PE8.38 The PCR-DGGE Analysis of The Microbial Ecology of Turkish Sausage (Sucuk) 422.00

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Abstract—In this paper, the application of denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR) amplified 16S rRNA gene, in the study of microbial ecology of commercially available traditional fermented Turkish sausage (sucuk) was described. DGGE analysis method was applied for the analysis of PCR products obtained from DNA extracted directly from the sausage/sucuk samples. Analysis of DGGE patterns and sequencing of the bands allowed profiling of the microbial populations present in the samples. The bacterial ecology was mainly characterized by the stable presence of Lactobacillus sakei and Wisella viridescens. Also several bacteria belong to Staphylococcus species were detected. Finally, PCR-DGGE analysis seemed to promise good potential for the evaluation of microbial diversity of the fermented sausage/sucuk. This will likely to be an important tool for the investigation of microbial diversity and monitoring the presence of technologically important species during the food manufacturing processes, with the ultimate objective of quality of the product

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Index Terms— PCR-DGGE, Turkish sausage, microbial ecology

I. INTRODUCTION

Sucuk is a traditionally fermented Turkish style sausage which is the most popular meat product produced in Turkey. It is prepared by mixing lamb and/or beef, beef fat or sheep tail fat, salt, sugar, nitrite/nitrate, garlic and various spices such as black pepper, red pepper, cummin [1]. In general, the qualitative characteristics of naturally fermented sausages are known to be largely dependent on the composition of the microbial population, originated from the environment, equipment and the raw materials used to manufacture the products. It has been well known that different genera and species and even strains have been confirmed to significantly affect the sensory traits of fermented sausages, therefore, the microbial composition of fermented foods has drawn an increasing attention over the recent decades [2]. The microbial ecology of fermented sausages has been complex and complicated to study, mostly due to the fact that large proportion of microbial population could be in a viable but nonculturable form [3], and conventional culture-dependent methods have some limit to isolate the cultivable species or strains that less abundant [4].

However, molecular techniques and culture-independent methods have been regarded capable of providing a more realistic insight for the microbial diversity in complex foods [2,5]. Among them, PCR combined with Denaturing Gradient Gel Electrophoresis (DGGE) analysis is one of the most suitable and widely used method to study complex bacterial communities in environmental and food materials [6,7,8,9,10].

This method has the advantage of directly profiling microbial populations present in specific environment by separating PCR products originated with universal primers based on the melting domain of the DNA fragments. DGGE represents a method to get species information through PCR analysis of the same length but with different sequences based on different mobility in an acrylamide gel system with increasing denaturant (urea and formamide) intensity. PCR combined DGGE procedure has been used for the evaluation of microbial ecology in natural environments [11] and to profile complex microbial communities [12] and to monitor microbial community variations according to environmental changes [13].

In this research, we tried to optimize a simple and fast molecular method to study the microbial ecology of fermented Turkish sausages purchased from Kayseri and Afyon provinces in Turkey. The method was based on amplification of the V3 region of the 16S ribosomal DNA (rDNA) and electrophoresis of the polymerase chain reaction (PCR) product in a polyacrylamide gel containing with increasing concentration of denaturants. Because, it is possible to determine

species-specific migration patterns which could be used for specific identification purposes due to the diferences in the DNA sequence amplified by PCR.

II. MATERIALS AND METHODS

A. Sampling Eleven fermented dry-sausages/sucuk samples made by traditional methods were collected aseptically from different producers from Kayseri and Afyon provinces in Turkey. Physicochemical analysis of sucuk samples showed that % moisture, salt content, pH and aw values average % 31.29, %3.09, 4.899 and 0.863 respectively.

B. Genomic DNA extraction The total genomic DNA was extracted from each sucuk samples using a commercial purification kit (Qiagen, Hilden, Germany) according to manufacturer's protocol and then analyzed by 0.8% agarose gel electrophoresis.

C. PCR amplification The primers V3f (5'-ACTCCTACGGGAGGC AGCAG-3') and V3r (5'-ATTACCGCGGCTGCT GG-3') targetting V3 region of the 16S rRNA gene were used. A GC-clamp was added to the forward primer, according to Ampe et al. [14]. The PCR reaction was performed in a final volume of 50µl using 25 µl of Quantitect Probe PCR mix (Qiagen, Hilden, Germany) 100ng DNA, 0.8 µM of forward primer with GC clamp and 0.4 µM reverse primer.

D. The amplifications were performed on a Line Gene II PCR device (Bioer Technology Co., Hangzhou, China) and thermal cycling protocol; initial denaturation at 95 °C for 4 min, followed by a touchdown procedure (in which the annealing temperature was lowered from 61 °C to 51 °C at a rate of 0.5 °C every cycle) and 10 additional annealing cycles at 51 °C. At each cycle, the elongation was carried out at 72 °C for 3 min. A final extension at 72 °C for 30 min was performed at the end of all of the amplification cycles for eliminate artifactual bands. Sizes and quantities of PCR products were determined by 2% agarose gel electrophoresis.

