

**Impact of process parameters on the sourdough microbiota,
selection of suitable starter strains, and description of the novel
yeast *Cryptococcus thermophilus* sp. nov.**

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Chapter I

Scope and outline

Scope

The composition of the sourdough microbiota plays an essential role for bread taste and quality. For this reason, scientific research focuses primarily on determination of the microbiota of sourdoughs fermented under different conditions; on the search for suitable starter cultures for artisanal and industrial use; and on metabolic properties of the sourdough microbiota. Despite intense research, it is still unclear to which extent the fermentation microbiota is influenced and selected by the kind of substrate and the process parameters like temperature, dough yield, redox potential, refreshment time, and number of propagation steps.

Furthermore, the formation of a competitive microbiota is influenced by interactions between the different microorganisms in the dough.

The first part of this dissertation deals with the question how the sourdough microbiota is influenced by the substrate, process conditions and interactions between microorganisms. In the first study, different flours were fermented with a starter sourdough mixture of numerous lactic acid bacteria and yeasts to examine the influence of the substrate on the microbiota. In the second study, the focus was laid on influence of substrate, refreshment time, temperature, amount of backslopping and competing species on two competitive microbial associations in sourdough. The microbiota was determined using bacteriological culture, RAPD-PCR, PCR-DGGE and sequencing.

The second part of the dissertation is a description of a new yeast species, *Cryptococcus thermophilus* sp. nov., isolated from cassava sourdough.

Outline of the thesis

Chapter II gives a general overview about the technology and microbiota of sourdoughs. Typical LAB and yeast species in the different types of wheat and rye sourdoughs are introduced, as well as in rice, maize, sorghum, millet, oat, barley, pseudocereal (amaranth, quinoa and buckwheat) as well as cassava fermentations. Technological, nutritional and ecological aspects are discussed. Furthermore, information about the actual methods for investigation of the sourdough microbiota is provided.

Chapter III describes the investigation of the competitive microbiota of cereal, pseudocereal and cassava sourdoughs. Eleven different flours were fermented with a combined started mixture. The microbiota was analyzed using PCR-DGGE as well as bacteriological culture and RAPD-PCR, followed by 16S/26S rRNA sequence analysis. Competitive strains were analyzed for their suitability as starter cultures for non-bread cereal and pseudocereal sourdoughs in a second fermentation.

This chapter has been published in International Journal of Food Microbiology:

Stephanie A. Vogelmann, Michael Seitter, Ulrike Singer, Markus J. Brandt, and Christian Hertel. 2009. Adaptability of lactic acid bacteria and yeasts to sourdoughs prepared from cereals, pseudocereals and cassava and use of competitive strains as starters. Int. J. Food Microbiol. 130:205-212.

Chapter IV describes the effect of ecological factors on two microbial associations in sourdoughs: *L. sanfranciscensis* – *C. humilis* and *L. reuteri* – *L. johnsonii* – *I. orientalis*. Sourdoughs were fermented under varying defined conditions using the two associations which have been shown to be competitive in rye sourdoughs. Predominant lactic acid bacteria and yeasts were characterized by bacteriological culture, RAPD-PCR and rRNA gene sequence analysis. The processing parameters for the competitiveness of the two associations were defined. In addition, it was found that *I. orientalis* could only grow with enough oxygen supply and was not competitive when using a small ratio of surface to volume and refreshment times of 12 h.

This chapter has been published in Food Microbiology:

Stephanie A. Vogelmann, and Christian Hertel. 2011. Impact of ecological factors on the stability of microbial associations in sourdough fermentation. Food Microbiol. 28:583-589.

Chapter V contains the description of the new yeast species *Cryptococcus thermophilus* sp. nov., which has been isolated from the cassava starter sourdough prepared for fermentation I in chapter III. This yeast has several specific characteristics that separate it from known species, like budding on small neck-like structures, no fermentative ability, and the production of green or blue fluorescent substances in the growth medium. The closest relatives are the yeasts belonging to the *Cryptococcus humicola* complex.

This chapter has been published in International Journal of Systematic and Evolutionary Microbiology:

Stephanie A. Vogelmann, Sandra Chaves, and Christian Hertel. 2011. *Cryptococcus thermophilus* sp. nov., isolated from cassava sourdough. Int. J Syst. Evol. Microbiol. 62:1715-1720.

During the works for this thesis, some yeast species were renamed. Amongst others, *Issatchenkia orientalis* was renamed as *Pichia kudriazevii*, and *Saccharomyces exiguus* as *Kazachstania exigua*. However, the old names are used throughout this thesis, as the publications where these yeast species are contained (Chapter III and IV) had been accepted and published before the renaming of the species.

Co-authors

This dissertation comprises studies that were carried out in collaboration with several researchers. The work was supervised by PD Dr. Christian Hertel.

Chapter III: Markus Brandt contributed to the conception of fermentation I. During sourdough fermentation I, Michael Seitter contributed equally to the following laboratory works: preparation of growth media; sourdough refreshment; sample plating; picking, multiplication and storage of isolates. Ulrike Singer helped partly with the identification of isolates and carried out fermentation II. Markus Kranz handled the sequencer.

Chapter IV: Claudia Lis helped with the preparation of some growth media, Markus Kranz handled the sequencer.

Chapter V: Claudia Lis helped with the preparation of some growth media, Markus Kranz did the 26S rRNA sequencing of the yeast. The identification of respiratory quinones was carried out by the Identification Service and Dr. B. J. Tindall, DSMZ, Braunschweig, Germany. Dr. Sandra Chaves, Lisbon University, Faculty of Sciences, Vegetal Biology Department and Centre for Biodiversity, Functional & Integrative Genomics calculated the phylogenetic tree.

Chapter II

Introduction

Sourdough

Sourdough is an intermediate product which has been used for the production of food, especially staple foods, all over the world for thousands of years. As soon as ground cereals or starchy raw materials come in contact with water, fermentation takes place over time. The origin of fermented products is supposed to be in the Middle East (Katz and Voigt, 1986). It is not known if the brewing of beer or sourdough was discovered first. The ancient Egyptians knew both the brewing of beer and the process of baking leavened bread with use of sourdough, as proved by wall paintings and analyses of desiccated bread loaves and beer remains (Rothe et al., 1973; Samuel, 1996). Today, sourdoughs are used worldwide for a huge variety of products: leavened bread, fermented gruels, alcoholic and/or acid fermented drinks, vinegar and fermented red rice grains (Hammes et al., 2005). Besides sourdough breads, examples are Sudanese kisra (sorghum), Ethiopian injera (teff), Mexican pozol, Ghanaian kenkey and Nigerian ogi (maize), Indian idli (rice, beans or chickpeas), Turkish bosa (wheat, maize, sorghum, millet and/or rice) or Nigerian gari or fufu (cassava) (Steinkraus, 1983). Fermentation of cereals brings along the following advantages (Steinkraus, 1983; Hammes et al., 2005; Brandt and Gänzle, 2006):

- Dough leavening;
- Enhancement of the sensory properties through the development of colours, flavours, aromas, and textures;
- Preservation of food through the formation of lactic acid, acetic acid, and alcohol;
- Enhancing food safety by inhibition of pathogens;
- Improvement of the nutritive value by removing antinutritive compounds (e.g. phytate, enzyme inhibitors, polyphenols, tannins), and enrichment of protein, essential amino acids, essential fatty acids, and vitamins;
- Detoxification by reduction of mycotoxins, endogenous toxins, cyanogenic compounds, flatulence producing carbohydrates;

- Decrease in cooking times and fuel requirements;
- Dough acidification, as it is technologically required for producing leavened rye bread to inhibit the rye flour amylases.
- Extension of the shelf life of bread through delaying the retrogradation of starch by acidification, hydrolysis of starch, proteolysis and production of exopolysaccharides (Tieking et al., 2003).

