**Application of the PCR-DGGE technique to the fungal community of traditional Wielkopolska
fried ripened curd cheese to determine its PGI authenticity**

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ABSTRACT

Fried cheese is a traditional dairy product from the Wielkopolska region in Poland made from cow's milk having a Protected Geographical Indication (PGI) status. In this study, the yeast population of Wielkopolska fried cheese was analyzed by the microbial culture-independent technique of PCR-DGGE to examine if there was a link between the yeasts community present in the product and the geographical origin of the cheese. PCR-DGGE was performed with total DNA isolated from five cheese samples from Wielkopolska and one from the Silesia, a region out of the PGI area. The dominant eukaryotic community of this traditional product, which was investigated here for the first time, was shown to be composed by species of *Galactomyces geotrichum*, *Kluyveromyces marxianus*, *Pichia kudriavzevii*, *Candida inconspicua*, and *Trichosporon* spp. To look for correlations between geographical origin and microbiota composition, the DGGE fingerprinting profiles were subjected to a multivariate analysis, which included k-means clustering and principal component analysis (PCA). Our results showed that PCR-DGGE is a promising method for the traceability of Wielkopolska fried cheese and for extension other microbial-enriched foodstuffs.

KEYWORDS:

Cheese, traditional dairy product, fried cheese, PCR-DGGE, Protected Designation of Origin, Protected Geographical Indication, 26S rDNA fingerprinting

1. INTRODUCTION

Nowadays, European consumers pay increasing importance to the quality and authenticity of foodstuffs. European Union (EU) regulations provide the scopes which allow preserving the food products specific characteristics and their geographical origin. The originality of agriculture products and foodstuffs may be protected from counterfeit by Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) or Traditional Specialty Guaranteed status under EU Council Regulations 509/2006 and 510/2006 [1,2].

In many cases the microbiota composition of foodstuffs is closely related to the place of growth, process or manufacture. Application of culture-independent techniques based on Polymerase Chain Reaction combined with Denaturing Gradient Gel Electrophoresis (PCR-DGGE) was used as a traceability tool to determine geographical origin of food (Arcuri et al. 2013, El Sheikha et al., 2009, Montet et al. 2008). The use of PCR-DGGE allowed to establish the geographical origin of Tilapia fish derived from three lakes in Cameroon (Maiwore et al. 2009; Tatsadjieu et al., 2010); Pangasius fish angled in aquaculture farm from Viet Nam (Le Nguyen et al. 2008); fruits (El Sheikha, 2011; El Sheikha et al., 2009; Le Nguyen et al., 2008; Montet et al., 2010, El Sheikha et al., 2012, Bigot et al., 2015), marine salt (Dufosse et al., 2013), coffee (Durand et al., 2013) and cocoa (Hamdouche et al., 2015).

There are few studies showing application of PCR-DGGE technique to link microbial profile of fermented dairy food with a product provenance. Arcuri et al. (2013) suggested that specific band profile of traditional Minas cheese microbiota from Brazil may be used as a biological bar code to discriminate its origin. Also Mauriello et al. (2003) and Ercolini, Frisso, Mauriello Salvatore, & Copolla (2008) using the PCR-DGGE technique found that natural whey cultures used for Italian Protected Designation of Origin (PDO) water-buffalo Mozzarella cheese and Caciocavallo Silano PDO cheese, respectively, were related to their respective area of production.

Fried cheese from Wielkopolska is one of the few traditional fermented products in Poland endowed with a Protected Geographical Indication (PGI) label and should not be manufactured in any other part of the country. Its traditional manufacturing process, maintained through the ages in Wielkopolska region, gives the product an original and specific character. The sensory properties of the fried cheese are closely related to the particular cheese manufacturing technology, but also to its associated microbiota which plays a key role in creating its typical aroma and taste. The curd ripened fried cheese is produced on the basis of acid Tvarog obtained from pasteurized cow milk previously treated with lactic acid bacteria (LAB) starter cultures (Cais-Sokolińska, & Pikul, 2009). During curd ripening process another proteolytic and lipolytic agents such as *Geotrichum candidum* and *Oidium lactis* appeared causing changes of pH from 4.4 to pH over 5.0 (Cais-Sokolińska, & Majcher, 2010). After the ripening process is accomplished, the cheese bulk is fried with butter until smooth texture is obtained.