E. DGGE analysis The Dcode universal mutation detection system (Bio-Rad Laboratories, Richmond, Calif.) was used for a DGGE analysis directly from fermented sausages. Electrophoresis was performed in a 0.8-mm polyacrylamide gel (8% [wt/vol] acrylamide-bisacrylamide [37.5:1]) by using two different ranges of denaturants to optimize separation of the PCR products. Denaturant gradients, from 15 to 55% (100% denaturant was 7 M urea plus 40% [wt/vol] formamide) increasing in the direction of electrophoresis. The gels were analyzed by electrophoresis for 10 min at 50 V and for 4 h at 200 V, stained with ethidium bromide for 15 min and rinsed for 20 min in distilled water and photographed under UV illumination.

F. Sequencing of DGGE bands Small pieces of selected DGGE fragments were punched from the gel with sterile pipette tips. Then the pieces were transferred into 50 µl of sterile water and incubated overnight at 4°C allow diffusion of the DNA. Two microliters of the eluted DNA were used for re-amplification with primer without the GC clamp. PCR products purified by using QIAquick PCR purification kit (QIAGEN, Milan, Italy) and sequences were carried out by Refgen (Ankara,Turkey).

G. Sequence analysis Searches in the GenBank with the BLAST program were performed to determine the closest known relatives of the partial 16S rDNA sequences obtained.

III. RESULTS AND DISCUSSION

In this research, the microbial diversity was assessed by a PCR-DGGE approach to identify the main populations involved in the fermentation process of the sausage/sucuk. The principal PCR-DGGE protocol based on the analysis of variable V3 region of the 16S rRNA gene was used and generally obtained good differentiation between the mixed populations analyzed after DNA extraction directly from sucuk samples. The results obtained from the analyses were shown in Fig. 1, and as could be seen multiple bands were detected in most of the samples.

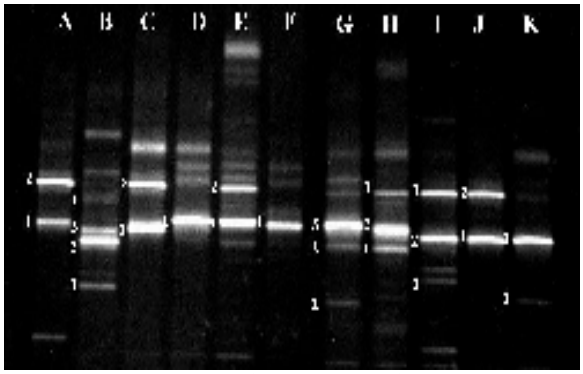

Figure 1. DGGE profiles of the V3 region of the 16S rRNA gene obtained by PCR amplification of DNA extracted directly from the 10 sucuk samples A, B, C, D, E, F, G, H, I, and J. identification of each band is presented in Table 1

All the bands were excised from the gel and reamplified prior to sequencing, and the results of the identification were presented in Table 1.

Table 1. Sequence information for DGGE bands obtained by analyzing the V3 region of 16S rRNA gene of DNA extracted directly from sucuk samples.

Bands No*	Closest relative	Accession number	Identy (%)
A1, C1, D1, E1, F1, G3,H2	*Lactobacillus sakei*	FJ040202.1	99
A2, C2, E2, H3, I3, J2	*Wisella viridescens*	DQ102386.1	99
B1, K1	*Brochothrix thermosphacta*	AY543029.1	100
B2	*Psychrobacter sp.*	FJ613613.1	98
B3, I2, J1, K2	*Lactococcus lactis/ Lactobacillus curvatus/ L.graminis*	FJ171328.1/E U621984.1/A B289145.1	100
B4	*Staphylococcus lentus*	AB210947.1	97
G1	*Lactobacillus namurensis/ L.acidifarinae /L.zymae/ L.spicheri*	AM259119.1/ AJ632158.1/ AJ638157.1/ AJ634844.1	97
G2, H1	*Staphylococcus succinus*	FJ155339.1	98
I1	*Staphylococcus pscifermentans/ S.condimenti /S.carnosus*	EU727184.1/ EU727183.1/ EU727182.1	99
I4	*Wisella halotolerans*	AB022926.1	99

*:The letters and the numbers correspond to the bands shown in Fig.1

Members of the Lactobacillus and Wisella genuses were predominant bacteria in the sausages/sucuk samples tested. Mainly Lactobacillus sakei was determined in seven out of eleven samples analyzed (A, C, D, E, F, G, and H) while the species of L.lactis/L. curvatus/L.graminis were detected in samples of B, I, J, and K. These species were probably responsible bacteria for the physical and organoleptic characteristics of the sausages/sucuk tested. Again, Wisella viridescens was detected in six samples (A, C, E, H, I and K) while W. halotolerans occured only in one sample (I). Members of the Staphylococcus genus (S. pscifermentans/ S.condimenti /S.carnosus) were found in sample I while S. succinus was detected in samples G and H.