The microbiota of sourdoughs

Hammes and Gänzle (1997) described the ecological factors affecting the microbiota of sourdough fermentations. They differentiate between endogenous and exogenous factors. Endogenous factors are the composition of the cereal substrate, as fermentable carbohydrates, N-sources, minerals, vitamins, lipids, enzyme activities, growth inhibitors, and the original microbiota of the grains. Exogenous factors are the applied process parameters like temperature, oxygen, dough yield, amount of backslopping dough, addition of sodium chloride, fermentation time, number of propagation steps and the used starter cultures. If these parameters are held constant for a longer period of time, their interplay induces the selection of a distinctive microbiota of LAB and yeasts. Cell counts of dominant LAB in rye sourdough exceed 10^9 CFU per gram (Kline and Sugihara, 1971b; Loenner and Ahrné, 1995; Vogel et al., 1994; Böcker et al., 1990; Müller et al., 2001), cell counts of competitive yeasts range two orders of magnitude below (Loenner and Ahrné, 1995; Gobbetti et al., 1994; Rossi, 1996). Numerous LAB and yeast species occur in sourdough, and in the past years, a lot of new *Lactobacillus* (*L.*) species were discovered in sourdough fermentations, for example *L. hammesii* (Valcheva et al., 2005), *L. rossiae* (Corsetti et al., 2005), *L. acidifarinae* and *L. zymae* (Vancanneyt et al., 2005), *L. nantensis* (Valcheva et al., 2006), *L. siliginis* (Aslam et al., 2006), *L. secaliphilus* (Ehrmann et al., 2007), *L. namurensis* (Scheirlinck et al., 2007a) and *L. crustorum* (Scheirlinck et al., 2007b).

Rye and wheat sourdoughs

Based on technological and microbiological aspects, Böcker et al. (1995) defined three different types of sourdoughs from rye flour. Including wheat sourdoughs into this classification, Brandt (2001) added a fourth type, type 0 sourdoughs, so-called “wheat

predoughs”, which are wheat sourdoughs started with baker’s yeast and propagated for 3-24 hours. These predoughs are used for achieving better dough and leavening characteristics and improved sensory properties of wheat bread. Product examples are French baguette and Italian ciabatta. The microbiota of type 0 doughs depends on the microbiota of the used baker’s yeast, as the production of baker’s yeast is not a sterile fermentation. Besides *Saccharomyces (S.) cerevisiae*, lactic acid bacteria play a role after a dough propagation of 8-16 hours, especially *L. plantarum*, but also *L. brevis*, *L. curvatus* and *L. sakei*, *Leuconostoc (Lcn.) lactis* and *Pediococcus (P.) pentosaceus* are often found (Brandt and Gänzle, 2006).

Type I sourdoughs are characterised by fermentation temperatures below 30 °C and continuous refreshments (every 4-24 hours) to keep microorganisms permanently active (Brandt, 2001). These doughs are used both for acidification and leavening, so that addition of baker’s yeast is not necessary. Products are panettone, San Francisco sourdough French bread (wheat) and German three-stage rye sourdough breads. The microbiota consists of LAB and yeasts, which may persist over years, if the process parameters remain constant (Böcker et al., 1990; Böcker, 1993). Frequent LAB of type I sourdoughs are *L. acidophilus*, *L. alimentarius*, *L. brevis*, *L. fructivorans*, *L. pentosus*, *L. paralimentarius*, *L. plantarum*, *L. pontis*, *L. spicheri* and *L. sanfranciscensis* as well as *Lcn. mesenteroides* and *Weissella (W.) confusa*; frequent yeasts are *S. exiguus* (recently renamed as *Kazachstania exigua*), *Candida (C.) humilis* and *S. cerevisiae* (Brandt and Gänzle, 2003). The most typical and well-studied association of LAB and yeasts in type I sourdoughs is *L. sanfranciscensis* and *C. humilis*. Already in 1924, Knudsen described a heterofermentative sourdough bacterium named *betabacterium* γ . In 1971, Kline and Sugihara (1971b) isolated *L. sanfrancisco* from ‘San Francisco’ wheat sourdough. In 1984, Spicher isolated a bacterium named *L. brevis* ssp. *lindneri* from sourdough for several times and expected it to be the same species as *betabacterium* γ . It was specialized for long-term rye sourdoughs, and breads baked from sourdoughs with this organism had optimal quality characteristics regarding acidity, elasticity of crumb, pore structure, and taste (Spicher et al., 1980). *L. brevis* ssp. *lindneri* was shown to be synonymous with *L. sanfrancisco* (Spicher and Schroeder, 1978) and *L. sanfrancisco* was renamed as *L. sanfranciscensis* in 1997 (Trüper and De Clari). In the past decades, *L. sanfranciscensis* has been isolated from a lot of traditional long-term wheat and rye sourdoughs (overview: Gobetti and Corsetti, 1997). It is considered to be the most important LAB of type I sourdoughs (Brandt and Gänzle, 2006; Gobetti and Corsetti, 1997). Recently, the genome of *L. sanfranciscensis* TMW 1.1304 was sequenced (Vogel et al., 2011). It was found that *L. sanfranciscensis* has a genome size of 1.3 Mbp and therefore contains the smallest genome

of all lactobacilli. In addition, it has the highest density of ribosomal RNA operons per Mbp genome among all known genomes of free-living bacteria, which allows high growth rates. Until now, *L. sanfranciscensis* has exclusively been found in sourdough (De Vuyst et al., 2009; own literature research) with one exception: Groenewald et al. (2006) found it in the gastro intestinal tract of vinegar flies. Vrancken et al. (2011) examined the microbiota of 4 spontaneously fermented type I wheat sourdoughs fermented under different conditions. *L. sanfranciscensis* was not present. As *L. sanfranciscensis* has never been found in semi-sterile laboratory fermented sourdoughs (Vrancken et al., 2011), but often in artisanal sourdoughs, the authors suggest a nonflour origin.

