Asian Journal of Chemistry Vol. 21, No. 4 (2009), 3257-3262

Identification of Microflora in Butter Samples from Turkey by Using the Microbial Identification System

S. ÖZTÜRK YILMAZ
Refik Saydam Hygiene Center, Ministry of Health, Food Security and Nutrition Research Directorate, 06100 Sihhiye, Ankara, Turkey
Fax: (90)(312)4582383; Tel: (90)(31204582206
E-mail: suzanoz1@yahoo.com

A total 138 bacterial strains and 52 fungi isolates were isolated from 53 butter samples collected from grocery stores in Erzurum, Turkey in the winter season of 2000. They were identified based on fatty acid methyl esters (FAMEs) analysis with microbial identification system (MIS; MIDI Inc., Newark, Del.). All of the isolates and 4 reference bacterial strains were identified at species level. The most abundant bacterial species found was *Enterococcus faecalis* followed by *Staphylococcus hominis, Micrococcus luteus, Pseudomonas putida, P. fluorescens, Brevibacillus brevis, Streptococcus sanguis, Weissella viridescence, Lactococcus lactis-lactis* and *Lactobacillus kefir*. However, only 73 % (38) of the fungi isolates tested could be identified with MIS at the genus or species level. The most prevalent fungi species was found to be *Aspergillus versicolor* followed by *A. flavus, Rhizophus* spp., *Penicillium* spp., *Fusarium* spp., *Exophiala salmonis, Torulomyces rubrum, Phoma* spp., *Ulocladium* spp., *Geotricum candidum* and *Paecilomyces variotii*. The remaining 14 fungi isolates could not be identified with MIS. Present MIS results are confirmed with conventional methods used for identification of the reference and randomly selected microorganisms isolated in this study. Present results suggest that MIS system is a rapid and reliable method for identification of microflora of butter samples.

Key Words: Butter, Bacteria, Fungi, Microflora, Microbial identification system.

INTRODUCTION

Butter spoilage has often been recognized as inconvenient and the most important concern by food technologists[1-4]. It is usually a result of microbial activity of a variety of microorganisms including fungi and bacteria[1,5-8]. The contamination of butter with microflora can take place at various stages of the production and the sale and the distribution of the product[5,9]. The development of microbial flora on butter, particularly during storage, depends on many parameters including water activity, pH, redox potential, nutrients, antimicrobial compounds, temperature, humidity and preservation. Due to the economical impacts of food spoilage and the consumer's demands for more natural products, a lot of attention has been paid to detect and identify the microorganisms that are able to proliferate in butter

products[1,7,8,10]. Better detection method offers opportunity to take all necessary precautions to avoid spoilage caused by the microorganisms[7,9]. This could be established either by changing the conditions of processing and storage of products or by preventing the entrance of the undesired microorganisms into the production chain[11-15].

So far only traditional techniques have been used to identify food-associated microorganisms. These techniques require the use of all conventional tests including morphological, physiological and biochemical procedures that are complex laborious and time consuming[7,10,16]. They are lack of discriminatory power that result in frequently misidentification of the Flora. Therefore, current practices in food microbiology rely on molecular techniques including PCR, SDS-PAGE and MIS[8,17-19]. According to literatures, fatty acid methyl ester (FAME) analysis using microbial identification system (MIDI, inc., Newark, DE) is highly predictive and promising technique for identification of food-associated bacterial microorganisms, but not for fungi species[17,18]. Recently, Microbial Identification System with software package (MIS Sherlock 4.5 MIDI, inc., Newark, DE) has been updated to improve the identification capacity of the microorganisms including bacteria and fungi.

Since there is no data on microflora of butters sold in Turkey, the aim of this study was to determine microflora of butter samples sold in markets in Erzurum, Turkey by using microbial identification system (MIS).

