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ISOLATION AND DIVERSITY OF FOOD SPOILAGE *YARROWIA* YEAST STRAINS FROM MEAT

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Yeasts of the *Yarrowia* clade frequently occur in meat and milk products. In many cases they cause spoilage due to their strong lipolytic and proteolytic activity, and their ability to produce brown pigments. Using conventional methods, some of these yeasts can be misidentified as *Yarrowia lipolytica*, but using molecular biological methods it can be stated that they are members of a complex group including several different species. The aims of this study were to find a proper method to collect *Yarrowia* strains from raw meat, and to assign them to groups based on their physiological and molecular characteristics, followed by sequence-based identification of the selected members of each group to determine the composition of the species. This study revealed three novel *Yarrowia* species. From 62 samples of different raw meats 291 yeast strains were isolated, from which 118 strains proved to be members of the *Yarrowia* group. They could be assigned to seven species. Four of them are described species, three of them are novel ones.

Keywords: *Yarrowia*, diversity, food spoilage

Yarrowia lipolytica is known as one of the most dangerous and one of the most common spoilage yeasts of the food industry (Deák, 2008). Members of the *Yarrowia* clade occur mainly in meat and milk products, and they cause spoilage in many cases, due to their strong lipolytic and proteolytic activity, and their ability to produce brown pigments (Roostita & Fleet, 1996; Suzzi et al., 2001; Gardini et al., 2006). They have also favourable effects, like for example *Y. lipolytica* has a high potential to be used for the production of citric acid, lipases, and single-cell oil. It is used as a production host for mannitol, erythritol (De Zeeuw & Tynan, 1973a, b; Tomaszewska et al., 2012), and carotenoids (Bailey et al., 2006, 2008; Sharpe et al., 2008). They also may have desirable effects on the ripening of cheeses (Wyder et al., 1999; Ferreira & Viljoen, 2003; Lanciotti et al., 2005) and meat products (Gardini et al., 2001; Patrignani et al., 2007, 2011a, b; Romano et al., 2006). Based on the extensive review of Groenewald and co-workers (2014), *Y. lipolytica* is 'safe-to-use' in feed or food, or when deployed as a production host for biotechnological applications.

Using conventional methods based on phenotypic characteristics for identification, some yeasts of the *Yarrowia* clade can be misidentified as *Y. lipolytica*, which was the only known member of the clade for a long time. However, using molecular biological methods, such as sequence analysis of the D1/D2 region of the large subunit rRNA gene (Bigey et al., 2003) and polyphasic approach (Knutsen et al., 2007), the heterogeneity of the species was recognized. The most important recent changes in the systematics of the yeasts in the *Yarrowia* clade were summarized in our previous study (Nagy et al., 2013). Since then a new anamorphic

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species, *Yarrowia keelungensis* f.a. from sea water (Chang et al., 2013) was described. The aims of this study were to find a proper method to isolate *Yarrowia* strains, and to assign them to groups based on their physiological and molecular characteristics, followed by sequence-based identification of the selected members of each group to determine the species composition of the *Yarrowia* group in meat.

1. Materials and methods

1.1. Isolation

Sixty-two different kinds of raw meat samples were investigated: 29 pork, 26 beef, 5 turkey samples and 2 samples of mixtures of minced beef and pork. They were obtained from a quality control laboratory in Budapest, Hungary. Before processing they were stored at 4 °C for 24–48 h. To efficiently isolate *Yarrowia* strains from meat, developing a selective isolation method was necessary. First a three-step enrichment was used (Nagy et al., 2013). The enrichment steps were carried out in liquid Yeast Nitrogen Base (SIGMA Y1250) medium pH 3.6, prepared with phosphate-citric acid buffer according to McIlvaine (1921), supplemented with 0.5% (v/v) hexadecane as a carbon source. The reduced pH of the medium served to inhibit bacterial growth and hexadecane was used because only about 10% of the known yeast species are able to grow with this compound as a sole carbon source (among the species tested for this character), including members of the *Yarrowia* clade (Kurtzman et al., 2011). In case of 44 meat samples, 10 g of raw meat sample was homogenized with 90 ml sterile peptone water (0.9% NaCl, 0.1% peptone) in a stomacher (2 min), then 1 ml homogenized sample suspension was added into 5 ml enrichment media in 16 mm culture tube. Incubation lasted for 7 days on a roller drum (30 r.p.m.) at 25 °C. After 1 week, 0.1 ml culture liquid was transferred to a 16 mm tube containing 5 ml of the same enrichment medium, the incubation conditions were also the same. After 1 week of incubation, a third enrichment step, identical to the second one was also made. In 18 cases, 10 g of raw meat samples were added into 100 ml of enrichment media in 250 ml flasks and incubated for 7 days on a horizontal rotary shaker (100 r.p.m., 25 °C). The second and third enrichment steps were the same as described above. After the enrichments, serial decimal dilutions and surface plating on Rose-Bengal chloramphenicol agar (MERCK 1.00467) were made. This medium is recommended and conventionally used for selective isolation of yeasts and moulds from food (Jarvis, 1973). Following the incubation at 25 °C for 5 days in darkness, strains representing different colony types were isolated and purified by repeated streaking on glucose-peptone-yeast extract agar (1% glucose, 1% peptone, 0.3% yeast extract, 2% agar).