Kline and Sugihara (1971a) described a yeast named *Torulopsis holmii*, the imperfect form of *S. exiguus*, associated with *L. sanfranciscensis* in San Francisco French bread sourdough. Yarrow (1978a,b) renamed *Torulopsis holmii* to *Candida holmii* and divided the species because of differing GC contents into *C. holmii* and the new yeast *C. milleri*. Kurtzmann und Robnett (1998) at last found the two yeasts *C. milleri* and *C. humilis* to be one species. So the yeast described by Kline and Sugihara (1971a) presumably also was *C. humilis*. The association of *L. sanfranciscensis* and *S. exiguus* was further described by Nout and Creemers-Molenaar (1987) in commercial Dutch wholemeal wheat sourdoughs. Gobbetti et al. (1994a) detected this association in sourdoughs for Panettone. Garofalo et al. (2008) identified *L. sanfranciscensis*, *L. brevis* and *C. humilis* as dominant microbiota in sourdoughs for panettone via cultivation and PCR-DGGE. Venturi et al. (2012) examined sourdoughs for Panettone and Lagaccio, where a stable microbial community of *L. sanfranciscensis*, *S. cerevisiae* and *C. humilis* occurred. Böcker (1993) found *L. sanfranciscensis* strains in 4 of five analyzed starter sourdoughs (mother sponges) which were propagated continuously for several years. In one mother sponge, *L. sanfranciscensis* was associated with *C. humilis* (Böcker et al., 1990). Regular refreshments keep the microorganisms in an active state and so cause an adaptation advantage for *L. sanfranciscensis* strains (Böcker, 1993). The reasons for the competitiveness of the association *L. sanfranciscensis* - *C. humilis* have been researched extensively (Gobbetti and Corsetti, 1997). *C. humilis* is a maltose-negative yeast, *L. sanfranciscensis* is maltose-positive (Sugihara et al., 1970). This prevents a competition for the main carbon source, maltose. In addition, *L. sanfranciscensis* transports maltose into its cells, cleaves it without expenditure of adenosine-triphosphate, and excretes glucose (Stolz et al., 1993), which results in glucose accumulation. The glucose is assimilated by *C. humilis*. In return to this glucose supply, *C. humilis* provides essential amino acids and peptides which stimulate growth of *L. sanfranciscensis* (Gobbetti et al., 1994b; Gobbetti, 1998), as well as

fructose by its invertase activity, which is used as electron acceptor by the lactobacilli to increase their energy yield (Gobbetti et al., 1994c). In contrast, the maltose-positive *S. cerevisiae*, on one hand, competes with *L. sanfranciscensis* for maltose. On the other hand, the maltose uptake of *S. cerevisiae* is repressed by the accumulated glucose. Nout and Creemers-Molenaar (1987) found that even by contaminating a sourdough containing *L. sanfranciscensis* and *C. humilis* with *S. cerevisiae*, the association *L. sanfranciscensis* - *C. humilis* was stable and *S. cerevisiae* fell under the detection limit after the second propagation step. Besides the maltose competition and the glucose repression, further explanations are discussed. *S. exiguus* and *C. humilis* are very tolerant against acetic acid (Gänzle et al., 1997; Suihko and Mäkinen, 1984), some strains of *S. cerevisiae* are not (Suihko and Mäkinen, 1984). Rosenheim (2004) compared the growth rates of *S. cerevisiae* and *C. humilis* in a rye sourdough with *L. sanfranciscensis*. The growth rate of *S. cerevisiae* was $\mu_{\max} = 0.21$, the growth rate of *C. humilis* was $\mu_{\max} = 0.311$. As the cell size of *S. cerevisiae* (8 μm) was twice the cell size of *C. humilis* (4-5 μm), the cell size seems to play a role for the growth rate, and the growth rate for the competitiveness in sourdoughs.

Type II sourdoughs are mainly used as acidifiers for bread doughs and thus are characterised by process temperatures above 30 °C, sometimes above 40 °C, high dough yields, and long fermentation periods (up to 5 days) (Böcker et al., 1995). Because of these process parameters, yeasts occur rarely. Meroth et al. (2003b) found *Issatchenkia (I). orientalis* (recently renamed to *Pichia kudriazevii* by Kurtzman et al. (2008)) in a stirred rye bran fermentation at 40 °C with a refreshment time of 48 hours. Rosenquist and Hansen (2000) detected *S. cerevisiae* in an industrial type II sourdough fermented by regular refreshments over 28 weeks at 30-34 °C. Böcker et al. (1993) did not find yeasts in silo sourdoughs. Frequently isolated LAB in type II sourdoughs are *L. panis* and *L. pontis* (Brandt and Gänzle, 2003). They are characterized by a high acid and temperature tolerance (Brandt and Gänzle, 2003; Wiese et al., 1996). Other LAB in type II sourdoughs are *L. acidophilus*, *L. crispatus*, *L. delbrueckii*, *L. fermentum*, *L. johnsonii* and *L. reuteri* (Brandt and Gänzle, 2003; Meroth et al., 2003a). Type I and II sourdoughs have in common that both doughs are inoculated with “ripe” sourdoughs so that the process can be continued over years. One reason for the competitiveness of *L. reuteri* may be that some strains produce several bacteriocins and similar compounds with antimicrobial activity, for example reuterin (Axelsson et al., 1989) and reutericyclin (Gänzle and Vogel, 2003; Gänzle, 2004).

Last, type III doughs are dried sourdoughs (mostly type II) which are used as dough acidifiers, aroma carriers or as starter for sourdoughs in bakeries. The microbiota mainly

consists of LAB which are resistant to drying and storage. Yeasts are rarely found which is a result of the high fermentation temperatures.

The organic acids produced by the LAB, especially lactic acid and acetic acid, are responsible for pH reduction, influence bread flavour and reduce spoilage caused by bacteria and molds. Moore et al. (2008) also reported antifungal activity of a *L. plantarum* strain, Ryan et al. (2011) of a *L. amylovorus* strain. Antimycotoxigenic activity is also reported for LAB strains (El-Nezami et al., 2002; Fuchs et al., 2008; Piotrowska and Zakowska, 2005). Furthermore, amino acids and peptides are generated by the proteolytic activity of LAB and yeasts as well as by cereal enzymes present in the flour (Hammes and Vogel, 1997; Thiele, 2002). Some strains even produce antioxidant peptides (Coda et al., 2012). Thiele et al. (2002; 2003) showed that proteolysis in dough is caused mostly by cereal enzymes and the low pH and only to a small part by proteolytic activities of yeasts and LAB. Especially the amino acids ornithine, leucine, phenylalanine and methionine act as key aroma precursors for the characteristic bread flavour (Schieberle, 1996). Czerny et al. (2003; 2005) reported that different LAB and yeast species produce specific concentrations of aroma compounds and aroma precursors, so that the bread flavor depends on the used fermentation microbiota.