EXPERIMENTAL

Sample collection, microorganism isolation and culture conditions: In the winter of 2000, 53 butter samples representing 21 different manufacturers were collected from grocery stores in Erzurum, Turkey. 10 g sample were taken from the interior of each butter samples and homogenized in 90 mL sterile peptone water by shaking in a Stomacher (Gerhardt, Germany) for 5 min. Then decimal dilutions of the resulting suspension were prepared in 9 % (w/v) NaCl solution and inoculated on potato dextrose (PDA) agar (Oxoid, Hampshire, UK) and sabouraund dextrose (SD) agar (Difco, Detroid, USA) for fungal isolation and Man Rogosa Sharpe (MRS) agar (Oxoid), violet red bile (VRB) agar (Oxoid), Baird-Paker agar (Difco) and nutrient (NA) agar (Acumedia, Baltimore, Maryland, USA) for bacterial isolation. All plates were incubated at 30 ºC for 3-5 d. After incubation period, the grown bacterial and fungal colonies were purified by sub-culturing on the same media used before for isolation step. A total of 138 bacterial strains and 52 fungi isolates were isolated in this study and stored for the further studies (Table-1). Four bacterial strains and a fungus isolate from American Type Culture Collection (ATCC) were used as reference strains in this study (Table-1). Reference bacterial strains were maintained for long-term storage in nutrient broth with 15 % glycerol at 80 ºC. Reference Fungi isolate were kept in PDA slants at 4 ºC in refrigerator.

Extraction and analysis of fatty acid methyl esters (FAMEs) and identification of fungal and bacterial species: Preparation and analysis of FAMEs of

whole cell fatty acids from bacterial strains and fungal isolates were performed according to the method described by manufacturing manual (Sherlock Microbial Identification System version 4.5, MIDI, inc., Newark, DE). Approximately 40 mg of living cells was harvested from each samples and added to 1 mL of 1.2 M NaOH in 50 % aqueous methanol in a screw cap tube with 5 glass beads (3 mm dia), then incubated at 100 ºC for 0.5 h in a water bath. After the saponification the samples were cooled at room temperature for 25 min. They were acidified and methylated by adding 2 mL 54 % 6 N HCl in 46 % aqueous methanol and incubated at 80 ºC for 10 min in a water bath. After rapid cooling, methylated fatty acids were extracted with 1.25 mL 50 % methyl-*tert* butyl ether (MTBE) in hexane. Each sample was mixed for 10 min and the bottom phase was removed with a Pasteur pipette. The top phase was washed with 3 mL of 0.3 M NaOH. After mixing for 5 min, the top phase was removed for analysis. Following the base wash step, the extract FAMEs were cleaned in anhydrous sodium sulfate and then transferred into a GC sample vial for analysis.

Fatty acid methyl esters were separated by gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA) with a fused-silica capillary column (25 m × 0.2 mm) and cross-linked 5 % phenylmethyl silicone. The operating parameters set for the study were controlled automatically by computer program. The chromatograms with peak retention times and areas were produced on the recording integrator and were electronically transferred to the computer for analysis, storage and report generation. Peak naming and column performance was achieved through the use of calibration standard mix (Microbial ID 1200-A) containing nC9-nC20 saturated and 2 and 3 hydroxy fatty acids. Cellular fatty acids were identified on the basis of equivalent chain length data. Fatty acid methyl esters profiles of each microorganism (bacteria or fungi) tested was identified by comparing the commercial databases (TSBA 40, yeast and fungi) with the MIS software package. Identity of the micro-organism was relieved by computer comparison of FAME profile of the unknown test strain/isolate with those in library.

Conventional methods have also been used for identification of 45 randomly selected microorganisms including 25 bacterial strains and 20 fungi isolates from present culture collection in addition to reference strains[7,10,16,20].

RESULTS AND DISCUSSION

A total of 138 bacterial strains isolated and reference strains were identified based on fatty acid analysis at the species level by using MIS with similarity indices ranging from 19 to 89 % (Table-1). *Enterococcus faecalis* was found to be the most prevalent bacterial species isolated from 83 % of the butter samples tested. The identity and incidences of the other bacterial species isolated were *Staphylococcus hominis* (43 %); *Micrococcus luteus* (36 %); *Pseudomonas putida* (28 %); *P. fluorescens* (21 %), *Brevibacillus brevis* and *Streptococcus sanguis* (13 %); *Weissella viridescence* (11 %), *Lactococcus lactis-lactis* and *Lactobacillus kefir* (6 %), respectively (Table-1). Only 38 (73 %) of 52 fungi isolates recovered from butter

TABLE-1
IDENTIFICATION OF BACTERIAL STRAINS ISOLATED FROM BUTTER SAMPLES FROM ERZURUM, TURKEY BY MICROBIAL IDENTIFICATION SYSTEM ON THE BASIS OF FATTY ACID METHYL ESTER ANALYSIS