1.2. Physiological characteristics

Before further investigation, the ability of the isolated strains to grow on hexadecane was confirmed by inoculating strains into a medium containing 0.5% hexadecane as a sole carbon source, and incubating at 25 °C. Growth was monitored weekly up to 3 weeks. Their glucose-fermentation and nitrate-assimilation were tested as described by Kurtzman and co-workers (2011), and the assimilation of 30 different carbon-sources by using API ID32 C tests (bioMérieux) were examined, following the manufacturer's recommendations.

1.3. PCR fingerprinting

A microsatellite primer $(GAC)_{5x}$ was used in PCR amplification reactions of the DNA extracted from the strains by using Qiagen DNA extraction kit (Qiagen 69106, Switzerland), according to manufacturer's instructions. For amplification the following conditions were applied: initial denaturation at 95 °C for 3 min followed up by 30 cycles of denaturation (96 °C, 30 sec), annealing (59 °C, 35 sec), and extension (73 °C, 55 sec). Amplification was finished with a final extension at 72 °C for 30 sec and cooling at 12 °C. The PCR products were separated by horizontal agarose gel electrophoresis on 1.4% agarose gel, in 1X TAE buffer (0.04 M Tris-acetate, 0.00l M EDTA - pH 8.0) at 5 V cm^{-1} and stained with ethidium bromide (7×10^{-5} μg μl^{-1}). The resulting DNA fragments and the molecular size marker (Sigma P-9577) were visualized by UV transillumination.

1.4. Amplification and sequencing of the D1/D2 region of the large subunit rRNA gene

Amplifications were made according to KURTZMAN and ROBNETT (1998). The amplicons were sequenced by commercial sequencing facilities (Bay Zoltán Nonprofit Ltd. for Applied Research, Hungary; Biomi Ltd., Hungary). Sequence similarity searches were performed against the GenBank sequence database using the BLAST 2.2.28 database search program (ZHANG et al., 2000).

2. Results and discussion

2.1. Isolation

By using the new isolation method described above, 291 yeast strains were isolated from 62 different kinds of raw meat samples, 133 from pork, 125 from beef, 10 from mixtures of minced pork and beef, and 23 from turkey.

2.2. Physiological characteristics

One hundred and fifty-six of the 291 strains could assimilate hexadecane. Using the ability to grow with hexadecane as a sole carbon-source was chosen as a selective enrichment parameter, because all recognized members of the *Yarrowia* group assimilate hexadecane, while only about 10% of the known yeast species with available hexadecane assimilation data possess this character (KURTZMAN et al., 2011). From the hexadecane positive strains the members of the *Yarrowia* group were selected by the results of additional physiological tests; the inability to ferment glucose and to assimilate nitrate, and the specific carbon-source assimilation profile obtained by using API ID 32 C tests. One hundred and eighteen strains (76% of the hexadecane positive strains) were assigned as members of the *Yarrowia* group. Only a smaller proportion, 38 strains (24%) of hexadecane-assimilating strains, is not member of the group.

2.3. PCR fingerprinting

Strains, selected based on their physiological characteristics as members of the *Yarrowia* group, were assigned to groups based on PCR fingerprinting, using the above mentioned microsatellite primer and conditions. The 118 strains formed seven groups.

