlled to ensure that a slow drying of the product proceeds.

Lb. plantarum and *Lb. pentosus* isolated from *wakalim* belonged to cluster 1. Other investigations on the 16S rDNA sequence analysis of the two strains showed the existence of more than 99% sequence similarity between the two species (Collins *et al*. 1991).

In this study, we employed both rep-PCR and 16S rDNA sequence analysis on representative isolates identified earlier in the study using API system (API 50CH). The methods generated reliable data concerning the identity and distribution of LAB in *wakalim* during fermentation. Most of the LAB that dominated *wakalim* fermentation was confirmed to be *Lb. plantarum* and *Ped. pentosaceus*.

There were some discrepancies between results of the biochemical and genetic methods used in the identification of our LAB isolates. This could be because of differences in the sensitivity and reliability between the phenotypic and molecular methods of characterization. Although biochemical tests are very useful methods for a preliminary identification of LAB, the readings may

be subjective while molecular methods are much less subjected to error, and yield more rapid and accurate results (Stiles and Holzapfel 1997). The rep-PCR fingerprinting technique using (GTG) 5-PCR is a rapid, easy-to-perform and reproducible tool for differentiation of a wide range of food-associated *lactobacilli* at the species, subspecies and potentially up to the strain level, with a single-performance protocol (Gevers *et al*. 2001).

All species of LAB, affiliated to *Lb. sakei subsp*. *carnosus* by 16S rDNA, were identified earlier either as *Lb. fermentum*, *Lb. delbrueckii* subsp. *delbrueckii* or *Lb. brevis* by API carbohydrate fermentation tests. *Lb. sakei* ssp. *carnosus* belong to the facultative heterofermentative group of LAB while *Lb. fermentum* are members of the obligate heterofermentative *lactobacilli*. Discrepancies between results of identification of strains based on phenotypic and genetic methods were reported earlier (Yeung *et al*. 2002).

Different explanations have been forwarded for variations between methods in speciation of isolates. It might be because of the loss and gain of plasmids, leading to inconsistency in metabolic traits of a strain, as most carbohydrate fermentation capacities are plasmid-encoded (Ahrne *et al*. 1989). The degree of completeness and accuracy of the databases could contribute to the discrepancies as different databases sometimes give different speciation results (Yeung *et al*. 2002). Moreover, some database sequences do not represent the most current nomenclature. In the absence of matching profile of a strain in the database, API WEB will not produce reliable identification (Conter *et al*. 2005).

In general, the findings of this study are the first of their kind from traditional Ethiopian fermented sausage and could have paramount importance as a baseline data for the large-scale production of the traditional product using defined starter cultures. The strains characterized in this study could be the candidate starter cultures.

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REFERENCES

AHRNE, S., MOLIN, G. and STAHL, S. 1989. Plasmids in *Lactobacillus* strains isolated from meat and meat products. Syst. Appl. Microbiol. *11*, 320–325.

ANTARA, N.S., SUJAYA, I.N., YOKOTA, A., ASANO, K., ARYANTA, W.R. and TOMITA, F. 2002. Identification and succession of lactic acid bacteria during fermentation of *Urtuan*, a Balinese indigenous fermented sausage. World J. Microbiol. Biotechnol. *18*, 255–262.

ANTONY, U. and CHANRDA, T.S. 1997. Microbial population and biochemical changes in fermenting finger millet (*Eleusine coracana*). World J. Microbiol. Biotechnol. *13*, 533–537.

BOZOGLU, T.F. and RAY, B. (eds.) 1996. *Lactic Acid Bacteria: Current Advances in Metabolism, Genetics and Applications*, Springer-Verlag, Berlin, Germany.

CEYLAN, E. and FUNG, D.Y.C. 2000. Destruction of *Yersinia enterocolitica* by *Lb. sakei* and *Ped. acidilactici* during low-temperature fermentation of Turkish dry sausage (*sucuk*). J. Food Sci. *65*, 876–879.

COLE, J.R., CHAI, B., FARRIS, R.J., WANG, Q., KULAM-SYED-MOHIDEEN, A.S., MCGARRELL, D.M., BANDELA, A.M., CARDENAS, E., GARRITY, G.M. and TIEDJE, J.M. 2007. The ribosomal database project (RDP-II): Introducing *myRDP* space and quality controlled public data. Nucleic Acids Res. *35*, D169–D172.

COLLINS, M.D., RODRIGUEZ, U., ASH, C., AGUIRREL, M., FARROW, J.A.E., MARTINEZ-MURCIA, A., PHILIPS, B.A., WILLIAMS, A.M. and WALLBANKS, S. 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. FEMS Microbiol. Lett. *77*, 5–12.

