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MOLECULAR ANALYSIS OF POULTRY MEAT SPOILING MICROBIOTA AND HETEROGENEITY OF THEIR PROTEOLYTIC AND LIPOLYTIC ENZYME ACTIVITIES

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Psychrotrophic *Pseudomonas* species *P. fluorescens*, *P. fragi* and *P. lundensis* were found as predominant bacteria of chicken meat stored at chill temperature, which showed high level of molecular diversity, while isolates of the psychrotrophic yeasts *Candida zeylanoides*, *Metschnikowia pulcherrima*, *Rhodotorula glutinis* and *Rhodotorula mucilaginosa* formed clusters of high level similarity within the different species as revealed by RAPD-PCR analysis.

Combination of multiplex PCR and sequencing of the *rpo*B gene resulted correct identification of the *Pseudomas* isolates, while the routine diagnostic tests led to improper identification in case of half of the isolates, which indicated the extended biochemical and physiological heterogeneity of the food-borne pseudomonads. Majority of *P. fluorescens* and *P. lundensis* isolates were strong protease and lipase producers, while *P. fragi* strains were week or negative from this respect. Proteolytic and lipolytic activities of the isolated yeast strains were species specific and protease production was less frequent than lipolytic activities.

Keywords: poultry meat, spoilage, *Pseudomonas*, yeasts, molecular characterisation, protease, lipase

Meat spoilage is determined by numerous environmental factors, although the metabolic activity of microorganisms plays the most important role. In case of fresh meat reduction of freshness and progress of spoilage can be traced back to the growth and increase of metabolic activity of different microorganisms, mainly bacteria (Gallo et al., 1998; Horváth et al., 2007b; Ercolini et al., 2009), although yeasts are also present in a substantial but much less number in the fresh and spoiled meat (Viljoen et al., 1998; Ismail et al., 2000).

Initial total aerobic bacterial count of poultry carcass is generally ranging from 10^2 to 10^6 CFU g^{-1} but it depends on several factors including origin of poultry, farm and processing hygiene, processing technology and external temperature (Geornaras et al., 1995). Increase in the sizes of bacterial populations is controlled mainly by the storage temperatures, therefore populations of psychrotrophic microorganisms are selected out during chilled storage of meat (McMeekin, 1982; Lucianez et al., 2010).

Predominant bacteria that are associated with the original spoiling microbiota of poultry meat mainly belong to different taxa of *Gammaproteobacteria*; the most important genera are *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Shewanella*, *Aeromonas* but certain genera of *Enterobacteriaceae* are also among the meat contaminating microbiota. Certain species of the genera *Flavobacterium* and *Chryseobacterium* – that belong to the Phylum *Bacteroidetes* – are also among the initial bacterial populations in considerable ratio (Jooste & Hugo, 1999).

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Population dynamics of the evolving microbiota is, however, highly influenced by the environmental conditions of storage (Mead, 1982; McMeekin, 1982; Horváth et al., 2007b). *Pseudomonas* species are predominant in the fresh and spoiled meat, while the diversity of other species is changing considerably as the storage is proceeding (Gallo et al., 1998). Psychrotrophic bacteria other than *Pseudomonas* that are also present in chill stored meat most frequently belong to *Flavobacterium*, *Schewanella*, *Aeromonas*, *Psychrobacter*, *Alcaligenes* and *Citrobacter* but all are unable to compete in growth with the pseudomonads during storage (McMeekin, 1982; Jay et al., 2003).

Reliable detection, characterisation and identification of pathogenic and food spoilage microbes in raw and processed foods are the prerequisites for fulfilling the demand for the production of safe food and protection of consumers' health (Horváth et al., 2007a; Deák, 2008; Jasson et al., 2010).

PCR-based molecular biological techniques have excellent specificity, sufficient sensitivity and because the time required for the analysis is much shorter than in the case of conventional methods they seem promising for the detection and identification of pathogenic and spoiling microorganisms (Baleiras Couto et al., 1995; Justé et al., 2008; Jasson et al., 2010). *P. fragi*, *P. lundensis*, *P. putida* and *P. fluorescens* are considered as being the most important psychrotrophic meat spoiling *Pseudomonas* species (Stanbridge & Davis, 1998), therefore Ercolini and co-workers (2007) developed a multiplex PCR assay for the simultaneous identification of the first three species based on the co-amplification of different regions of the *car*A gene.

While sequence analysis of the 16S rRNA gene is widely used in molecular identification of bacteria, the sequence differences within this region are not discriminatory enough in the case of *Pseudomonas* for species level identification (Anzai et al., 2000). Tayeb and co-workers (2005) found that the phylogenetic resolution of the *rpo*B tree was approximately three times higher than that of the 16S rDNA tree, which allowed more exact identification of a high number of *Pseudomonas* isolates studied.

Yeast microbiota of fresh poultry meat comprises species belonging to either Ascomycetes or Basidiomycetes but strongly fermentative species are sparse. *Yarrowia lipolytica* and *Candida zeylanoides* were reported being the predominant species in most cases. They comprise as much as 55–65% of the total yeast isolates originated from different raw and processed poultry meat but other *Candida* and different *Cryptococcus*, *Rhodotorula*, *Debaryomyces* and *Trichosporon* species have also been detected in considerable ratio (Viljoen et al., 1998; Ismail et al., 2000). Molecular identification and typing techniques proved to be much more reliable tools in the identification and characterisation of yeasts than the traditional phenotype-based techniques (Baleiras Couto et al., 1995; Kurtzman & Robnett, 1998; Majoros et al., 2003; Lopandic et al., 2006) but phenotypic characterisation is still very important, especially if used for studying the physiology and enzyme activities of natural yeast strains (Boekhout & Robert, 2003).

Extracellular enzymes of spoiling microorganisms like proteases and lipases play an important role in the spoilage process (Gilmour & Rowe, 1990). Raw milk and dairy products spoiling *Pseudomonas* spp. were reported producing different hydrolytic enzymes mainly heat-resistant proteases and lipases at chill temperatures that are highly responsible for the spoilage of heat-processed dairy products (Rajmohan et al., 2002; Dogan & Boor, 2003; Marchand et al., 2009). Proteases of meat spoiling pseudomonads were extensively studied and their role in the production of volatile organic compounds responsible for the quality

decrease of spoiled meat has been proved (Vanderzant & Ousley, 1963; Ercolini et al., 2009).

Protease production by food-borne yeasts is far less well documented than that of the food spoiling bacteria. Studies concerned mainly the importance of protease activity in fermentations of alcoholic beverages and just a few yeast proteases have been studied for potential applications (Abranches et al., 1997).