2.4. Amplification and sequencing of the D1/D2 region of the large subunit rRNA gene

To determine the composition of the species, selected members of each group were identified by amplifying and sequencing the D1/D2 region of the ribosomal RNA's large subunit coding gene.

Using the above mentioned molecular biological methods, yeast strains of the *Yarrowia* group from raw meat were assigned to seven species. Four of them (*Yarrowia lipolytica, Y. deformans, Candida galli,* and *C. alimentaria*) are earlier described species, three of them proved to be novel ones. We proposed the first new species revealed in this study, *Y. divulgata* f.a., a new anamorphic member of the genus in a study published last year (Nagy et al., 2013) to accommodate some animal related yeast strains. The description of two additional new *Yarrowia* species, *Y. porcina* and *Y. bubula* f.a., also revealed in this study was published very recently (Nagy et al., 2014), increasing the number of the species of the *Yarrowia* group to thirteen species. Earlier, based on conventional phenotypic tests, the majority of the isolated strains would have been identified as *Y. lipolytica*, though actually only 20% of them (24 strains) belong to this species. The predominant *Yarrowia* species recovered from raw meat samples during this study was *Y. deformans*. Forty-five percent of the yeast strains (the strains of *Y. deformans, Yarrowia divulgata,* and *Yarrowia porcina*) isolated during this experiment would have been misidentified (as *Y. lipolytica*). Thirty-five percent of the isolated yeast strains of the *Yarrowia* group belonged to *Y. deformans* (41 strains), 15% of them to *C. galli* (17 strains), 10% of them to *C. alimentaria* (12 strains), and 20% of them could be assigned to three new, recently described species, 10% to *Y. bubula,* 5% to *Y. porcina* (Nagy et al., 2014), and 5% to *Y. divulgata* (Nagy et al., 2013) (Fig. 1).

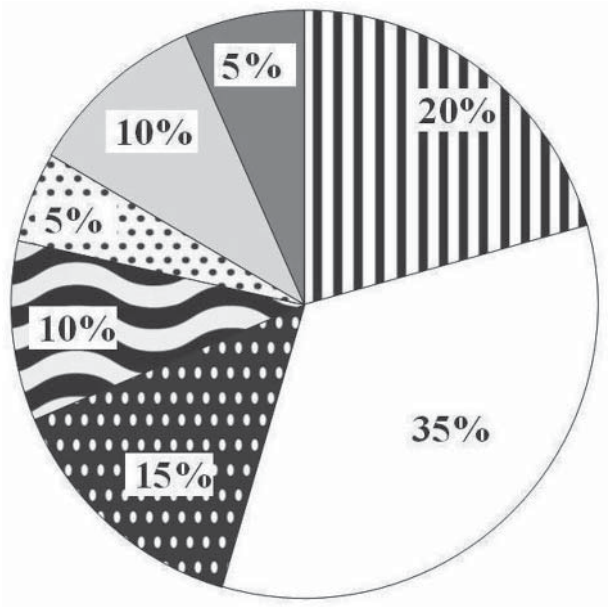

Fig. 1. Composition of the species of *Yarrowia* group isolated from raw meat.
Sector with vertical stripes: *Y. lipolytica*; white sector: *Y. deformans*; white spotty sector: *C. galli*; sector with wavy stripes: *C. alimentaria*; black spotty sector: *Y. divulgata*; light grey sector: *Y. porcina*; dark grey sector: *Y. bubula*

3. Conclusions

The newly developed isolation method can be used effectively to isolate yeast strains of the *Yarrowia* group. The method is efficient, but not fully selective.

Phenotype-based tests are very important, but the identification based only on physiological characteristics is often not reliable. Nevertheless, studying the physiological characteristics of the isolated strains can contribute to the cognition both of their advantageous and disadvantageous role in the food industry, and to the estimation of their potential industrial or biotechnological applicability.

Yarrowia lipolytica is widely reported as a food spoilage microorganism, however in some of the cases only phenotypic identification was applied, so it is very likely that some other species of the group also contribute to the spoilage.

Based on this study, at least seven species, which is more than half of the currently known species of the group, can be isolated from raw meat.

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