Species of bacteria	Origin*	No. of samples	Range of similarity indices (%)
Isolated strains			
Enterococcus faecalis	Butter/this study	44/53	19-76
Staphylococcus homini	Butter/this study	23/53	32-71
Micrococcus luteus	Butter/this study	19/53	35-69
Pseudomonas putida	Butter/this study	15/53	22-89
Pseudomonas fluorescens	Butter/this study	11/53	36-78
Brevibacillus brevis	Butter/this study	7/53	44-68
Streptococcus sanguis	Butter/this study	7/53	21-53
Weissella viridescence	Butter/this study	6/53	71-82
Lactococcus lactis-lactis	Butter/this study	3/53	40-77
Lactobacillus kefir	Butter/this study	3/53	26-81
Reference strains			
Bacillus substilis	ATCC-6633	1	78
Enterococcus faecalis	ATCC-29212	1	83
Staphylococcus aureus	ATCC-29213	1	63
Pseudomonas fluorescens	ATCC-49838	1	72

*Origin = Sources of bacterial strains isolated;
ATCC = American type culture collection, USA.

samples could be identified at the genus and/or species level by MIS with similarity indices of 32 to 91 % (Table-2). They belong to 12 fungi species in 10 different genera in which the most abundant species identified is *Aspergillus versicolor* (19 %) followed by *A. flavus* (13 %), *Rhizophus* spp. and *Penicillium* spp. (9 %); *Fusarium* spp (6 %) and *Exophiala salmonis* (4 %). The other 6 fungi species including *Torulomyces rubrum, Phoma* spp., *Ulocladium* spp., *A. terreus, Geotricum candidum* and *Paecilomyces variotii* were represented by a single isolate.

Present results were confirmed by a number of previous studies reporting the existence of the bacterial species in the genera of *Enterococcus, Staphylococcus, Pseudomonas, Streptococcus, Lactococcus* and *Lactobacillus* and fungal species in the genera of *Aspergillus, Rhizophus* and *Penicillium* as microbial flora causal spoilage agents in butter[1-6,8,21]. However, the bacteria belong to the genus of *Micrococcus, Brevibacillus* and *Weissella* and the fungi isolates in the species of *Exophiala, Torulomyces, Phoma, Ulocladium, Fusarium, Geotricum* and *Paecilomyces* could not be isolated from butter samples in the previous studies[1-5,8,21]. Therefore the presence of these bacterial and fungal species as microflora of butter is the first time demonstrated in this study. This is also the first study for detection and identification of microbial flora in butter by using MIS technique on based FAME analysis. The results of MIS identification of randomly selected 25 bacterial strains including 4 reference strains and 20 fungi isolates were confirmed by using conventional methods including morphological, physiological and biochemical tests[7,10,16,20]. However,

TABLE-2
IDENTIFICATION OF FUNGAL ISOLATES FROM BUTTER SAMPLES FROM ERZURUM, TURKEY BY MIS ON THE BASIS OF FAME ANALYSIS

Species of fungi	Origin*	No. of samples	Range of similarity indices (%)
Test isolated			
Aspergillus versicolor	Butter/this study	10/53	52-88
Aspergillus flavus	Butter/this study	7/53	63-91
Rhizophus spp.	Butter/this study	5/53	32-66
Penicillium spp.	Butter/this study	5/53	56-81
Fusarium spp.	Butter/this study	3/53	41-87
Exophiala salmonis	Butter/this study	2/53	64-75
Torulomyces rubrum	Butter/this study	1/53	71
Phoma spp.	Butter/this study	1/53	57
Ulocladium spp.	Butter/this study	1/53	66
Aspergillus terreus	Butter/this study	1/53	87
Geotricum candidum	Butter/this study	1/53	49
Paecilomyces variotii	Butter/this study	1/53	53
Unidentified	Butter/this study	14/53	No match
Reference isolates			
Aspergillus versicolor	ATCC-96920	1	79

*Origin = Sources of fungi species isolated;
ATCC= American type culture collection, USA, Bottom of form 1.

14 (27 %) of the fungi isolates could not be identified by MIS system nor by microscopic examination due to lack of spore formation on standard fungi growing media (Water agar, PDA or Sabouraund dextrose agar).