To eliminate unfair competition and misleading of consumers by promoting non-genuine products, it is necessary to create an effective traceability system of food commodities. According to the UE regulation 178/2002 traceability is "the ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing and distribution".

The main aim of this study was to investigate whether curd ripened fried cheese samples from Wielkopolska region and Silesia can be differentiated by the PCR-DGGE profiles of their yeast population, thus providing an invariable molecular code which could be applied as marker for the distinction of PGI status authenticity and geographical origin. Seasonal variations in the microbiota composition were also considered. Moreover, the yeasts constituting the majority populations as revealed by DGGE were then identified by sequencing and sequence comparison.

2. MATERIAL and METHODS

2.1. Samples

Samples of curd ripened fried cheese produced in Wielkopolska region and Silesia were purchased at the market. From six group of samples, five came from Wielkopolska region and one from Silesia (Fig. 1). Fresh samples were examined by culture, and samples refrigerated at – 20 °C were analyzed by non-culture DNA-based techniques.

Fig. 1 Origins of fried cheese samples, 1 – Wielkopolska region (province) divided into counties, 2- Poland, (A – sample from Silesia, B,C,D,E,F,G – samples from Wielkopolska) 3 – grayscale of Protected Geographical Indication (PGI) logos, English and Polish version (the maps were created with Open Source Geographic Information System, QGIS 2.4.0 2014)

2.2. Culture based analysis

Ten grams of cheese samples were homogenized with 90 mL of a 2% (w/v) sterile sodium citrate solution at 40°C in a BagMixer 400W stomacher (Interscience, France). Serial of 10-fold dilutions were made in 0.85% (w/v) sodium chloride solution and plated in duplicate on to Yeast-Extract Glucose Chloramphenicol agar (BTL, Poland). Yeast and moulds were enumerated after 3-5 days of incubation at 25°C.

2.3. Total DNA extraction

DNA extraction from the cheese samples was performed in a unique step according to Arcuri et al., (2013) using phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation.

106

107 *2.4. DNA amplification*

108

109 The fragment of the D1/D2 region of the 26S rRNA gene was amplified using eukaryotic universal
110 primers: forward, NL1GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC ATA
111 TCA ATA AGC GGA GGA AAA G-3'; Sigma); reverse, LS2 (5'-AAT CCC AAA CAA CTC GAC TC-
112 3'; Sigma). A 30 bp GC clamp was linked to the forward primer in order to insure that the fragment
113 of DNA remain partially-stranded and that region screened is in the lowest melting domain
114 (Sheffield et al., 1989).

115 PCR was performed in a Thermal Cycler S1000 (Bio-Rad) in 50 µL volumes containing REDTaq
116 ReadyMix PCR ReactionMix, with MgCl₂ (Sigma, USA), 0.2 µM of the primers and approximately
117 100 ng of template DNA. The amplification was carried out as follows: An initial denaturation at
118 95°C for 5 min, 30 cycles of 95°C for 60 s, 52°C for 2 min, 72°C for 2 min and a final extension at
119 72°C for 7 min. PCR products were visualised by conventional electrophoresis in a 0.8% agarose
120 gel with TAE 1X buffer (40 mM Tris-HCl pH 7.4, 20 mM sodium acetate, 1.0 mM Na₂-EDTA) (w/v)
121 and stained with ethidium bromide.

122

123 *2.5. Denaturing gradient gel electrophoresis conditions*

124 DGGE was performed using a Bio-Rad DCode™ universal mutation detection system (Bio-Rad,
125 USA) at 60°C and 8% polyacrylamide gels (acrylamide/N,N'-methylene bisacrylamide, 37/5/1,
126 Serva) with a denaturing gradient urea formamide ranging from 30% to 60% (100% corresponded
127 to 7 M of urea and 40% [v/v] formamide, Sigma). Electrophoresis was carried out in 1X TAE buffer
128 (40 mM Tris-HCl pH 7.4, 20 mM sodium acetate, 1.0 mM Na₂-EDTA) at 20 V for 20 min and then
129 80 V for 12 h. After electrophoresis, the gels were stained for 25 min with ethidium bromide
130 (Sigma) and rinsed for 20 min in distilled water and then photographed on a UV transilluminator
131 with the GelDoc System (Bio-Rad, USA).