Numerous different yeast species occur in sourdoughs, amongst others belonging to the genera *Saccharomyces*, *Candida*, *Debaryomyces*, *Dekkera*, *Pichia* (P.), *Hansenula* and *Torulaspora* (Rossi, 1996; Brandt and Hammes, 2003). However, some yeasts are not well adapted to the substrate sourdough, as for example some *Pichia* species. They are obligate aerobic yeasts and therefore are not competitive in the anaerobic milieu of long-term sourdoughs. The most frequent yeast species in sourdough are *S. cerevisiae*, *S. exiguus*, *C. humilis*, *Pichia kudriazevii* (formerly *I. orientalis*) or its anamorphic form *C. krusei*, *Wickerhamomyces anomalus* (formerly *P. anomala*), and *P. subpelliculosa* (Pulvirenti et al., 2004). The main function of the yeasts in breadmaking is the production of CO₂. Further important functions are modification of the dough rheology and production of aroma precursors and aroma compounds (Jenson, 1998; Suihko and Mäkinen, 1984). Sourdough yeasts are characterized by their acid tolerance, whereas *S. cerevisiae* is much more acid sensitive than for example *S. exiguus* (Rossi, 1996; Suihko and Mäkinen, 1984). Vogel (1997) supposed that the competitiveness of *C. krusei* (= anamorphic form of *I. orientalis*) and *C. humilis* in sourdoughs is caused by their tolerance to undissociated acetic acid and the fact that they are maltose-negative. *S. cerevisiae* is a frequently found sourdough yeast, although several authors reported that it is not successful in continuously propagated sourdoughs (Vogel, 1997; Nout and Creemers-Molenaar, 1987). The yeast species can metabolize

different types of carbohydrates, which is important for interrelationships with other present yeasts and LAB in the dough. Especially the competition for maltose plays a role for the ability of a species or strain to establish in the environment or not (Rossi, 1996).

Although the microbiota of sourdoughs has been intensely studied during the last decades, it is still not clear how the composition of the microbiota is influenced by the substrate, the process factors and interactions between microorganisms. The kind of flour and flour quality seem to play a major role but to which extent is not evident until now (De Vuyst et al., 2009). The bakery environment also plays a role, as recently demonstrated by Scheirlinck et al. (2009). A well-studied process factor is the fermentation temperature. Generally, while low fermentation temperatures stimulate yeast growth, higher temperatures promote growth of LAB and acidification (Stephan, 1982). The different types of sourdoughs are mainly based on fermentation temperatures (see above). This has also been shown by Meroth et al. (2003a,b), where sourdoughs fermented at different temperatures contained a unique microbiota each. Several investigations have been made concerning influence of temperature, pH, inoculum size, ionic strength, organic acids, and NaCl and sucrose concentrations on the sourdough microbiota (Brandt et al., 2004; Gänzle et al., 1998; Passos et al., 1994; Spicher, 1961; Simonson et al., 2003). The buffering capacity of the dough influences the pH and thus growth of LAB (Rohrlich, 1960). Gänzle and Vogel (2003) showed that *L. reuteri* is a competitive type II sourdough LAB due to its production of reutericyclin. Interactions between LAB and yeasts are important for the stability of the sourdough microbiota (Gobbetti, 1998; Brandt et al., 2004). A well-studied association is between *L. sanfranciscensis* and *C. humilis* (Gobbetti et al., 1994b,c; Brandt et al., 2004; Gänzle et al., 1998). Despite all these investigations, there is a lack of information about the competitiveness of LAB and yeast species or strains in sourdoughs and how it can be influenced.

Recently, new demands have emerged concerning breads and bakery products in general. On one hand, smaller bakeries suffer from high competition pressure through market saturation by cheap bakery goods produced by the baking industry and thus have to offer special and niche products to keep their competitiveness and attract the clients. This leads to a revival of sourdough fermented rye bread and wheat breads with type 0 predoughs as well as to experimentation with new ingredients and tastes. On the other hand, consumers ask for a greater variety of baked goods and often have special demands, for example gluten free products. As the amount of people suffering from celiac disease is one of every 100 to 250 people in Europe (Hoffenberg et al., 2004), gluten free products of good quality are widely

requested. Coeliac disease is caused by an autoimmune response to peptides contained in gluten (Helms, 2005). When coming in contact with gluten, the intestinal villi are destroyed, resulting in malabsorption of food, anemia, diarrhea, abdominal pain, failure to thrive and delay of puberty (Hoffenberg et al., 2004; Helms, 2005). The only remedy is a lifelong gluten free diet. Several investigations have been conducted to identify and eliminate the problematic peptides in gluten (Rizzello et al., 2006; Hartmann et al., 2006), or to apply sourdough fermentations with special LAB that excrete peptidases to digest the problematic peptides (Di Cagno et al., 2002; 2004; 2005). The alternative is the exclusive use of gluten free cereals like maize, rice, sorghum and millets, and pseudocereals. Unfortunately, the gluten free baked goods are mostly of poor quality (Arendt, 2009). Sourdough fermentation can help to improve the quality of gluten free breads (Moore et al., 2007; Arendt, 2009; Brandt and Bode, 2009).

Sourdoughs and fermented products from other cereals and pseudocereals

Fermentation of cereal substrates is applied all over the world. Especially rice, maize, sorghum, millet and cassava are fermented into a huge variety of products, which often constitute a major part of the people's diet. In Africa and South America, fermentations are often started spontaneously and conducted in small scales in private households. Other fermentations are performed in huge scales, for example the industrialized fermentation of rice for sake production. As fermentation conditions and substrates are different to traditional rye and wheat sourdoughs, the microbiota also consists partly of different species. Dominant LAB belong to the genera *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus*, and *Weissella*, dominant yeast genera are *Saccharomyces* and *Candida*. The most competitive species in those studies are *L. fermentum*, *L. plantarum*, *S. cerevisiae* and *I. orientalis*. *L. fermentum* is often a competitive LAB in traditional millet and sorghum sourdoughs (Hamad et al., 1997) and fermentations (Lei and Jakobsen, 2004; Mugula et al., 2003a,b) as well as in maize dough (Hayford et al., 1999). In cassava fermentations, *L. fermentum* occurs but not always. For example, Lacerda et al. (2005) found *L. fermentum* in sour cassava starch, Mante et al. (2003), and Miambi et al. (2003) in fermented cassava dough. Oyevole and Odunfa (1990) examined LAB in fermented cassava, where *L. fermentum* was not present. In a study about LAB in 5 fermented cassava probes for Gari from Benin, Kostinek et al. (2005) found that *L. fermentum* was the third frequent LAB with 18 % of all isolates. The most frequent LAB was *L. plantarum*. This is congruent with several other studies about the

microflora of cassava sourdoughs, where *L. plantarum* was always the predominant LAB (Mante et al., 2003; Miambi et al. 2003; Amoa-Awua et al., 1996). In the past, *L. plantarum* was also often isolated from millet fermentations (Mugula et al., 2003a; Gashe, 1985, Muyanja et al., 2003), but not from sorghum sourdoughs (Hamad et al., 1992, 1997; Gasseem, 1999). *S. cerevisiae* is a common yeast in wheat and rye sourdoughs, as well as in fermentations from non-bread cereals. It was described as competitive yeast in sorghum beer fermentations (van der Aa Kühle et al., 2001; Glover et al., 2005), in maize sourdoughs (Rocha and Malcata, 1999; Hayford and Jespersen, 1999; Obiri-Danso, 1994; Jespersen et al., 1994), in starters for rice wine fermentation (Dung et al., 2007), and in fermented cassava (Oyewole, 2001). *I. orientalis* has often been found in maize sourdoughs (Hayford and Jakobsen, 1999; Obiri-Danso, 1994; Jespersen et al., 1994). Furthermore, it is a common yeast in fermented sorghum and millet (Hamad et al., 1992; Mugula et al., 2003a).