Lipolytic enzymes produced by microorganisms belong to (i) carboxylesterases (EC 3.1.1.1) which hydrolyse small ester containing molecules at least partly soluble in water, (ii) true lipases (EC 3.1.1.3) that hydrolyse water insoluble triglycerides and (iii) different types of phospholipases. In case of *Pseudomonas* the first two types of enzymes are responsible mainly for the hydrolysis of meat lipids (Arpigni & Jeager, 1999; Gupta et al., 2004), while phospholipases are important virulence factors of pathogenic *Pseudomonas* spp. (Kulasakara et al., 2006). Yeasts are important sources of lipases produced for industrial and biotechnological purposes and several yeast species were reported as sources of commercial lipases. Lipase producers belong mainly to the *Candida* genus but *Yarrowia lipolytica* and certain *Pichia* and *Rhodotorula* spp. were also published as producers of extracellular lipases (Corzo & Revah, 1999; Sharma et al., 2001). Ismail and co-workers (2000) found that majority of *C. zeylanoides* and *Y. lipolytica* strains which were isolated from fresh and spoiled poultry meat possessed both protease and lipase activities, however, their role in the spoiling process is difficult to estimate, because concentration of yeast cells is two to four orders of magnitude lower than that of the bacteria.

Our aim was to compare the identification potential of the traditional miniaturised bacterial and yeast identification systems with that of the molecular identification approaches based on the PCR analysis of phylogenetically relevant genes. By the application of the selected most reliable molecular identification techniques we wanted to get an insight into the diversity of poultry meat spoiling pseudomonads and yeasts, and to study the population dynamics of the most frequently occurring species. Moreover, by the determination of the proteolytic and lipolytic activities of the correctly identified and typed food isolates we wanted to assess the spoiling potential of the different *Pseudomonas* and yeast species.

1. Materials and methods

1.1. Isolation and characterization of bacterial and yeast strains

Table 1 contains the type and reference strains of bacteria and yeasts that were used as controls in this study.

Isolation of bacteria and yeasts from chicken meat was as follows. Chicken upper leg samples were stored at 4 °C for 5 days (until shelf-life expiration) and samples were taken in regular intervals from the homogenised upper leg (without bones) and from the separated skin. Ten grams of samples were pummelled in 90 ml of 0.1% saline – peptone water with BagMixer (Interscience, France) and decimal dilutions were made in 0.1% saline – peptone water. In case of bacteria the CFU g^{-1} were determined by pour plate technique using TGYN agar [TGY (0.5% w/v peptone, 0.1% w/v glucose, 0.25% w/v yeast extract and 1.5% w/v agar) supplemented with nystatin (0.01% w/v)], while CFU g^{-1} of yeasts and moulds were defined by spread plate technique on YPDC agar plates [YPD (0.5% w/v yeast extract, 0.5% w/v peptone, 1% w/v glucose and 1.5% w/v agar) supplemented with chloramphenicol (0.01% w/v)]. After inoculation the TGYN and YPDC plates were incubated at 30 °C and

Table 1. List of type and reference strains used as controls in this study

Species	Strain	Origin
BACTERIA		
P. lundensis	CCM 3503	CCM[a]
P. fragi	CCM 3903	CCM
P. putida	DSM 291	DSMZ[b]
P. taetrolens	CCM 1982[T]	CCM
P. mendocina	CCM 3590[T]	CCM
P. fragi	CCM 3704	CCM
P. stutzeri	CCM 4557[T]	CCM
P. lundensis	CCM 3907	CCM
P. fluorescens	CCM 3899	CCM
P. diminuta	B01118[T]	NCAIM[c]
P. aeruginosa	ATCC 10145[T]	ATCC[d]
P. putida	B.01634[T]	NCAIM
Burkholderia cepacia (syn.: *P. cepacia)*	B.01621	NCAIM
Aeromonas sobria	CCM 2807[T]	CCM
Aeromonas hydrophila	CCM 7232[T]	CCM
E. coli	ATCC 8739	ATCC
E. coli (O157:H7)	MBT-E1	CUB[e]
Campylobacter jejuni	CCM 6214[T]	CCM
Yersinia enterocolitica	HNCMB 98001	NCE[f]
Listeria monocytogenes (4ab)	MBT-L1	CUB
Bacillus subtilis	MBT-B1	CUB
YEASTS		
Rhodotorula glutinis	Y.01235	NCAIM
Candida zeylanoides	Y.01264	NCAIM
Rhodotorula mucilaginosa	Y.01318	NCAIM
Cryptococcus curvatus	Y.01354	NCAIM
Cryptococcus laurentii	Y.01321	NCAIM
Trichosporon inkin	Y.01410	NCAIM
Trichosporon asahii	Y.01412	NCAIM
Debaryomyces hansenii (Candida famata)	Y.01579	NCAIM
Rhodotorula minuta	Y.01612	NCAIM
Metschnikowia pulcherrima	CBS 5833	CBS[g]

[a]Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic
[b]Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany
[c]National Collection of Agricultural and Industrial Microorganisms, Corvinus University of Budapest, Budapest, Hungary
[d]American Type Culture Collection, USA
[e]Corvinus University of Budapest, Department of Microbiology and Biotechnology, Budapest, Hungary
[f]National Center for Epidemiology, Budapest, Hungary
[g]Centraalbureau voor Schimmelcultures

25 °C, respectively, for 24–48 h and CFU g^{-1} of the original samples has been determined. At the end of storage the skin was carefully removed from the upper legs and samples from the muscle were taken for the isolation of bacteria and yeasts. Samples were treated as described earlier.

Representative bacterial and yeast colonies were isolated from the TGYN (T) and YPDC (Y) plates, respectively, which originated from the skin (S), homogenized chicken leg (C) and muscles (M). Codes of the isolates have been defined to indicate their origin.

Bacterial isolates were characterized by morphological examination, Gram-staining, biochemical tests (oxidase, catalase and nitrate reduction tests, glucose assimilation, carbohydrate oxidation/fermentation, citrate utilization, methyl red and Voges–Proskauer test, urease test, Eijkman reaction and indole probes).

Yeast isolates were characterized morphologically by colony morphology on YPD and WL nutrient agar (Atlas, 1995) and microscopic examination. Urease activity was determined in rapid urea broth (Atlas, 1995).

Cultures of the isolates were maintained at –80 ºC.

During the following studies – if not stated otherwise – bacterial isolates were cultured in TGY and yeasts isolates in YPD media, while temperature of incubation was 30 and 25 °C, respectively.

Temperature dependence of growth was determined by inoculating the bacterial and yeast isolates in streaks into TGY and YPD plates, respectively, followed by incubation at 5, 10, 20, 25, 37 and 42 °C.

Routine identification of the Gram-negative and Gram-positive bacteria has been performed by API 20E, API 20NE (BioMerieux) and BBL Crystal (BD Diagnostics), while ID 32C (BioMerieux) was used for the identification of yeasts.