132 *2.6. Sequencing of DGGE bands*

133 Bands on the polyacrylamide gels were excised using a sterile blade and allowed DNA to diffuse
134 overnight into distilled water. This solution was used as a template for re-amplification reaction
135 using the same primer pairs but without GC clamp. Amplicons were then purified through
136 GenElute PCR clean up kit (Sigma, USA) and sequenced with one of the amplified primers.
137 Sequences were compared to those in the GeneBank database
138 (<http://www.ncbi.nlm.nih.gov/BLAST/>) and those of the Ribosomal Database Project
139 (<http://rdp.cme.msu.edu/index.jsp>) using the blast program (Altschul et al., 1997). Sequences with
140 a percentage identity of 97% or greater were considered to belong to the same species (Palys et
141 al., 1997).

142 *2.7. Statistical gel analysis*

The individual lanes of the gel images were aligned and proceed using GelAnalyser 2010a freeware. This software permitted to identify the bands and their relative position (retention factor, R_f) compared with the molecular weight marker as reference profile. Banding patterns were standardized with four reference patterns: *Galactomyces geotrichum* DNA, *Kluyveromyces marxianus* DNA, *Candida inconspicua* DNA and *Pichia kudravzevii* DNA that were included in all gels. The standardized banding patterns matrix was generated, the bands represent different yeast species. The DGGE fingerprints were manually scored by the presence (1) of absence (0) of co-migrating band, independent of intensity. A similarity matrix between each pair of standardized banding patterns was calculated using the Dice similarity coefficient (S_D) according to formula $2n_{AB}/(n_A + n_B)$ with n_{AB} , the number of bands common for pattern A and B, n_A , the total number of bands in pattern A, and n_B the total number of bands in pattern B. The standardized banding patterns matrix (retention factors, R_f) and the Dice similarity coefficient (S_D) matrix were used for statistical gel analysis (Tatsadjieu et al., 2010). Hierarchical cluster analysis was performed using unweighted pair-group centroid amalgamation rules with the Euclidean distance (d) measure. The tree plots were scaled to a standardized scale ($dlink/dmax*100$) and were expressed as percentage of similarity. Non-hierarchical cluster analysis (k -means clustering) was performed to form a grouping of DGGE fingerprints matrixes, into a pre-determined number of two groups. The discriminant analysis (LDA) was used in order to determine which variables discriminate samples. Principal component analysis (PCA) technique was used to dimensionality reduction of data and to present the samples in new coordinate system. The statistical analysis was performed using Statistica software, Version 10, StatSoft Inc., OK-US.

3. RESULTS

3.1. Viable fungi population

Enumeration of yeasts and moulds in cheese samples were done. Viable fungi were not detected in curd ripened fried cheese samples cultured on the YGC counting plates. This indicates that the final stage of the technological process (frying) ensures the sterility of the product.

3.2. PCR-DGGE fingerprinting and sequencing

In this study genetic diversity of the fungi in curd ripened fried cheeses was determined by means of PCR–DGGE technique. Amplification of D1/D2 region of 26S rDNA was performed by conventional PCR using universal primer. PCR products that had the same number of bases were separated by denaturing gradient gel electrophoresis allowing extracting single bands reflecting individual microorganism present in examined sample (Fig. 2). Previously extracted, re-amplified and purified DNA was sequenced.

The results of DGGE analysis and sequencing are shown in Fig. 2. Previously isolated, purified and sequenced DNA from the gel was used as the marker. There was lack of band 1 in the lane A

referring to the sample from Silesia region. Band 1 corresponding to *Galactomyces geotrichum* (GenBank accession number KC844829, homology 99%) was present in all of analyzed samples from Wielkopolska region. Band 2 corresponding to *Kluyveromyces marxianus* (GenBank accession number KC512907, homology 99%) appeared on almost all lanes except lane G. *Trichosporon sp.* (GenBank accession number JF781334, homology 100%), *Pichia kudriavzevii* (KF247215, homology 100%), *Candida incospicua* (AB179767.1, homology 99%) were present in the lane G, D, B, respectively.