These results suggest that this species may play an important role in the ripening process of traditional fermented-meat products, as already reported by Rantsiou et al. [15]. Finally, S. lentus was found in sample B while Brochothrix thermosphacta was determined in the samples of B and K and Psychrobacter sp. only in one sample (B). Some earlier studies showed that different species may yield PCR products which comigrate in the DGGE gels due to the fact that many bacteria species are closely related, and the 16S rDNA fragment may not contain major differences to allow the separation in DGGE system [13. 16, 17]. Therefore, it can be suggested that more detailed studies might be needed and/or should be performed by investigating the other regions of the 16S rDNA gene for different species having the similar melting position. In the present study, the group of L. lactis/ L. curvatus/ L. graminis and S. pscifermentans/ S. condimenti /S. carnosus species were shown at the same DGGE profil.

In conclusion, it can be proposed that PCR-DGGE protocol clearly has super potential in the analysis of LAB communities from the fermented Turkish dry-sausage/sucuk. This method is likely to become an important tool for the investigation of microbial diversity and monitoring the presence of technologically important species during the food manufacturing processes, with the ultimate objective of quality of the product.

REFERENCES

[1] Aksu and Kaya, 2004 M.I. Aksu and M. Kaya, Effect of usage Urtica dioica L. on microbiological properties of sucuk, a Turkish dry-fermented sausage, Food Control 15 (2004) (8), pp. 591–595

[2] Randazzo CL, Torriani S, Akkermans ADL, de Vos WM and Vaughan EE .2002. Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA Analysis. Appl. Environ. Microbiol, Vol. 68, 1882-1892.

[3] Millet,V., Lonvaud-Funel, A., 2000. The viable but non-culturable state of wine micro-organisms during storage. Lett. Appl. Microbiol. 30, 136-141.

[4] Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.

[5] ben Omar N & Ampe F (2001) Microbial community dynamics during production of the Mexican fermented maize dough Pozol. Appl Environ Microbiol 66: 3664–3673.

[6] Muyzer, G., 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. Curr. Opin. Microbiol. 2, 317–322.

[7] Cocolin, L., Bisson, L.F., Mills, D.A., 2000. Direct profiling of the yeast dynamics in wine fermentations. FEMS Microbiol. Lett. 189, 81– 87.

[8] Cocolin, L., Manzano, M., Cantoni, C., Comi, G., 2001. Denaturing gradient gel electrophoresis analysis of the 16S rRNA

gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. Appl. Environ. Microbiol. 67, 5113– 5121.

[9] Norris, T.B., Wraith, J.M., Castenholz, R.W., McDermott, T.R., 2002. Soil microbial community structure across a thermal gradient following a geothermal heating event. Appl. Environ. Microbiol. 68, 6300–6309

[10] Fontana, C., G. Vignolo, and P. Cocconcelli. 2005. PCR-DGGE analysis for the identification of microbial populations from Argentinean dry fermented sausages. J. Microbiol. Methods 63:254–263.

[11] Coppola, S., Blaiotta, G., Ercolini, D., Moschetti, G., 2001. Molecular evaluation of microbial diversity occurring in different types of Mozzarella cheese. J. Appl. Microbiol. 90, 414–420.

[12] Ferris, M.J., Muyzer, G., Ward, D.M., 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA defined populations inhabiting a hot spring microbial mat community. Appl. Environ. Microbiol. 62, 340–346.

[13] Meroth, C., Walter, J., Hertel, C., Brendt, M., Hammes, W., 2003. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 69, 475– 482.

[14] Ampe, F., Ben Omar, N., Moizan, C., Wacher C. and Guyot, J.P. 1999. Polyphasic study of the spatial distribution of microorganisms in Mexican Pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations, Appl. Environ. Microbioli 65, 5464–5473.

[15] Rantsiou K., Cocolin L. 2006. New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: a review. International Journal of Food Microbiology, 108, 255-267.

[16] Ercolini, D., Moschetti, G., Blaiotta, G., Coppola, S., 2001. Behavior of variable V3 region from 16S rDNA of important lactic acid bacteria in denaturing gradient gel electrophoresis. Curr. Microbiol. 42, 199– 202.

[17] Ercolini, D., Hill, P.J., Dodd, C.E.R., 2003. Bacterial communities structure and location in Stilton cheese. Appl. Environ. Microbiol. 69, 3540–3548.