1.2. Genomic DNA isolation

DNA extraction from bacterial and yeast cells was done by the method of Hoffman and Winston (1987) with slight modifications. Cells of overnight cultures were transferred into Eppendorf tubes containing 1.0 ml sterile ultrapure water and centrifuged at 14 000 r.p.m. for 5 min. The supernatant was discarded and 200 µl breaking buffer (2% v/v triton X-100, 1% w/v SDS, 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA; pH 8.0), 0.3 g glass beads (0.425–0.6 mm, Sartorius) and 200 µl PCIA (buffered phenol/chlorophorm/isoamyl-alcohol, v:v:v=25:24:1) were added to each tube. After vigorous mixing for 3 min 200 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) was pipetted into the samples and mixed shortly. Following the centrifugation at 14 000 r.p.m. for 5 min the upper phase was transferred to a new Eppendorf tube and 800 µl of 96% ice-cold ethanol was added to the samples. The tubes were placed into a –20 °C freezer for 10 min. The precipitated nucleic acids were centrifuged and after discarding the supernatant 50 µl TE buffer and 0.6 mg ml^{-1} RNase (Sigma) was added. The digestion of RNA molecules was performed by incubation at 60 °C for 30 min. The enzymatic reaction was stopped by adding 100 µl of 96% ice-cold ethanol to the tubes that were put into the freezer for a short time. The repeated spinning was followed by drying the DNA samples in vacuum dryer (DNA mini, Heto) and 30 µl of TE buffer was added. Until use the DNA samples were stored at –20 °C.

1.3. Typing of bacterial isolates by RAPD-PCR

For RAPD-PCR fingerprinting OPA 4 and P272 oligonucleotide primers were used in case of the *Pseudomonas* isolates while for the other bacterial isolates M13, OPA 4 and OPE 19 primers (Table 2) were applied. The 25 µl of PCR mixture contained 1× DNA polymerase buffer, 1.25 mM $MgCl_2$, 0.3 mM dNTP, 0.2 µM of primer, 0.6 U *Taq* DNA polymerase (DyNAzyme, Finnzymes) and 1 µl of the template DNA. Conditions of the PCR were the following: DNA denaturation for 5 min at 95 °C; amplification (35 cycles): pre-denaturation at 95 °C for 30 s, annealing at 40 °C for M13 and 37 °C for the other primers for 30 s and extension at 72 °C for 1.5 min; final extension took 5 min at 72 °C. The amplicons were separated by gel electrophoresis applying 1.5% agarose gel and analysed by GelCompare II (AppliedMaths, Belgium) software. Cluster analysis of the pairwise values was generated using UPGMA algorithm.

1.4. Typing of yeast isolates by RAPD-PCR

Diversity of the yeast isolates at strain level was determined by RAPD-PCR analysis. The 30 µl PCR reaction mixture contained 1× DNA polymerase buffer, 1.5 mM of $MgCl_2$, 0.17 mM dNTP, 0.5 µM of primer M13, $(GTG)_3$ or OPA 10 (Table 2), 1 U *Taq* DNA polymerase (DyNAzyme, Finnzymes) and 2.5 µl template DNA. PCR reaction was run as follows: pre-denaturation for 5 min at 95 °C; amplification (35 cycles): 95 °C for 30 s, annealing at 40 °C for M13 and $(GTG)_3$ primers while 36 ºC for OPA 10 primer for 30 s, and extension at 72 °C for 90 s; and final extension at 72 °C for 7 min. The amplicons were separated by gel electrophoresis applying 1.5% agarose gel and analysed by GelCompare II (AppliedMaths, Belgium) software. Cluster analysis of the pairwise values was generated using UPGMA algorithm.

Table 2. List of primers used in PCR reactions

Application	Primers	**Sequences (**5'-3')	Reference
P. fragi specific PCR	fra-F	CGTCAGCACCGAAAAAGCC	Ercolini et al., 2007
P. lundensis specific PCR	lun-F	TGTGGCGATTGCAGGCATT	
P. putida specific PCR	put-F	ATGCTGGTTGCYCGTGGC	
P. species specific PCR	*carA*-R	TGATGRCCSAGGCAGATRCC	
rpoB gene PCR (RFLP)	LAPS	TGGCCGAGAACCAGTTCCGCGT	Tayeb et al., 2005
	LAPS27	CGGCTTCGTCCAGCTTGTTCAG	
RAPD-PCR	OPA4	AATCGGGCTG	
	OPA10	GTGATCGCAG	Operon Technologies, Inc., USA
	OPE19	ACGGCGTATG	
	P272	AGCGGGCCAA	Mahenthiralingam et al., 1996
	M13	GAGGGTGGNGGNTCT	Vassart et al., 1987
	$(GTG)_3$	GTGGTGGTG	Senses-Ergul et at., 2006
PCR amplification of 18S rDNA for RFLP	NS1	GTAGTCATATGCTTGTCTC	White et al., 1990
	ITS2	GCTGCGTTCTTCATCGATGC	
LSU rDNA D1/D2 domain sequencing	NL1	GCATATCAATAAGCGGAGGAAAAG	Kurtzman & Robnett, 1998
	NL4	GGTCCGTGTTTCAAGACGG	

1.5. Identification of Pseudomonas lundensis, P. fragi *and* P. putida *isolates by species-specific PCR*

Distinct sequences of the carbamoyl phosphate synthase gene (*car*A) were amplified with species-specific primer sets (Table 2) for the identification of *P. lundensis*, *P. putida* and *P. fragi* isolates as described by Ercolini and co-workers (2007) with slight modifications as follows: The reaction mixtures contained 1× DNA polymerase buffer, 1.25 mM $MgCl_2$, 0.1 mM dNTP, 0.2 µM of each primer, 0.6 U *Taq* DNA polymerase (DyNAzyme, Finnzyme), and 1 µl of template DNA. The final volume of the reaction mixtures was 25 µl. The conditions of the PCR were the following: pre-denaturation at 95 °C for 4 min; amplification (25 cycles): denaturation at 94 °C for 30 s, primer annealing at 58 °C for 20 s and primer extension at 72 °C for 40 s; final extension was performed at 72 °C for 3 min. The amplicons were detected by gel electrophoresis using 1% agarose gel.

1.6. Direct sequencing of the Pseudomonas rpo*B gene amplicons*

Amplification of the *rpo*B gene using the LAPS and LAPS27 primers (Table 2) was done by the modified protocol of Tayeb and co-workers (2005). The reaction mixtures contained 1× DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 0.2 µM of each primer, 0.6 U *Taq* DNA polymerase (DyNAzyme, Finnzyme) and 1 µl of template DNA. The final volume of the reaction mixture was 25 µl. The conditions of the PCR were the following: pre-denaturation at 94 °C for 1.5 min, amplification (40 cycles): denaturation at 94 °C for 10 s, primer annealing at 55 °C for 20 s and primer extension at 72 °C for 50 s; final extension at 72 °C for 5 min. The amplified DNA was purified using PCR-Advenced™ PCR Clean Up System (Viogene) and the sequencing was performed by the BayGen Institute (Szeged, Hungary) using the LAPS and LAPS27 PCR primers.