Figure 2. PCR-DGGE 26S rDNA band profiles of curd ripened fried cheese from 7 manufacturers (summer batch), A – from Silesia, B – G from Wielkopolska region, M – DNA marker (1- *Galactomyces geotrichum*, 2 – *Kluyveromyces marxianus*, 3 – *Candida incospicua*, 4 – *Pichia kudriavzevii*), lane G, band no. 5 – *Trichosporon sp.*)

3.3. Statistical analysis

197 The DGGE 26S rDNA band profiles of curd ripened fried cheese from 7 manufacturers were
 198 compared. The standardized banding patterns matrix (retention factors (R_f) of raw tracks, presented in
 199 Fig. 2) and the Dice similarity coefficient (S_D) matrix (Tab. 1) were used for comparison of fungi
 200 profiles of cheeses. Two hypotheses were tested: the possibility to use the results of PCR-DGGE
 201 technique as a traceability tool, and usefulness of retention factor matrix to distinguish of two groups
 202 (samples from Silesia and Wielkopolska region). This type of cheese, registered as 'Wielkopolski ser
 203 smażony' (commission regulation (EC) No 323/2009), is produced in two provinces in Poland. The
 204 areas of production of registered cheese (PGI) and non-original one are close to each other. Different
 205 techniques were used to demonstrate the differences between the above-mentioned groups. The Dice
 206 similarity coefficient (S_D) is the best estimator for calculation of genetic divergence using DNA
 207 fragment patterns (Heyndrickx et al., 1996) but more convenient is to use the retention factor (R_f)
 208 matrix when any absolute reference product is not available. The S_D factor is not useful to categorise a
 209 new sample when the boundaries of the similarities are not fixed, additionally the reference point is not
 210 stable (DGGE patterns of registered cheese samples (PGI) are not the same). Calculation of statistical
 211 model based on the raw coefficients (R_f) which allows to distinguish samples of cheese and gives
 212 opportunity to add unknown sample to this model is more convenient. Different multivariate exploratory
 213 techniques were used to verify the hypotheses assumed.

214
 215 Table 1 The Dice similarity coefficient (S_D) matrix between each pair of standardized banding patterns
 216 (DGGE fingerprints of the fungi); origin of fried cheese samples (Poland): A – sample from Silesia
 217 region; B,C,D,E,F,G – samples from Wielkopolska region; the numerical values under the diagonal are
 218 a mirror image of the values encoded above the diagonal.

S_D set number	1(A)	2(B)	3(C)	4(D)	5(E)	6(F)	7(G)
1(A)	1	AB	AC	AD	AE	AF	AG
2(B)	0.5	1	BC	BD	BE	BF	BG
3(C)	0.29	0.55	1	CD	CE	CF	CG
4(D)	0.33	0.6	0.89	1	DE	DF	DG
5(E)	0.5	0.67	0.73	0.8	1	EF	EG
6(F)	0.33	0.4	0.44	0.5	0.6	1	FG
7(G)	0.57	0.73	0.4	0.5	0.55	0.67	1

219
 220 The cluster analysis was used to organize observed data into meaningful structures, the variation in
 221 DGGE fingerprint patterns was considered. Cluster analysis works by attempting to group objects with
 222 the closest relationships through exploratory data analysis. Those without any close connection are
 223 not grouped. This technique was utilised to seek out patterns and connections in data without giving
 224 understanding of the rules. The variations of fried cheese pieces were assayed, (Fig. 3). The analysis
 225 of hierarchical tree showed that the fungi profile of sample A is similar to F and G, and these samples

formed one cluster when the standardized banding pattern (R_f) was recognised. The sample A was the least similar to others when the Dice similarity coefficient (S_D) matrix was used. This confirms the statement that S_D coefficient is the best estimator for calculation of genetic divergence but unfortunately the results are not satisfactory. The sample A, which is a non-registered cheese (PGI), tends to be the most dissimilar but discrimination of this sample with hierarchical cluster analysis is not unequivocal.

Figure 3 Dendrogram based on the fungi DGGE fingerprints clustering of standardized banding pattern (R_f) (A) and the Dice similarity coefficient (S_D) matrix (B), origin of fried cheese samples (Poland): A – sample from Silesia region; B,C,D,E,F,G – samples from Wielkopolska region

Using the k-means algorithm, results could be divided into two groups (1 – DGGE patterns of non-registered, 2 – DGGE patterns of registered cheese samples). The variables with discriminant power were ascertained using the ANOVA (Analysis of variance) results, which provide information about the dissimilarities in the mean averages of the continuous variables, Tab. 2 and Tab. 3. It was noted that *Galactomyces geotrichum* and *Pichia kudravzevii* ($p < 0.05$) from variables set presented in Tab. 2 and S_D number 1(A) from variables set showed in Tab. 3 had sufficient discrimination power. The final classification was different for these data sets. The samples were correctly assigned when the Dice similarity coefficient (S_D) was used. The discriminant analysis (LDA) showed that it was possible to discriminate samples with the retention factors (R_f). The variable *Kluyveromyces marxianus* properly discriminate cheese samples using LDA results, the rest of the variables were not in the model. The LDA model was not considered because its discriminatory power was based on one variable only.