Present data suggested that MIS system can be utilized as rapid and reliable method for detection and identification of microflora in foods. However, the fungi database of MIS has showed that MIS need to be improved in oder to identify all fungi species of food-associated microflora. This is the first study demonstrated the presence of the bacterial species of *Brevibacillus* and *Weissella* and fungi species of *Exophiala, Torulomyces, Ulocladium, Geotricum* and *Paecilomyces* in the butter microflora. None of the bacteria, but some fungi (*Fusarium* spp., *Aspergillus* spp., *Rhizopus* spp. and *Penicillium* spp.) species isolated from butter samples in present study were known to be the causal agents of food spoilage. In this study, the microbial flora found in the butter samples may come from the source (milk) and/or be contaminated at various stages of the production, sale and distribution of the product. Therefore, a further studies need to be conducted in order to test the entrance of the undesired microorganisms into the chain of the production of butter and other dairy products.

ACKNOWLEDGEMENT

The author thankful to Prof. F. Sahin, Head, Department of Genetics and Bioengineering, Yeditepe University for his help in carrying out this research work.

REFERENCES

1. W.B. Cooke and A.R. Brazis, *Mycopathologia at Mycologia Applicata*, **35**, 281 (1968).
2. F. Amato, L. Becheroni and A. Dracos, *Nuovi Annali d'Igiene e Microbiologia*, **21**, 341 (1970).
3. T.E. Minor and E.H. Marth, *J. Dairy Sci.*, **55**, 1410 (1972).
4. S.K. Garg and B.K. Mital, *Crit. Rev. Microbiol.*, **18**, 15 (1991).
5. S.A. Dos Reis Filho and S.T. Iaria, *Revista de Saude Publica*, **15**, 418 (1981).
6. S.J. Walker, *J. Soc. Dairy Technol.*, **41**, 91 (1988).
7. J.I. Pitt and A.D. Hocking, Fungi and Food Spoilage, Blackie Academic & Professional, London, edn. 2 (1997).
8. F. Cappa and P.S. Cocconcelli, *Int. J. Food Microbiol.*, **69**, 157 (2001).
9. T. Deak and L.R. Beuchat, Handbook of Food Spoilage, CRC Press, New York (1996).
10. R.B. Larry, Food and Beverage Mycology, Aspen Publishers, USA, edn. 2 (1999).
11. Y. Montarjemi, F. Kaferstein, G. Moy, S. Miyagawa and K. Miyagishima, *Food Control*, **72**, 77 (1996).
12. O. Sagdiç, M. Arici and O. Simsek, *Food Microbiol.*, **19**, 303 (2002).
13. A.A. Ali and M.R. Fisher, *Food Rev. Int.*, **21**, 189 (2005).
14. M. Kacem and N.E. Karam, *Grasas Y. Aceites*, **57**, 198 (2006).
15. G. La Salandra, A. Dambrosio, N.C. Quaglia, M. Corrente, A. Parisi, G. Santagada, A. Firinu, E. Crisetti and G.V. Celano, *Int. J. Food Microbiol.*, **115**, 290 (2007).
16. J.G. Holt, N.R. Krieg, P.H.A. Sneath, J.T. Staley and S.T. Williams, Bergey's Manual of Determinative Bacteriology, Lippincott Williams & Wilkins, Baltimore, Maryland, edn. 9 (1994).
17. S. Lin, H. Schraft, J.A. Odumeru and M.W. Griffiths, *Int. J. Food Microbiol.*, **43**, 159 (1998).
18. J.A. Odumeru, M. Steele, L. Fruhner, C. Larkin, J.D. Jiang, E. Mann and W.B. McNab, *J. Clin. Microbiol.*, **37**, 944 (1999).
19. I. Sanchez, S. Sesena and L. Palop, *Int. J. Food Microbiol.*, **82**, 181 (2003).
20. B.A. Forbes, D.F. Sahm and A.S. Weissfeld, Bailey and Scott's Diagnostic Microbiology, Mosby Inc., St. Louis, Missouri. edn. 11 (1998).
21. M. Milohnoja, *Nordisk Veterinaermedicin*, **24**, 139 (1972).

(*Received*: 25 November 2008; *Accepted*: 24 January 2009) AJC-7184