1.7. rDNA-RFLP analysis of the yeast isolates

The PCR reaction for rDNA amplification was performed in 30 µl reaction volume. The reaction mixture contained 1× DNA polymerase buffer, 1.5 mM of $MgCl_2$, 0.17 mM dNTP, 0.5 µM of each of the primers NS1 and ITS2 (Table 2), 1 U DNA polymerase (DyNAzyme, Finnzymes) and 2.5 µl template DNA. PCR reaction was as follows: DNA pre-denaturation for 5 min at 95 °C; amplification (35 cycles): 95 °C for 30 s, 58 °C for 30 s and 72 °C for 90 s, and final extension: 72 °C for 7 min. Amplicons of approximately 2000 bp were digested with the restriction enzymes *Rsa*I, *Msp*I, *Hae*III and *ScrF*I at 37 °C for 4 h and the fragments were separated by electrophoresis using 1.5% agarose gels. The bands were visualized using ethidium-bromide staining and UV transillumination. The electrophoretic patterns were analysed by GelCompare II (AppliedMaths, Belgium) software.

1.8. Amplification and sequencing of the LSU rDNA D1/D2 domain of yeasts

Amplification of a distinct part of the D1/D2 domain of the 26S rDNA was performed using the NL1-NL4 primer pair (Kurtzman & Robnett, 1998; Table 2). Composition of the PCR reaction mixture was the same as in 1.7. Condition of PCR reaction was as follows: DNA denaturation for 5 min at 95 °C; amplification (30 cycles): 95 °C for 30 s, 53.5 °C for 30 s and 72 °C for 90 s; and final extension: 72 °C for 7 min. For nucleotide sequence determination the amplified DNA was purified using the PCR-Advenced™ PCR Clean Up System (Viogene). Sequencing was performed by using the ABI 3100 sequencer (BIOMI Ltd, Gödöllő, Hungary).

1.9. Detection of proteolytic activity

For determination of protease activity SMC (standard methods caseinate) agar and SM (skim milk) agar (ATLAS, 1995) were used. Protease activity of the isolates was determined by inoculation of the cells in the form of macrocolony onto the surface of the agar plates as follows. Ten µl of cell suspensions (10^7–10^8 cells ml^{-1}) were dropped to SMC and SM plates in duplicates. Plates were incubated for three days and diameters of the zones were measured every day. Tests were repeated twice.

1.10. Detection of lipolytic activity

For detection of esterase activity Tween™ 80 (polyoxyethylen sorbitan monooleate) Hydrolysis Medium (PAT-80) was used (ATLAS, 1995). Lipase activity was checked by the application of plate count agar (PCA; ATLAS, 1995) supplemented with tributyrin (PCATB). Bacterial and yeast strains were cultivated and esterase or lipase activity were tested as described in 1.9 but PAT-80 and PCATB agar plates were used for testing the enzyme activities. Plates were incubated for three days and diameters of turbid or clearing zones in case of PAT-80 and PCATB plates, respectively, were measured. Tests were repeated twice.

2. Results and discussion

2.1. Population dynamics of bacteria and yeasts during storage of chicken raw meat at chill temperature

Changes in the numbers of aerobic bacteria and yeasts during refrigerated storage of chicken meat samples at 4 °C until the expiration date (5 days) are shown in Table 3. The number of bacteria increased from the initial 4–5 $\log_{10}$ CFU g^{-1} to 6–8 $\log_{10}$ CFU g^{-1} during the 5 days of storage. Bacterial count of the fresh skin was more than one order of magnitude higher than that of the homogenized upper leg but this difference almost disappeared by the end of storage, what indicated that the majority of bacteria penetrated through the skin and the muscle. Skin especially supports the colonisation and growth of bacteria, because a fluid layer is evolving on the skin after immersion into water. This layer contains proteins and amino acids which provide an excellent growth medium for the spoiling microbial associations (THOMAS & MCMEEKIN, 1984). THOMAS and co-workers (1987) found that penetration of bacteria into the chicken breast muscle was the concerted action of motile, invasive and proteolytic bacterial strains, although in mixed culture with non-motile, non-invasive and non-proteolytic bacteria the formers supported penetration of the last mentioned types of bacteria.

In fresh chicken the average number of yeasts was two orders of magnitude lower than that of the bacteria (Table 3). The average CFU g^{-1} was around 2–3 $\log_{10}$, that increased slightly during the first two days of storage, but after three additional days bacteria overgrew them. GALLO and co-workers (1998) and ISMAIL and co-workers (2000) found similar tendency in the change of yeast populations during storage, especially above the expiration date, but in some cases the initial yeast count was above 4 $\log_{10}$ CFU g^{-1} (JAY & MARGARITIC, 1981; HSIEH & JAY, 1984). Even in the latter cases yeasts were not considered as important agents causing spoilage (DEÁK, 2008).

Table 3. Population dynamics of bacteria and yeasts during refrigerated storage of chicken raw meat

Storage (days)	Bacteria (log_{10} CFU g^{-1})				Yeasts (log_{10} CFU g^{-1})			
	Skin		Homogenized meat		Skin		Homogenized meat	
	1st storage	2nd storage	1st storage	2nd storage	1st storage	2nd storage	1st storage	2nd storage
0	4.90	5.13	3.50	4.53	2.87	3.21	2.00	2.86
2	4.67	6.06	3.68	5.90	2.98	4.71	2.00	4.61
5	6.43	7.87	5.90	7.74	3.94	5.32	3.19	5.20

2.2. Isolation and characterization of bacteria from chilled chicken meat

From TGYN agar plates 47 representative bacterial colonies were isolated. Selection of the isolates based mainly on the difference in colony morphology that became more uniform as the storage proceeded. However, at the later phases of storage more than one colony was isolated aiming to assess the diversity of dominant bacteria at species or strain level.

In order to determine the temperature limits of growth the isolates were incubated at six different temperatures (5, 10, 20, 25, 37 and 42 °C) on TGY agar plates for 4 days. All but two of the isolates could grow relatively well at 5 and 10 °C and the optimum growing temperature was between 20 and 25 °C. Some of the isolates had good growth at 37 °C and two of them even at 42 °C. This indicated that most of the isolates were psychrotrophic as it is typical to the spoiling bacterial population of meat stored at chill temperature (Huis In't Veld, 1996; Ercolini et al., 2009).

2.3. Identification of bacterial isolates by API and BBL Crystal tests

Preliminary identification of the 47 bacterial isolates has been performed by the commercial miniaturised tests API and BBL Crystal that are frequently used for routine identification of bacteria. API 20E and API 20NE (BioMerieux) were used for the identification of Gram-negative, while BBL Crystal (BD Diagnostics) was used for the Gram-positive isolates. Results of identification are shown in Table 4. Forty-five out of the 47 isolates were identified as Gram-negative bacteria belonging to the genera of *Pseudomonas* (64%), *Aeromonas* (24%) and *Hafnia* (4%), while the genera of *Citrobacter* and *Serratia* were represented by single isolates. The two Gram-positive isolates were identified as *Staphylococcus capitis* and *Corynebacterium* sp. Half of the *Pseudomonas* and *Aeromonas* isolates could be identified by the API 20NE at genus level only, what can be attributed to the biochemical and physiological heterogeneity of the food-borne bacterial isolates belonging to a given species. All the identified genera and species can be considered as stable constituents of typical chilled poultry meat spoiling bacterial microbiota. *Pseudomonas* and *Aeromonas* spp. are generally predominating in the fresh and spoiled meat, although number of *Pseudomonas* spp. are gradually increasing over the *Aeromonas* spp. (Daud et al., 1979; Ercolini et al., 2007; Jay et al., 2003; Lucianez et al., 2010).