Table 2 The *k*-means clustering results based on standardized banding pattern (R_f values), computed with the fungi DGGE fingerprints, origin of fried cheese samples (Poland): A – sample from Silesia region; B,C,D,E,F,G – samples from Wielkopolska region

ANOVA results			Final cluster members		
Reference pattern	F	p value	Sample	Final classification	Distance to centroid
<i>Galactomyces geotrichum</i>	9.2	0.0289			
<i>Galactomyces geotrichum</i>	0.0	0.8496			
unidentified	0.0	0.8508	A	1	1.15
unidentified	6.4	0.0522	F	1	1.50
<i>Trichosporon sp.</i>	0.1	0.7200	G	1	1.38
<i>Kluyveromyces marxianus</i>	1.4	0.2856	B	2	1.44
<i>Candida intermedia</i>	0.7	0.4366	C	2	1.44
<i>Candida inconspicua</i>	5.7	0.0624	D	2	1.25
unidentified	0.7	0.4366	E	2	1.45
<i>Pichia kudravzevii</i>	450676.8	0.0000			

Table 3 The *k*-means clustering results based on the Dice similarity coefficient (S_D) matrix computed with the fungi DGGE fingerprints clustering of standardized banding pattern, origin of fried cheese samples (Poland): A – sample from Silesia region; B,C,D,E,F,G – samples from Wielkopolska region

ANOVA results			Final cluster members		
S_D set number matrix	F	p value	sample	Final classification	Distance to centroid
1(A)	20.9	0.0060	A	1	0.00
2(B)	0.5	0.5055	B	2	0.72
3(C)	2.1	0.2080	C	2	0.76
4(D)	2.8	0.1563	D	2	0.64
5(E)	1.6	0.2601	E	2	0.61
6(F)	1.3	0.3067	F	2	0.90
7(G)	0.1	0.7766	G	2	0.89

The interdependence of the variables and the way they effected the organisation of data was described using a Principal Component Analysis (PCA). Each of the main components forms a linear group of variables and these groups are perpendicular to each other. PCA was used in this study to decrease the amount of variables rather than its more common application as a method of dimensional reduction of the data. Loadings plot is the name often given to the principle components' (PC) coordinates and interpreting the (PCs) of the variables was greatly simplified through the plotting of these data. These graphs (Fig. 4A, 5A) were drawn on the plan of sphere (x-loadings on PCs). The largest principal component coordinate (variable-PC correlation) which can arise, as a result of the

present analysis being based on correlations, is 1.0. By projecting the new variables onto the sphere plan, analysis of the original variables, how they related to each other and their influence over the new system of coordinates, was simplified.

The three new variables (PCs) were introduced, these PCs explained about 79 and 91% of the data variance, for the standardized banding pattern (retention factors, R_f) (Fig. 3) and the Dice similarity coefficient (S_D) matrix (Fig. 4) respectively. As observed in Fig. 3B and 4B, it was possible to distinguish two groups. One cluster represented sample (A) from Silesia region, the second one included samples from Wielkopolska region (B,C,D,E,F,G). It was indicated that parameters for these groups were definitely different. The simultaneous comparison loadings plot (Fig. 3A, 4A) with appropriate score plot (Fig. 3B, 4B) allowed to identify the relationships between samples and variables. The position of sample A was determined by low/absence of R_f values of three variables, namely: *Galactomyces geotrichum*, *Kluyveromyces marxianus*, *Pichia kudravzevii* (Fig. 3). The PCA combined the results obtained by the *k*-means algorithm (*Galactomyces geotrichum* and *Pichia kudravzevii* were discriminant variables), and LDA (*Kluyveromyces marxianus* variable properly discriminated cheese samples). The position of sample A (Fig. 4) was determined by 1(A) variable (S_D set number 1(A), Tab. 1), that means that curd ripened fried cheese A (from Silesia) was the least similar to other samples from 6 manufacturers from Wielkopolska region. It can be concluded on the basis of statistical analysis that it was possible to clearly distinguish the samples of curd ripened fried cheeses by using DGGE fingerprints of fungi. The PCA calculated models showed the possibility to distinguish samples by using of raw coefficients (retention factors, R_f) and the Dice similarity coefficient (S_D) matrix.