The microbiota of those fermentations is widely studied, but not systematically. Knowledge about starter cultures and fermentation conditions is often not existent, so that microbial studies are not reproducible and comparable with others. In addition, the microbiota is mostly identified only by bacteriological culture combined with physiological and physicochemical criteria, which is not sufficient for a reliable species identification.

This chapter gives an overview about common fermentation practices, products and the appropriate microbiota of fermented alternative cereals maize, rice, sorghum and millet and the starchy root cassava as well as information about oat and barley and the pseudocereals buckwheat (*Fagopyrum esculentum* Mönch), amaranth (*Amaranthus caudatus* L.) and quinoa (*Chenopodium quinoa* Willd). However, information about the microbiota of fermentations with buckwheat, amaranth, quinoa, oat and barley is rare.

Fermented maize

Maize (*Zea mays*) belongs to the plant family of *Poaceae* (*Gramineae*), subfamily of *Panicoideae*. It was originally cultivated in Central America since 4500 BC and was the staple food of the Incas (Peru), Mayas and Aztecs (Mexico). Columbus brought maize to Southern Europe, where it was cultured and included into the diet (FAO, 1999). Traditional Portugese maize sourdough was studied by Rocha and Malcata (1999). They found a huge and unmanageable variety of bacteria and yeasts. As they identified species with API, results are furthermore not reliable. Almeida and Pais (1996) investigated yeasts in Portugese bread

doughs made with rye and maize. Dominating yeast was *S. cerevisiae*, followed by *I. orientalis*, *P. membranaefaciens* and *Torulaspora (T.) delbrueckii*. A very popular and well-studied fermented maize product is Pozol, made by fermenting and diluting maize dough in small household scales. The origins of this product trace back to the Mayas (Ulloa and Herrera, 1986). Maize kernels are boiled, peeled and rinsed, ground, shaped into balls and fermented in banana leaves for 1 to 5 days (Ulloa and Herrera, 1986). For consumption, the product is diluted with water. Several studies have been conducted in regard to the microbiota of pozol (Ampe et al., 1999a,b; Ben Omar and Ampe, 2000; Escalante et al., 2001; Diaz-Ruiz et al., 2003). Ampe et al. (1999b) only distinguished bacteria to the genus level. Ampe et al. (1999a) used 16S rRNA-targeted oligonucleotide probes, 16S rDNA gene sequencing and PCR-DGGE for determination of the microbiota. They found that *L. fermentum* and *L. plantarum* were the dominant organisms, together with members of the genera *Streptococcus*, *Leuconostoc* and *Weissella*. Ben Omar and Ampe (2000) expanded the investigations and found *L. casei* and *L. delbrueckii* besides *L. fermentum* and *L. plantarum* as dominant LAB. Escalante et al. (2001) used extraction of total DNA and PCR amplification as methods. They found *Lactococcus (Lc.) lactis* and *Streptococcus (S.) suis* as well as *L. plantarum*, *L. alimentarius*, *L. casei* and *L. delbrueckii* as dominant organisms. No fermentation conditions were depicted. Diaz-Ruiz et al. (2003) only determined amylolytic LAB strains and found *S. bovis* strains to be dominant. All studies have in common that pozol balls or nixtamal dough samples (cooked, ground raw material) were bought on markets so that nothing is known about preceding handlings and contaminations. Kenkey, a fermented maize dough in Ghana, was studied by Halm et al. (1993), Jespersen et al. (1994), Obiri-Danso (1994), Olsen et al. (1995), Hayford and Jespersen (1999) and others. Kenkey and other fermented maize dough products are a staple food in Ghana. It is produced in small scales under poor hygienic conditions. All fermentations are spontaneous. The corn is cleaned, steeped, wetmilled into grist, mixed with water and fermented for 1-3 days (Obiri-Danso, 1994). Halm et al. (1993) and Olsen et al. (1995) found *L. fermentum* and *L. reuteri* as dominating LAB microbiota. Jespersen et al. (1994) and Obiri-Danso (1994) studied yeasts in kenkey and found *C. krusei*, *S. cerevisiae*, *C. tropicalis* and *C. kefir* to be dominating. Hayford and Jespersen (1999) isolated and characterized *S. cerevisiae* strains, Hayford and Jakobsen (1999) strains of *C. krusei* from maize dough. *L. fermentum* was isolated from maize dough by Hayford et al. (1999) as dominant species. A lot of other fermented maize products exist, which have been studied more or less, for example chicha (South America), ogi (West Africa) (see below at sorghum/millet), mahewu (South Africa), mawe (Benin)

(FAO, 1999; Campbell-Platt, 1987; Steinkraus, 1983), and different types of maize beer (Morris, 1979; FAO, 1999).

Fermented rice

Rice (*Oryza sativa*) has its origins in Asia, has been cultivated since about 4500 BC and is the second most abundant cereal crop after wheat (FAO, 1999). It belongs to the subfamily of *Oryzoideae* within the family of *Poaceae* (*Gramineae*). 90 % of the world rice crop is grown in Asia (FAO, 1999). Rice fermentations are mostly conducted with yeasts and molds, lactic acid fermentations are rare and play a minor role (FAO, 1999). For example, Chinese red rice (also called Angkak) is inoculated and fermented with *Monascus purpureus* (Steinkraus, 1983). In Vietnamese rice wine fermentation starters, yeasts and molds dominated, LAB were present at relatively low levels. Dominating yeast was *S. cerevisiae*, the LAB species were not identified (Dung et al., 2007). In Balinese rice wine, *P. pentosaceus* and *Weissella* spp. were the dominant LAB (Sujaya et al., 2002). A further product is idli, which is widely used in the South of India. It is produced from rice and black gram. Mukherjee et al. (1965) determined *Lcn. mesenteroides*, *S. faecalis* and *P. cerevisiae* as dominating LAB. In the Philippines, a product named puto is similar to idli, but contains no legume. The microbiota is also similar (Steinkraus, 1983). In Asia, different types of rice beer are produced, depending on regional customs. The fermentation microbiota mainly consists of different yeasts and molds (Pederson, 1979). Furthermore, plenty of different fermented rice cakes are produced in Asia such as Indonesian tapé ketan. The basic product is ragi. It is generally fermented by *Amylomyces rouxii* and different yeasts like *C. pelliculosa*, *Endomycopsis burtonii*, *S. cerevisiae*, other *Saccharomyces* and *Hansenula* spec., LAB do not play a role (Steinkraus, 1983; Rose, 1982; Ardhana and Fleet, 1989; FAO, 1999). A similar product is poko, a fermented rice product in Nepal, where the starter is called murcha. In the poko fermentation, yeasts play the major role, followed by LAB and molds. Dominant yeasts are *S. cerevisiae* and *C. versatilis*, dominant LAB is *P. pentosaceus* (Shrestha et al., 2002). The first study of rice sourdoughs was conducted by Meroth et al. (2004), using PCR-DGGE. The most competitive LAB in one of the two sourdoughs was the newly described *L. spicheri* with shares of about 80 %. Other LAB were *L. paracasei*, *L. perolens* and *L. paralimentarius*, and in the last fermentation step also *L. pontis*. The unique yeast was *S. cerevisiae*. In the second rice sourdough, the microbiota consisted of *L. fermentum*, *L. gallinarum*, *L. kimchii*, *L. plantarum* and *L. pontis*. Besides the dominant yeast *S. cerevisiae*, *I. orientalis* and