2.4. Molecular typing of bacterial isolates by RAPD-PCR

For genotypic characterisation of the bacterial microbiota *Pseudomonas* isolates were fingerprinted by RAPD-PCR analysis using the OPA 4 and P272 oligonucleotide primers, while for the non-*Pseudomonas* isolates M13, OPA 4 and OPE 19 primers were applied

Table 4. Bacterial isolates originated from chilled chicken meat as identified by API 20E and API 20NE (Gram-negative isolates), BBL Crystal (Gram-positive isolates), multiplex PCR and sequencing the *rpo*B gene. Extracellular protease and lipase activities of the bacteria were measured by inoculating the cells onto the test media

Code	Identification by API and BBL Crystal kits	Species specific multiplex PCR	Sequencing of *rpo*B gene	Protease activity	Lipolytic activity	
				SM	PAT-80	PCATB
TM-51	*Pseudomonas* sp.	Negative	*P. fluorescens*	0.0[a]	0.0	6.0
TS-12	*Pseudomonas* sp.	Negative	*P. fluorescens*	10.3	5.8	6.5
TC-4	*Pseudomonas* sp.	Negative	*P. fluorescens*	10.0	5.8	6.5
TC-61	*P. fluorescens*	Negative	*P. fluorescens*	10.0	6.3	9.5
TS-201	*P. fluorescens*	Negative	*P. fluorescens*	5.0	3.5	3.0
TS-211	*Pseudomonas* sp.	Negative	*P. fluorescens*	4.5	0.5	6.5
TM-131	*Pseudomonas* sp.	Negative	*P. fluorescens*	8.5	2.8	5.0
TM-141	*Pseudomonas* sp.	Negative	*P. fluorescens*	9.0	4.0	7.0
TS-17	*Pseudomonas* sp.	Negative	*P. fluorescens*	9.0	6.0	7.5
TS-351	*P. fluorescens*	Negative	*P. fluorescens*	0.0	0.0	9.5
TM-4	*Pseudomonas* sp.	Negative	*P. fluorescens*	9.8	8.8	6.5
TM-7	*P. fluorescens*	Negative	*P. fluorescens*	11.3	4.5	7.5
TS-271	*Pseudomonas* sp.	Negative	*P. fluorescens*	5.0	5.0	7.0
TS-281	*Pseudomonas* sp.	Negative	*P. fluorescens*	12.0	4.8	6.5
TM-161	*Pseudomonas* sp.	Negative	*P. fluorescens*	13.8	4.5	7.5
TM-171	*Pseudomonas* sp.	Negative	*P. fluorescens*	3.5	0.0	7.5
TS-3	*P. putida*	*P. fragi*	*P. fragi*	0.0	0.0	7.5
TC-3	*P. putida*	*P. fragi*	*P. fragi*	0.0	0.0	5.5
TS-13	*P. fluorescens*	*P. fragi*	*P. fragi*	10.0	0.0	7.5
TS-15	*Pseudomonas* sp.	*P. fragi*	*P. fragi*	5.0	0.0	7.0
TS-16	*P. putida*	*P. fragi*	*P. fragi*	0.0	0.0	7.5
TS-18	*Pseudomonas* sp.	*P. fragi*	*P. fragi*	3.0	0.0	1.5
TC-6	*Pseudomonas* sp.	*P. fragi*	*P. fragi*	5.0	6.3	6.0
TM-6	*P. putida*	*P. fragi*	*P. fragi*	0.0	0.0	1.5
TM-9	*P. fluorescens*	*P. fragi*	*P. fragi*	0.0	0.0	2.5
TS-291	*P. fluorescens*	Negative	*P. gessardii*	8.5	4.8	8.0
TS-5	*Pseudomonas* sp.	*P. lundensis*	*P. lundensis*	3.5	0.0	6.5
TS-14	*Pseudomonas* sp.	*P. lundensis*	*P. lundensis*	15.5	3.3	8.5
TM-10	*P. putida*	*P. lundensis*	*P. lundensis*	7.3	0.0	7.0
TS-181	*P. putida*	Negative	*P. taetrolens*	0.0	0.0	6.5
TM-1	*Aeromonas hydrophila*	ND	ND	ND	ND	ND
TS-31	*Aeromonas* sp.	ND	ND	ND	ND	ND
TS-1	*Aeromonas* sp.	ND	ND	ND	ND	ND
TS-2	*Aeromonas hydrophila*	ND	ND	ND	ND	ND
TS-4	*Aeromonas sobria*	ND	ND	ND	ND	ND
TS-6	*Aeromonas sobria*	ND	ND	ND	ND	ND
TC-1	*Aeromonas hydrophila*	ND	ND	ND	ND	ND
TM-2	*Aeromonas* sp.	ND	ND	ND	ND	ND

Table 4. (continued)

Code	Identification by API and BBL Crystal kits	Species specific multiplex PCR	Sequencing of *rpo*B gene	Protease activity	Lipolytic activity	
				SM	PAT-80	PCATB
TS-9	*Aeromonas* sp.	ND	ND	ND	ND	ND
TS-10	*Aeromonas sobria*	ND	ND	ND	ND	ND
TS-11	*Aeromonas sobria*	ND	ND	ND	ND	ND
TM-5	*Hafnia alvei*	ND	ND	ND	ND	ND
TM-8	*Hafnia alvei*	ND	ND	ND	ND	ND
TS-8	*Citrobacter braakii*	ND	ND	ND	ND	ND
TM-3	*Serratia liquefaciens*	ND	ND	ND	ND	ND
TC-2	*Staphylococcus capitis*	ND	ND	ND	ND	ND
TC-5	*Corynebacterium* sp.	ND	ND	ND	ND	ND

SM: skim milk agar medium; PCATB: plate count agar supplemented with trybutirin; PAT-80: Tween™ 80 hydrolysis medium; ND: not determined; [a]values are indicating the width of the diffusion zone in mm

(Table 2) as described in 1.3. The aim of this analysis was to demonstrate the rate of similarity between the isolates that would indicate the identity or difference at strain level. High level similarity groups comprise strains belonging to the same species with high probability, therefore selection of a representative strain is generally a good strategy for further species level identification. Dendograms of RAPD-PCR analysis of the 30 *Pseudomonas* and 17 non-*Pseudomonas* isolates are shown in Fig. 1, what indicated very divergent RAPD patterns. This made probable that majority of the isolates represented different strains. Only two *Pseudomonas* isolates (TC-3 and TS-13) originated from the same sample showed ca. 90% similarity, what indicated their very close clonal relationship.