Figure 3 Three-dimensional plot representing the PCA of loadings plot (A) and score plot (B) for tested curd ripened fried cheese, computed with standardized banding pattern (R_f values) of the fungi DGGE fingerprints, loading plot variables (DNA markers): 1, 7- *Galactomyces geotrichum*, 2 – *Kluyveromyces marxianus*, 3 – *Candida inconspicua*, 4 – *Pichia kudravzevii*, 5 – *Trichosporon* sp., 8 – unidentified),

score plot – origin of fried cheese samples (Poland): A – sample from Silesia region; B,C,D,E,F,G – samples from Wielkopolska region

Figure 4 Three-dimensional plot representing the PCA of loadings plot (A) and score plot (B) for tested curd ripened fried cheese, computed with the Dice similarity coefficient (S_D) matrix of the fungi DGGE fingerprints, loading plot variables (DNA markers): 1, 7- *Galactomyces geotrichum*, 2 – *Kluyveromyces marxianus*, 3 – *Candida inconspicua*, 4 – *Pichia kudravzevii*, 5 – *Trichosporon* sp., 8 – unidentified), score plot – origin of fried cheese samples (Poland): A – sample from Silesia region; B,C,D,E,F,G – samples from Wielkopolska region

4. DISCUSSION

DGGE fingerprinting was used in this work for the geographical origin determination of curd ripened fried cheeses from two regions. The application of this technique to discriminate the origin of traditional fermented dairy products such as Minas cheese (Arcuri et al., 2013), Castelmagno PDO cheese (Dolci et al., 2010), Fontina PDO cheese (Dolci et al., 2013), Danish surface ripened cheese (Gori et al., 2013), May bryndza cheese (Pangallo et al., 2014) and Stilton PDO cheese (Gkatzionis et al., 2014) has been already shown. PCR-DGGE is one of the culture-independent methods that in comparison with culture-dependent methods is less time-consuming, more accurate, specific and allows to identify unculturable microorganisms (Ercolini, 2004). This work shows that applying only culture-dependent methods is not sufficient to discover all microbiota present in final product. It is worth noting that cheese frying, which is the final production stage, reduces the amount of fungi below the detection limit. Most of microorganisms identified during the production of curd ripened fried cheese are not hazardous, however they do not have GRAS status (Generally Recognized As Safe). PCR-DGGE is a valuable method in terms of food safety and food quality and it is useful for

traceability of food. This type of research is important to prevent food adulteration in the future. Recently some reported cases of food adulteration and strategies to monitor food chains were reviewed by Manning and Soon, 2014.

There are few papers concerning the study of Polish fried cottage cheese. It is the first work describing its yeast microbiota. Majcher et al. (2014) analysed its specific aroma compounds (the highest concentration of 2,3-butanedione, phenylacetic acid and 2-phenylethanol). In our study the cheese sample from Silesia differentiated considerably from cheese samples from Wielkopolska. The samples from Wielkopolska are distinguished by the presence of *Galactomyces geotrichum*, microorganism also found in products such as Slovak bryndza cheese (Chebeňová-Turcovská et al., 2011), Cotija cheese (Flores-Magallón et al., 2011), Argentinian autochthonal cheese starters (Binetti et al., 2013) and boza (Bulgarian cereal-based fermented beverage) (Osimani et al., 2015). It is also responsible for the flavour formation of fried cheese (Majcher et al., 2014). PCR-DGGE has also indicated minor differences within Wielkopolska region which samples had more diverse microbiota while the band representing cheese from Silesia showed only the presence of *Kluyveromyces marxianus*. Results also highlighted the appearance of *Candida inconspicua*, *Pichia kudriavzevii* and *Trichosporon* sp. in cheese from Wielkopolska. *Kluyveromyces marxianus*, *Candida inconspicua* and *Trichosporon* are autochthonous among fungi that can be found in raw milk (Delavenne et al., 2011).