P. membranifaciens were present (Meroth et al., 2004). Other studies about rice sourdoughs do not exist to date.

Fermented sorghum/millet

Sorghum and millets consist of several genera and species. Within the family of *Poaceae* (*Graminae*), sorghum and most of the millets belong to the subfamily of *Panicoidae*. Within the huge amount of sorghum species, *Sorghum bicolor* (L.) Moench plays the most important role. Sorghum is a tropical cereal grass, similar to maize and rice. Its origins lay in Africa 3000 to 5000 years ago (Belton and Taylor, 2002; FAO, 1999). The plant is very draught tolerant and therefore an important food crop in dry landscapes. Main producer is the USA, where sweet sorghum is also used for production of bioethanol (Belton and Taylor, 2002; Mamma et al., 1996). “The millets” is a collective term for different plant genera, which have in common small, spelted seeds. Common millets are pearl millet (*Pennisetum glaucum*), having bigger seeds, and the small or minor millets foxtail millet (*Setaria italica*), true or proso millet (*Panicum miliaceum*), finger millet (*Eleusine coracana*), barnyard millet (*Echinochloa crus-galli*), kodo millet (*Paspalum scrobiculatum*), white fonio (*Digitaria exilis*), black fonio (*Digitaria iburua*), and teff (*Eragrostis tef*). They are an important staple food in the dry zones of Africa, especially in the Sahel (Belton and Taylor, 2002). Millets contain antinutritional factors as phytic acid, amylase inhibitors, trypsin inhibitors, tannins and polyphenols, which can be reduced by fermentation (Sharma and Kapoor, 1996; Anthony and Chandra, 1998; Elyas et al., 2002). Sorghum is deficient in lysine and sulphur containing amino acids. Fermentation of sorghum increases its nutritive quality, especially the contents of lysine and methionine (Au and Fields, 1981).

A popular fermented sorghum product is kisra, a flat bread which constitutes a major part of the staple diet of the people in Sudan. Several authors examined sorghum sourdoughs for kisra production (Hamad et al., 1992, 1997; Mohammed et al., 1991; Gasseem, 1999). Mohammed et al. (1991) studied the population of a spontaneously fermented sorghum sourdough. After 3 refreshment steps, *P. pentosaceus* was the dominant organism, other bacteria were *Enterococcus* (*E.*) *faecium*, *L. brevis* and *L. confusus*. The dominant yeasts were *C. intermedia* and *Debaryomyces* (*D.*) *hansenii*. Hamad et al. (1992) fermented sorghum flour in the traditional way for kisra production. Dominant LAB were *L. fermentum*, *L. reuteri* and *L. amylovorus*, dominant yeast was *C. krusei*. However, identification of LAB was conducted with the api 50 system and other physiological characteristics. Own results, based on 16S

rRNA sequencing, showed that the strains identified by Hamad et al. (1992) as *L. fermentum* and *L. amylovorus* were in reality *L. pontis* and *L. helveticus*, respectively (see chapter III). This shows the unreliability of physiological identification. In a later investigation, Hamad et al. (1997) studied sorghum flour and sorghum sourdoughs. In that study, *L. fermentum*, *L. reuteri*, *Lc. lactis* and *E. faecalis* as well as *L. vaginalis* and *L. helveticus* were the dominant LAB. These are the only studies where *L. helveticus* was found in cereal fermentations and constituted a major part of the microbiota. Gassem (1999) fermented sorghum flour for khamir bread production and found after 24h of fermentation *L. brevis*, *Lc. lactis*, *Lc. cellobiosus*, *C. parapsilosis*, *C. norvegensis*, *Rhodotorula glutinis* and several different contaminating bacteria and molds. In sorghum flour and fermented sorghum porridge, the dominant species was *L. plantarum* (Kunene et al., 2000). Other LAB were *Lcn. mesenteroides*, *L. sake/curvatus*, *P. pentosaceus*, *P. acidilactici*, and *Lc. lactis*. Ogi is a fermented cereal porridge which serves as staple and weaning food in West Africa. The production is similar to that of kenkey, the fermented porridge is cooked before consumption. Ogi is produced from maize, sorghum or millet or a mixture of them. Dominating LAB is *L. plantarum*, dominating yeasts are *C. mycoderma* and *S. cerevisiae*. Furthermore, *Corynebacterium* and *Aerobacter*, as well as some mold species are involved (FAO, 1999). Johansson et al. (1995) examined LAB in Nigerian ogi from sorghum and maize and other fermented sorghum and maize products by API 50CH. Out of these LAB, *L. plantarum* was the dominant organism. Sanni et al. (1994) used several of these LAB for ogi fermentation and found *L. plantarum* to be the best starter for fermentation of ogi. In Beninese ogi from maize, *L. fermentum* and *L. brevis* were dominant together with *C. humicola*, *C. krusei* and *Geotrichum* spp.. The differences in the microbiota are probably caused by process differences (Nago et al., 1998). In togwa, a fermented porridge from sorghum, millet, maize and maize-sorghum, *L. plantarum* and *I. orientalis* dominated the final stages of fermentation (Mugula et al., 2003a) and suited best for further togwa fermentation (Mugula et al., 2003b). *P. cerevisiae*, *L. brevis*, *L. plantarum* and *L. fermentum* constituted the dominating microbiota of teff fermentation for Ethiopian injera, a sour fermented sorghum bread (Gashe, 1985). Lei and Jakobsen (2004) found *W. confusa* and *L. fermentum* as dominating organisms in Koko and Koko sour water, a millet porridge and drink. A popular drink made of sorghum and millets is beer. In Pito, a type of sorghum beer in Nigeria, Togo and Ghana, *S. cerevisiae* was predominating (Demuyakor and Ohta, 1991; Sefa-Dedeh et al., 1999; Glover et al., 2005), as well as in other West African sorghum beers (van der Aa Kuhle et al., 2001). Sawadogo-Lingani et al. (2007) also studied the LAB community of pito and dolo wort for sorghum

beer. *L. fermentum* was the dominant LAB. *L. fermentum* also was the dominant LAB in Kimere, a pearl millet sourdough in Kenya (Njeru et al., 2010), and in Fura, a fermented millet dumpling in West Africa (Owusu-Kwarteng et al., 2012). *L. sanfranciscensis* did not grow in sorghum sourdoughs (Sekwati-Monang et al., 2012).