2.5. Identification of Pseudomonas *isolates by species specific primers*

Presence of *P. fragi*, *P. putida* and *P. lundensis* among the *Pseudomonas* isolates was determined by the application of a multiplex PCR that is able to detect and distinguish any of the three species (Ercolini et al., 2007). This multiplex PCR reaction resulted amplicons of the expected sizes as follows: 370, 530 and 230 bp in the case of *P. fragi*, *P. lundensis* and *P. putida*, respectively. From the 30 *Pseudomonas* isolates nine were identified as *P. fragi* and three as *P. lundensis*. No amplicon typical to *P. putida* was generated in spite of that six isolates were identified as *P. putida* by the API 20NE kit. Comparing the results of the API 20NE test and the multiplex PCR-based identification (Table 4) it can be concluded that API 20NE frequently led to misidentification at species level, which confirms the more reliable identification potential of the PCR-based techniques (Ercolini et al., 2007; Jasson et al., 2010).

2.6. Identification of Pseudomonas *isolates by direct sequencing of the* rpo*B gene*

Because eighteen *Pseudomonas* isolates were negative in the multiplex PCR, direct sequencing of the *rpo*B gene was used for the confirmation of positive results obtained and identification purposes as described in *1.6.* After amplifying a distinct region of the *rpo*B gene of the *Pseudomonas* isolates the generated DNA fragments were sequenced. Results of the species

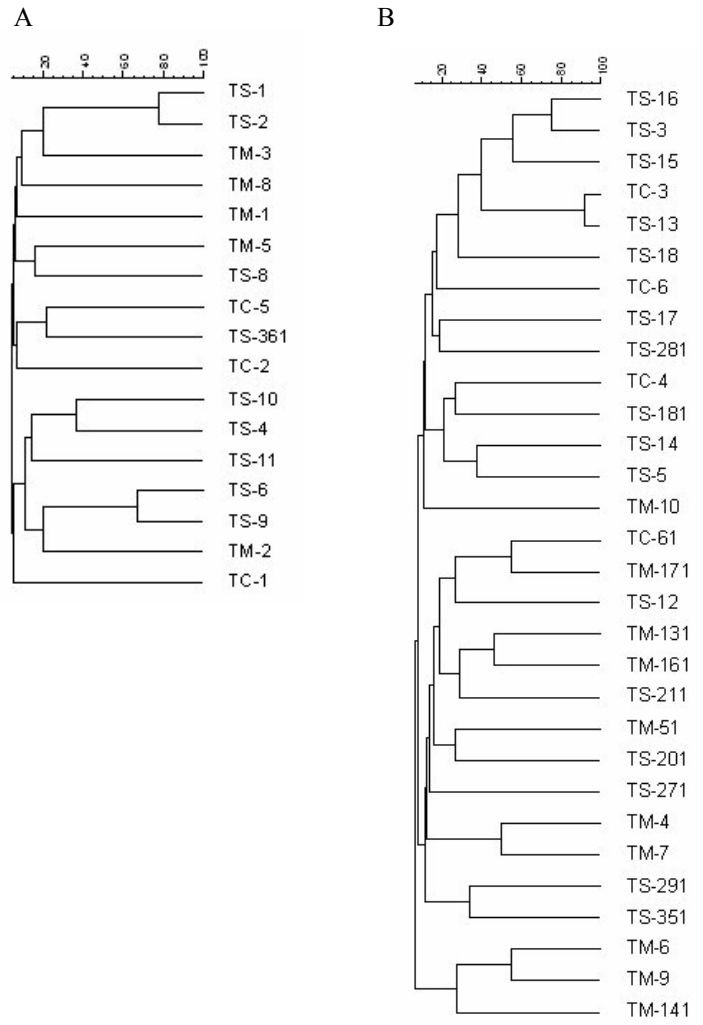

Fig. 1. Dendogram of RAPD-PCR patterns of bacterial isolates. A: RAPD-PCR analysis of non-*Pseudomonas* isolates was done by OPA 4, OPE 19 and M13 primers; B: RAPD-PCR analysis of *Pseudomonas* isolates was done by OPA 4 and P272 primers. Cluster analysis of the pairwise values was generated using UPGMA algorithm

level identification based on the sequence alignment are shown in Table 4 which confirmed the results of multiplex PCR-based identification (Ercolini et al., 2007) in the case of *P. fragi* and *P. lundensis* isolates and so this confirmed its applicability for identification of the food borne *P. fragi* and *P. lundensis* strains. None of the isolates have been identified as *P. putida* by the *rpo*B gene sequencing therefore identification of six isolates by API 20NE as *P. putida* should be considered as misidentification.

From the eighteen isolates being negative in the multiplex PCR reaction sixteen proved to be *P. fluorescens*, while two were identified as *P. taetrolens* and *P. gessardii*. This indicates the necessity for further development of the species specific PCR-based identification related

to the *Pseudomonas* genus aiming to be able to recognise food-borne *P. fluorescens* strains. However, because ERCOLINI and co-workers (2007) found that *P. fluorescens* strains belonging to different biotypes show very high sequence variability within the *car*A gene selection of an another gene for identification purposes seems to be necessary.

2.7. Isolation, characterization and identification of yeasts

Altogether 36 yeast isolates were selected from YPDC plates for further analysis. Colony morphology was analysed by inoculating the isolates into YPD and WL agar and – as it was expected – WL nutrient agar provided better discrimination between the isolates as compared to YPD. Thirteen isolates were positive in the urea test. Based on the differences in colony morphology on WL agar, microscopic cell morphology and urease reaction the 36 isolates were separated into 11 phenotypic groups (designated as PG1 – PG11; Table 5). One representative isolate from each group was chosen for identification by the ID 32C test. Results shown in Table 5 indicate that nine out of the eleven representative strains elected from the eleven phenotypic groups belonged to different species, while two phenotypic groups (PG2 and PG3) comprised the same species.

As regards the temperature limits of growth majority of the isolates (32 out of 36) could grow at 10 °C, moreover, nine could grow even at 5 °C. In two cases very good growth was observed at 37 °C. This means that majority of the isolates proved to be psychrotrophic.

Table 5. Yeast isolates originated from chilled chicken meat. Comparison of identification results obtained by ID 32C, ribotyping (rDNA-RFLP) and sequencing of the LSU rDNA D1/D2 domain

Code	Phenotype groups	% of isolates	Identification by ID 32C	Identification by		Protease activity	Lipolytic activity	
				Ribotyping (rDNA-RFLP)	rDNA D1/D2 sequencing	SM	PAT-80	PCATB
YS-7	PG 1	22	*Candida zeylanoides*	*Candida zeylanoides*	ND	0.0	0.0	3.5[a]
YS-19						0.0	0.0	5.0
YS-26						0.0	0.0	4.0
YM-2						0.0	1.5	4.0
YM-7						0.0	2.0	5.0
YM-15						0.0	0.0	3.5
YC-1						0.0	4.0	6.5
YC-4						0.0	3.5	4.5
YM-1	PG 2	14	*Cryptococcus curvatus*	Negative	*Cryptococcus curvatus*	0.0	3.5	6.5
YS-5						0.0	0.5	7.0
YS-11						0.0	5.0	5.0
YS-12						0.0	4.5	10.0
YS-37						0.0	6.0	6.0
YS-31	PG 3	5.5	*Cryptococcus curvatus*	*Cryptococcus curvatus*	ND	0.0	3.5	4.0
YC-5						0.0	4.5	10.0