Kluyveromyces marxianus is thermotolerant yeast and it is able to utilize lactose and inulin (Lane, Morrissey, 2010). It is the dominant species in dairy products such as Gouda cheese (Welthagen, Viljoen, 1999), Pecorino di Farindola cheese (Tofalo et al., 2014), Camembert cheese (Baudrit et al., 2010), Serro Minas cheese (Cardoso et al., 2015), kefir (Gao et al., 2012) and Koumiss (Mu et al., 2012). It is one of the major species found during the fermentation of cocoa (Ho et al., 2014). *K. marxianus* was associated with the production of acids, ethyl decanoate, 1-propanol and benzaldehyde in traditional ewes' and goats' cheeses (Padilla et al., 2014). It is also capable of synthesizing 2-phenylethanol, 2-phenylethyl acetate and ethyl acetate and has a wide range of application in industry (Morrissey et al., 2015).

Pichia kudriavzevii (formerly *Issatchenkia orientalis*, anamorph *Candida krusei*) can be found in various foodstuffs such as dairy products (Cardoso et al., 2015, Gao et al., 2012), fermented cassava products (Coulin et al., 2006, Oguntinyinbo et al., 2011, Freire et al., 2014, Ramos et al., 2015), cocoa (Papalexandratou, De Vuyst, 2011, Hamdouche et al., 2015) and is important for chocolate flavour formation (Ho et al., 2014). It was also reported that *P. kudriavzevii* is responsible for producing off-flavour odours and texture softening in kimchi (Moon et al., 2014), is one of the yeast producing terpenoids in Chinese light-style liquor (Wu et al., 2015). Both *P. kudriavzevii* and *G. geotrichum* are present in Chinese liquor fermentation starter Daqu and they participate in liquor flavour formation (Zhang et al., 2014).

Yeast play an important role in cheese ripening being responsible for deacidification, flavour formation and texture development (Beresford et al., 2001). Based on our results we can assume that the identified yeast can be associated with the characteristic flavour of fried cheese from Wielkopolska.

To show the influence of the place where the cheese was manufactured multivariate analysis was conducted. K-means clustering was used for the first time to diversify cheese samples into separate groups. Depending on used pattern matrix (R_f or S_D) different clusters were obtained. The analysis using the standardized banding pattern (R_f) showed that sample from Silesia was similar to two samples from Wielkopolska, whereas the analysis using Dice similarity coefficient (S_D) showed significant difference of sample from Silesia from the others. PCA was used because only one variable in LDA model had discriminatory power (such model is not sufficiently robust). PCA permitted to group samples according to their origin, two groups were distinguished, one with sample from Silesia and second one with samples from Wielkopolska. PCA is rarely used for the analysis of PCR-DGGE data. Recent papers applying this method include analysing the relationships between bacterial species in traditional fermentation starters for Hong Qu glutinous rice wine (Lv et al., 2012), microorganisms in Shanxi aged vinegar (Nie et al., 2015), microorganisms in the rhizosphere soil of Bt and non-Bt cottons in China (Zhang et al., 2015), microbial community in different Pu'er tea samples (Tian et al., 2013), bacteria present in different stages of the spontaneous ngari fermentation (dry fermented fish product) (Devi et al., 2015), correlation between volatile compounds and microorganisms during fermentation of yakupa (Brazilian fermented cassava beverage) (Freire et al., 2014), phyllosphere of lettuce (Zwiehler et al., 2008) and to discriminate farming types of nectarines and peaches (Bigot et al., 2015).

PCA is a valuable tool to discriminate samples into groups and to show correlation between samples. PCA allows to reduce the dataset and is sensitive to outliers that may change the result. So far mostly factorial variance analysis (FVA) was used to compare the similarities of the microbial communities (Le Nguyen et al., 2008, El Sheikha et al., 2009, El Sheikha et al., 2012, Arcuri et al., 2013). PCA was not sufficient during the analysis of DGGE fingerprints of nectarines and peaches so PLS-DA was used for more accurate results (Bigot et al., 2015).

5. CONCLUSION

The application of PCR-DGGE to determine geographical location of cheese certified with PGI status is the first work showing possible use of microbes as the marker. It may be a rapid analytical tool to study the dynamics of microbiota specific for food product and to obtain biological bar code.

Results showed in this paper indicate the existence of invariable microbes in curd ripened fried cheeses from Wielkopolska region that can have an application as a marker to determine the authenticity of certified foodstuffs.

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