CONTER, M., MUSCARIELLO, T., ZANARDI, E., GHIDINI, S., VERGARA, A., CAMPANINI, G. and IANIERI, A. 2005. Characterization of lactic acid bacteria isolated from an Italian dry fermented sausage. Ann. Fac. Med. Vet. Parma *25*, 167–174.

DROSINOS, E.H., MATARAGAS, M., XIRAPHI, N., MOSCHONAS, G., GAITIS, F. and METAXOPOULOS, J. 2005. Characterization of the microbial flora from traditional Greek fermented sausage. Meat Sci. *69*, 307–317.

ERKKILA, S. 2001. *Bioprotective and probiotic meat starter cultures for the fermentation of dry sausages*. Doctoral thesis, 64 pp, University of Helsinki, Department of Food Technology.

GEVERS, D., HUYS, G. and SWINGS, J. 2001. Applicability of rep-PCR finger printing for identification of *Lactobacillus* species. FEMS Microbiol. Lett. *205*, 31–36.

GREGERSON, T. 1978. Rapid method for distinction of Gram-negative and Gram-positive bacteria. Eur. J. Appl. Microbiol. *5*, 123–127.

HAMMES, W.P. and KNAUF, H.J. 1994. Starters in processing of meat products. Meat Sci. *36*, 155–168.

HUERTA, R., JORDANO, R., MEDINA, L.M. and LOPEZ, C. 2004. Population dynamics of the constitutive biota of French dry sausages in a pilot-scale ripening chamber. J. Food Prot. *67*, 2306–2309.

HUGAS, M., GARRIGA, M., AYMERICH, T. and MONFORT, J.M. 1993. Biochemical characterization of lactobacilli isolated from dry fermented sausages. Int. J. Food Microbiol. *18*, 107–113.

HUGH, R. and LEIFSON, E. 1953. The taxonomic significance of fermentative versus oxidative Gram-negative bacteria. J. Bacteriol. *66*, 24–26.

KABAN, G. and KAYA, M. 2006. Effect of starter culture on growth of *Staphylococcus aureus* in *sucuk*. Food Control *17*, 797–801.

KOVACS, N. 1956. Identification of *Pseudomonas pyocyanae* by the oxidase reaction. Nature *178*, 703.

LUCKE, F.-K. 1998. Fermented sausages. In *Microbiology of Fermented Foods* (B.J.B. Wood, ed.) pp. 441–483, Blackie Academic and Professional, London, UK.

MENDIOLEA, R., GUERREROB, I. and TAYLOR, A.J. 1995. Changes in sugars during storage of sausages. Meat Sci. *39*, 34–361.

PALUDAN-MULLER, C., VALYASEVI, R., HUSS, H.H. and GRAM, L. 2002. Genotypic and phenotypic characterization of garlic-fermenting LAB isolated from *som-fak*, a Thai low-salt fermented fish product. J. Appl. Microbiol. *92*, 307–314.

PAPAMANOLI, E., TZANETAKIS, N., LIPOPOULOU-TZANETAKI, E. and KOTZEKIDOU, P. 2003. Characterization of lactic acid bacteria isolated from a Greek dry fermented sausage in respect of their technological and probiotic properties. Meat Sci. *65*, 859–867.

RANTSIOU, K. and COCOLIN, L. 2006. New developments in the study of the microbiota of naturally fermented sausage as determined by molecular methods. A review. Int. J. Food Microbiol. *108*, 225–267.

REBECCHI, A., CRIVORI, S., SARRA, P.G. and COCCONCELLI, P.S. 1998. Physiological and molecular techniques for the study of bacterial community development in sausage fermentation. J. Appl. Microbiol. *84*, 1043–1049.

SANZ, B., SELGES, D., PAREJO, I. and ORDONEZ, J.A. 1988. Characteristics of *lactobacilli* isolated from dry fermented sausages. Int. J. Food Microbiol. *6*, 199–205.

STILES, M.E. and HOLZAPFEL, W.H. 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. *36*, 1–29.

VERSALOVIC, J., SCHNEIDER, M., DE BRUIJN, F.J. and LUPSKI, J.R. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol. Cell. Biol. *5*, 25–40.

WOLTER, H., LAING, E. and VILJOEN, B.C. 2000. Isolation and identification of yeasts associated with intermediate moisture meats. Food Technol. Biotechnol. *38*, 69–75.

YEUNG, P.S.M., SANDERS, M.E. and KITTS, C.L. 2002. Species-specific identification of commercial probiotic strains. J. Dairy Sci. *85*, 1039–1051.