Table 5. (continued)

Code	Phenotype groups	% of isolates	Identification by ID 32C	Identification by		Protease activity	Lipolytic activity	
				Ribotyping (rDNA-RFLP)	rDNA D1/D2 sequencing	SM	PAT-80	PCATB
YS-30	PG 4	3	*Cryptococcus laurentii*	Negative	*Cryptococcus mucoides*	0.0	3.0	5.5
YS-34	PG 5	3	*Debaryomyces hansenii*	*Debaryomyces hansenii*	ND	0.0	2.5	4.0
YS-4	PG 6	8	*Metschnikowia pulcherrima*	*Metschnikowia pulcherrima*	ND	12.0	2.0	5.0
YS-16						12.0	2.5	4.5
YS-22						8.0	2.0	4.0
YS-32	PG 7	8	*Rhodotorula glutinis*	*Rhodotorula glutinis*	ND	0.0	3.5	9.0
YS-33						0.0	5.0	9.0
YC-3						0.0	4.5	11.0
YM-4	PG 8	5.5	*Rhodotorula minuta*	*Rhodotorula minuta*	ND	0.0	5.0	5.5
YM-9						0.0	3.5	5.5
YS-2	PG 9	14	*Rhodotorula mucilaginosa*	*Rhodotorula mucilaginosa*	ND	0.0	2.0	5.0
YS-8						0.0	1.0	4.5
YS-14						0.0	4.0	5.0
YS-17						0.0	4.0	5.5
YM-12						0.0	4.5	6.5
YS-25	PG 10.0	3	*Trichosporon asahii*	*Trichosporon asahii*	ND	0.0	2.0	7.0
YS-1	PG 11	14	*Trichosporon inkin*	Negative	*Trichosporon montevideense*	0.0	2.5	4.5
YS-6						0.0	4.0	4.0
YS-13						0.0	2.0	6.0
YS-23						0.0	6.0	5.5
YM-3						0.0	2.5	3.0

ND: not determined; SM: skim milk agar medium; PAT-80: plate count agar supplemented with TWEEN-80; PCATB: plate count agar supplemented with trybutirin; [a]values are indicating the width of the diffusion zones in mm

2.8. Molecular typing of the yeast isolates

In order to investigate the molecular diversity of yeast isolates RAPD analysis with the application of M13, $(GTG)_3$, and OPA 10 primers has been performed. Results shown in Fig. 2 indicate that eleven isolates formed two big clusters (five and six in each), while ten isolates grouped into five clusters with 100% similarity in pairs and fifteen isolates showed individual RAPD patterns. Isolates that were grouped in pairs always originated from the same series of

chicken meat samples, while the two big clusters, that were preliminary identified as *Candida zeylanoides* and *Cryptococcus curvatus,* harboured isolates originated from different series of samples.

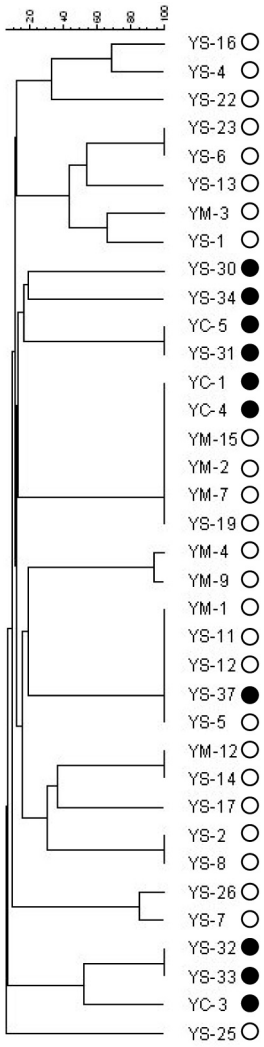

Fig. 2. Dendogram of RAPD-PCR analysis of yeast isolates. RAPD-PCR analysis was done by M13, $(GTG)_3$ and OPA 10 primers. ○: Isolates from the 1st series of storage; ●: Isolates from the 2nd series of storage

2.9. Identification of yeasts by rDNA RFLP (ribotyping) and sequencing the LRU rDNA D1/D2 domains

Amplicons from the SRU rDNA were generated with PCR by the application of NS1-ITS2 primer pair (Kurtzman & Robnett, 1998) that was followed by digestion with four different restriction endonucleases as described in 1.7. Combined dendogram of the RFLP patterns is shown in Fig. 3. The RFLP clusters corresponded completely to the different phenotypic