Fermented cassava

Cassava (*Manihot esculenta* Crantz) is a shrub from the family of *Euphorbiaceae* (spurge family) where the starchy roots are used for nutrition. The plant was domesticated 10,000 years ago along the southern border of the Amazon basin (Olsen and Schaal, 1999). The roots have high starch and low protein (1-2 %) contents (Steinkraus, 1983). The raw roots are poisonous because of their content of cyanogenic glycosides, especially linamarin and lotaustralin (Pedersen, 1979; Kostinek et al, 2005). “Sweet” cultivars only contain low levels of linamarine. Fermenting and cooking are two ways of eliminating the cyanogenic glucosides.

Fermented cassava products are widespread in the tropical zones of Africa and South America. Probably the most important cassava product in West Africa, especially in Nigeria, is gari, fermented and dried ready-to-eat cassava flour. Cassava roots are cleaned, peeled, grated and fermented and dewatered for up to 96 hours in cloth bags, toasted, dried, and sieved or milled (Kostinek et al, 2005; Steinkraus, 1983). A similar product is agbelima. The most abundant LAB species fermenting Gari and Agbelima were *L. plantarum*, followed by *Lcn. fallax* and *L. fermentum* (Kostinek et al, 2005). Other authors also reported that *L. plantarum* was the dominant species (Amoa-Awua et al., 1996; Mante et al., 2003); followed by other LAB as *L. brevis* and *Lcn. mesenteroides*. The same species and *L. cellobiosus* were also dominant in fufu, a fermented cassava flour similar to gari in South America, but soaked before fermentation (Oyewole and Odunfa, 1990). Competitive yeasts in fufu were *C. krusei*, *C. tropicalis* and *Zygosaccharomyces bailii* (Oyewole, 2001). In addition, *C. krusei* enhanced growth of *L. plantarum*. Brauman et al. (1996) also studied the bacterial populations in fermented cassava for fufu and found that *Lc. lactis*, *Lcn. mesenteroides* and *L. plantarum* replaced each other during the fermentation of 8 days. Amoa-Awua et al. (1997) studied yeasts and molds in inocula for agbelima dough. The same yeast species as Oyewole (2001) found in fufu, *C. krusei*, *C. tropicalis* and other *Zygosaccharomyces* spp., were prevalent in all 4 investigated types of inocula. Molds like *Penicillium* (*P.*) *sclerotiorum*, *P. citrinum*, *P. nodulum* and *Geotrichum candidum* were dominant in one inoculum. In Ghana

and Ivory Coast, a similar product to fufu is akyeke or attieke. Obilie et al. (2004) identified *L. plantarum*, *L. brevis* and *Lcn. mesenteroides* subsp. *cremoris* as dominant LAB. Coulin et al. (2005) came to a different result. *Lcn. mesenteroides* subsp. *mesenteroides* was dominant at the beginning, *L. salivarius*, *L. delbrueckii* subsp. *delbrueckii*, *L. fermentum* and *L. confusus* at the end of the fermentation. *C. tropicalis* was the prevalent yeast. Padonou et al. (2009) studied the microbiota of lafun, another fermented cassava flour in West Africa. *L. fermentum* was the dominant LAB followed by *L. plantarum* and *W. confusa*. The predominant yeast species associated with lafun fermentation were *S. cerevisiae*, *P. scutulata*, and *Kluyveromyces marxianus*. Sour cassava starch is produced in South America, especially in Colombia and Brazil. It is produced from grated cassava by watering, fermentation for 20-70 days and sun-drying (Lacerda et al., 2005). Lacerda et al. (2005) investigated the microbiota of sour cassava starch from two factories in Brazil via plating and PCR techniques as well as sequencing. The dominant LAB were *L. plantarum* and *L. fermentum*; *Galactomyces geotrichum* and *Issatchenkia* sp. were the dominant yeasts. Ampe et al. (2001) investigated the prokaryotic microbiota of sour cassava starch using denaturing gradient gel electrophoresis (DGGE). They found *Bifidobacterium minimum*, *Lc. lactis*, *L. panis* or *L. pontis*, *L. plantarum*, *L. manihotivorans*, *Lcn. citreum*, *Lcn. mesenteroides*, *E. saccharolyticus* and *Streptococcus* spec.. Shares of LAB were not determined except for *L. manihotivorans* with up to 13 %. *L. manihotivorans* is one of only few amylolytic LAB and was isolated during a sour cassava starch fermentation by Morlon-Guyot et al. (1998). Miambi et al. (2003) investigated fermented cassava dough with DGGE and culturing methods. Within a huge variety of species, *L. plantarum* was dominant together with *L. delbrueckii*. Other Lab were *L. fermentum*, *L. crispatus*, *L. delbrueckii* and *L. manihotivorans*. Some LAB strains isolated from different fermented cassava products produce linamarase, which is important for detoxification of cassava, for example strains of *Lcn. mesenteroides* (Gueguen et al., 1997), strains of *L. plantarum*, *L. brevis*, *Lcn. mesenteroides*, *L. salivarius* and *L. fermentum* (Obilie et al, 2004). Linamarase activity was found in all yeasts and molds isolated from inocula for agbelima cassava dough (Amoa-Awua, 1997). Oyewole (2001) discovered only *C. krusei* to produce linamarase.

Fermented oat and barley

Oat (*Avena sativa*) and barley (*Hordeum vulgare*) belong to the subfamily of *Poaceae* (*Gramineae*). The origins of barley are around 7000 BC in the Near East, whereas oat is a

much younger cereal crop, cultivated since about 400 BC in Europe (FAO, 1999). Oat has the difference to the other cereals to grow in panicles, not an ear. Oat is mostly used as rolled oats for food and feed, in small amounts for bread baking. In Great Britain it is used for brewing beer. Barley is used preferably for animal feed, food uses are alcoholic beverages (beer and whiskey) and flour used for porridges and baking. No information about oat fermentations or even of the microbiota is available except for one recent study about oat sourdoughs by Huettner et al. (2010). They found in spontaneously fermented oat sourdoughs the following LAB to be predominant: *Leuconostoc argentinum*, *Pedicoccus pentosaceus* and *Weissella cibaria* at 28 °C, *Lactobacillus coryniformis* at 37 °C. Some reports about barley sourdoughs exist (Marklinder and Johansson, 1995; Marklinder et al., 1996a,b), but without focus on the microbiota. Recently, Zannini et al. (2009) prepared barley sourdough by daily refreshment for two months with a multi-strain starter culture and analyzed the microbiota via culture techniques and PCR-DGGE. Dominant microorganisms were *L. plantarum*, *L. brevis* and *S. cerevisiae*.