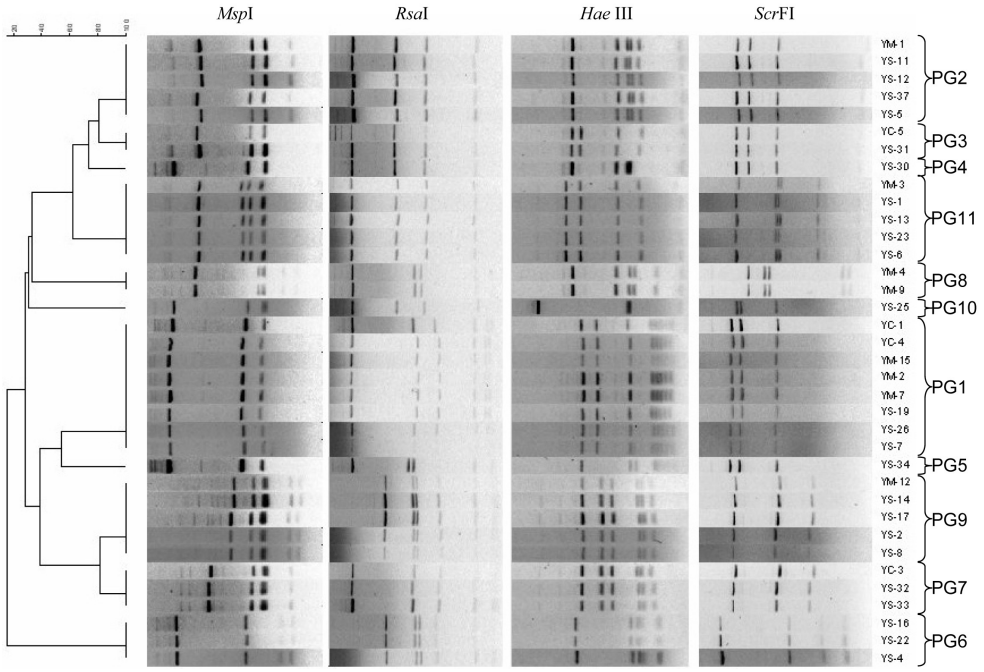

Fig. 3. Cluster analysis of rDNA-RFLP patterns of yeast isolates

groups, indicating that in spite of the extended molecular heterogeneity of the isolates – as revealed by RAPD analysis – phenotypic differences and rDNA sequence heterogeneity of the amplicons correlated very well. Identity of the rDNA RFLP clusters at species level has been determined by ribotyping, which means that the rDNA RFLP patterns of the isolates belonging to the individual clusters were compared with that of the type strains of the identified species. In case if the rDNA RFLP pattern of the type strain was different from that of the isolates belonging to the given cluster species identity was determined by sequencing the NL1-NL4 flanked amplicons of the D1/D2 domain. Results shown in Table 5 indicate that ribotyping confirmed the identity of PG 1 isolates as *Candida zeylanoides,* PG 3 isolates as *Cryptococcus curvatus,* PG 5 isolate as *Debaryomyces hansenii,* PG 6 isolates as *Metschnikowia pulcherrima*, PG 7 isolates as *Rhodotorula glutinis,* PG 8 isolates as *Rhodotorula minuta,* PG 9 isolates as *Rhodotorula mucilaginousa* and PG10 as *Trichosporon asahii.* Sequencing of the NL1-NL4 flanked amplicons of the D1/D2 domain revealed that PG2 group comprised *Cryptococcus curvatus* isolates and so the identification by ID 32C was confirmed. Isolates belonging to PG 4 were identified as *Cryptococcus mucoides* and PG11 isolates as *Trichosporon montevideense*. From the ratio of isolates belonging to the different species it can be concluded that most of them belonged to *Candida zeylanoides* and the genera of *Cryptococcus, Rhodotorula* and *Trichosporon*, but one of the most common poultry spoiling yeasts *Yarrowia lipolytica* (Hsieh & Jay, 1984; Viljoen et al., 1998; Ismail et al., 2000; Deák, 2008) was not isolated during our study. According to these authors *Candida zeylanoides* and *Yarrowia lipolytica* are the prominent yeast species of the fresh and spoiled meat and meat products but there are no animous opinions and findings which species is

associated more with the fresh and the spoiled meat. It is highly probable that the type of meat and temperature of storage have also influence on the occurrence and numbers of these and other yeast species. Similar to our result VILJOEN and co-workers (1998) found *Candida zeylanoides* as the predominant species in fresh poultry (35%), although it was present in a similarly high ratio (45.5%) in the spoiled products. The incidence of *Yarrowia lipolytica* was much lower in the fresh poultry meat (2.5%) and its ratio increased during spoilage (11.4%). Opposite to this ISMAIL and co-workers (2000) recovered *Yarrowia lipolytica* in higher ratio in the fresh poultry products than *Candida zeylanoides* (40 and 28.6%, respectively) and the percentage of both species slightly decreased till the end of the expiration date. HSIEH and JAY (1984) isolated also *Yarrowia lipolytica* in higher ratio from the fresh ground beef than *Candida zeylanoides* but frequency of both species decreased during the 15 days of chilled storage. They found, however, other yeast species being predominant in the fresh and stored ground beef when different samples were involved in the analysis. BARNES and co-workers (1978) stored poultry carcasses in subzero temperatures and they observed a considerable increase in the population of *Candida zeylanoides* during storage that could be the consequence of the difference in the minimum temperature limit of growth of these two yeast species. It seems that these two species are prominent not only in meat and meat products but LOPANDIC and co-workers (2006) found their high incidence (20%) in dairy products stored under refrigerated conditions, as well.

2.10. Proteolytic and lipolytic activities of Pseudomonas *isolates*

Protease activity of the 30 *Pseudomonas* isolates was checked using Standard Methods Caseinate (SMC) and skim milk (SM) agar media as described in 1.9. SM agar resulted clearly distinguishable clearing (proteolytic) zones around the macrocolonies, while proteolytic zones were not uniform when SMC agar was used. In the latter case narrow clearing zones were surrounded by opaque zones or only opaque zones were generated, which was probably the result of partial proteolysis of sodium caseinate or production of caseinate denaturing metabolites. Therefore test results obtained on SM agar were taken into consideration for characterization and quantification of proteolytic activity of the isolates. As shown in Table 4 proteolytic enzyme activity was detected in 22 *Pseudomonas* isolates on SM plates. Majority of *P. fluorescens* and *P. lundensis* strains had high proteolytic activity, while *P. fragi* strains were poor and instable protease producers.

Esterase and lipase activities of the *Pseudomonas* isolates were detected on PAT-80 and PCATB plates, respectively, as described in 1.10. Half of the 30 isolates showed esterase activity when inoculated into PAT-80 agar, while 27 isolates produced lipase on PCATB plates (Table 4). All the nine *P. fragi* strains were negative for the esterase activity but six of them were lipase producers.

Low proteolytic and lipolytic activity of *P. fragi* strains could explain why this species is considered as an important primary colonizer in meat (SASAHARA & ZOTTOLA, 1993) and the high enzyme activities of *P. fluorescens* isolates would contribute to the predominance of *P. fluorescens* populations in the later stage of spoiling (MICHIELS et al., 1997). ERCOLINI and co-workers (2009) found that *Pseudomonas* meat isolates were mostly negative for protease activity at 7 °C, but the protease positive *P. fragi* produced the highest concentrations of volatile alcohols and ketons, which contribute to the production of off-odour considerably.

2.11. Proteolytic and lipolytic activities of yeasts

Protease activity of yeast isolates was much less frequent than in the case of *Pseudomonas* isolates and protease negativity or positivity was species dependent (Table 5). Isolates belonging to *Rhodotorula glutinis* and *R. mucilaginosa* produced turbid zones on SMC agar, which could not be considered as the typical result of protease activity, it might have been the consequence of the production of protein denaturing metabolites. No zones were generated on SM agar by the *Rhodotorula* isolates. All of the three *Metschnikowia pulcherrima* strains exhibited protease activity on SM plates, sizes of the clearing zones were as big as that of the best *Pseudomonas* isolates.

Majority of yeast isolates had both esterase and lipase activities as checked on PAT-80 or PCATB agar plates, respectively, but in some *Candida zeylanoides* isolates only the lipase activity could be detected (Table 5).

Comparing the proteolytic and lipolytic activities of the isolated yeasts it can be concluded that both enzyme activities are species specific and protease production is far less frequent than lipolytic activity. It is highly probable that production of these enzymes is important in the persistence of the isolated yeasts in the fresh and the spoiled meat and they endow selection powers in the growth and enlarging the numbers during refrigerated storage of poultry meat because *Metschnikowia pulcherrima* strains which had both enzyme activities persisted in a considerable ratio during the whole storage period (data not shown). Lipase activity of the isolates seems to be a pre-requisite factor for the spoiling potential not only in the case of *Candida zeylanoides* and *Yarrowia lipolytica* as reported by Ismail and co-workers (2000) but also for the other Ascomycetes and Basidiomycetes yeasts.

3. Conclusions

This study demonstrates that the application of the polyphasic approach is a powerful tool in the characterisation and identification of the poultry meat spoilage microbial associations, because the traditional phenotype-based methodology provides an insight into the morphology, physiology, and enzyme production of the prominent bacteria and yeasts, while the molecular biological (especially the PCR-based) techniques are very reliable for the correct identification, typing and population analysis of the spoiling microbiota.

Determination of the spoiling potential, population dynamics and interaction of different bacteria and yeasts that contribute to the spoiling process could provide aids for working out new antimicrobial treatments and decreasing the loss of meat as the consequence of spoilage.

*

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