Fermented pseudocereals

Buckwheat (*Fagopyrum esculentum* Mönch), amaranth (*Amaranthus caudatus* L.) and quinoa (*Chenopodium quinoa* Willd), belong to the dicotyledonous plants and thus are no cereals, but are named pseudocereals because of the similar composition of their seeds. They do not contain gluten and therefore are not able to retain gas, so that baking of leavened bread is not possible without additives.

Buckwheat belongs to the family of *Polygonaceae*, is an annual plant, 60-70 cm high, with small triangular seeds. It is originated in Central and North East Asia (Pomeranz and Lorenz, 1983) and now cultivated mostly in Asia and Central and Eastern Europe, largest producer is China (Wijngaard and Arendt, 2006). The seeds are dehulled before milling to flour. Buckwheat contains tannins (polyphenols) and flavonoids like rutin, hyperin and quercetin which act as antioxidants and in higher amounts as antinutritional compounds, and proteinase inhibitors as further antinutritional substances. Total polyphenol contents and antioxidant activity increase during fermentation (Đorđević et al., 2010). Colour comes from anthocyanin pigments (Pomeranz and Lorenz, 1983). Buckwheat also contains high concentrations of D-Chiro-inositol and its derivate fagopyritol B1, which reduces serum glucose concentrations in rats. Thus buckwheat concentrate is suggested as a natural product in helping to treat diabetes (Kawa et al., 2003; Aufhammer, 2000). The nutritional quality of buckwheat protein is much

higher than that of wheat, the only limiting amino acid is leucine (Wijngaard and Arendt, 2006) or rather methionine (Pomeranz and Lorenz, 1983). Buckwheat is usually sold as flour to make pancakes or as grouts. Fermented buckwheat products are uncommon and no information about them or their microbiota is available except for a recent study about buckwheat and teff sourdoughs from Moroni et al. (2011). *L. plantarum* was the dominating LAB in the buckwheat sourdoughs. At 25 °C, the yeast *Kazachstania barnetti* was solely found. At 35 °C, no yeast was present.

Amaranth belongs to the family of *Amaranthaceae* and is distributed worldwide. All grain amaranth species, where the seeds are used, originate from Middle and South America. Cultivated amaranth is characterised by pale instead of dark brown or black seeds, findings in Mexico date back until 4000 B.C.. Three different grain amaranth species originate in separate regions in Middle and South America and are cultivated there: *Amaranthus hypochondriacus* (Mexico), *A. cruentus* (Guatemala), and *A. caudatus* (Andes and Argentina) (Aufhammer, 2000). *A. hypochondriacus* is cultivated in heights until 3500m.

The origins of quinoa lay around the lake Titicaca in the Altiplano region in Peru and Bolivia around 5000 B.C.. It grows in heights to 3800 m and is an important food crop in this area (Aufhammer, 2000). The plant belongs to the *Chenopodiaceae*. For seed production, only the species *Chenopodium quinoa* is used.

Especially quinoa contains bitter tasting saponins as antinutritive component. Varieties with less saponin content are on the market. Saponins have to be washed out of the grains or the outer layers have to be removed by polishing the grains. Amaranth grains also have saponin contents, but the bright grains contain only minimal amounts of saponins that do not influence taste (Aufhammer, 2000; Belton and Taylor, 2002). Amaranth and quinoa also contain trypsin inhibitors and phytate as antinutritive components, in addition, amaranth contains lectins and tannins. Furthermore, amaranth grains contain squalene, tocotrienol and gamma linoleic acid, which are part of the fat fraction and are supposed to contribute together with fibers to lowering of the blood plasma cholesterol level (Aufhammer, 2000). Crude protein, raw fat and fiber contents in pseudocereals are generally higher than in cereals (Aufhammer, 2000).

Both amaranth and quinoa grains are usually ground to flour and used for flat breads, sweet gruels, soups or drinks. Popped grains are used for muesli, sweets and desserts. Quinoa is used in South America to make chicha, a fermented drink similar to beer (Simmonds, 1965; Belton and Taylor, 2002), but information about the microbiota is not available. Information on other fermented quinoa products is also missing. Fermentation essays with amaranth have

been tried for ogi, soy sauce, beer and spirit without determination of the microbiota (Belton and Taylor, 2002). Sterr et al. (2009) examined the microbiota of spontaneous amaranth fermentations. *Pediococcus pentosaceus* was dominant in all 4 fermentations, followed by *L. sakei*, *L. plantarum* and *L. paralimentarius*. Without the latter, all species were suitable as starter culture for amaranth fermentation between 25 and 35 °C.

Methods for investigation of the sourdough microbiota

Traditionally, the microbiota of sourdoughs was determined with cultivation methods followed by phenotypic species identification. For example, yeasts were classified by investigating growth requirements, assimilation and fermentation of sugars, nitrate and other substrates, by microscopic appearance such as cell shape and formation of mycelia or pseudomycelia, and by some specific reactions. As these methods were found to be insufficient for a reasonable identification and taxonomic classification of bacterial and yeast species, DNA-based methods were developed. Today, identification and phylogenetic taxonomy of bacterial and yeast species by rRNA gene sequencing is state of the art. Further common techniques based on cultivation and DNA fingerprinting are: amplified ribosomal DNA restriction analysis (ARDRA), randomly amplified polymorphic DNA (RAPD), pulsed field gel electrophoresis (PFGE), repetitive element sequence (rep)-PCR fingerprinting, ribotyping, monoplex or multiplex PCR and multi-locus sequence analysis techniques (Satokari et al., 2003; De Vuyst et al., 2009). Recently, Scheirlinck et al. (2009) described a real-time PCR method for quantitative detection of LAB in sourdough. RAPD was originally introduced by Williams et al. (1990). Müller et al. (2001) first applied the method for LAB using primer M13V. DNA is amplified under relaxed conditions with an unspecific primer that detects polymorphisms, which leads to a mixture of DNA strands of different length. Separation by agarose gel electrophoresis results in a specific band pattern for each species or even different strains (Vogel et al., 1996; Tynkkynen et al., 1999). An advantage of cultural techniques is that isolates can be collected and stored which can be used for further studies. However, culturing techniques combined with DNA isolation, PCR, and sequencing or the above methods are time-consuming and are only suitable for a limited number of samples. Furthermore, regarding complex microbial communities, only those species can be determined which are capable to grow on the used culture medium. In 1993, Muyzer et al. presented a new DNA-based method, denaturing gradient gel electrophoresis (DGGE). It is a culture-independent determination technique for the microbiota of an ecosystem which is

based on DNA extraction from the habitat and amplification of a variable region of the rRNA gene, preferably 16S or 28S rRNA. Total DNA is extracted from the sample. For amplification of rRNA fragments, a special primer pair is used: a GC-rich sequence (GC clamp) of 30-40 bases is attached to one primer which insures that the DNA fragments will remain partially double stranded even under strong denaturing conditions. The PCR products have the same length (<500bp) but differing sequences. The mixture is subjected to polyacrylamide gel electrophoresis by using a denaturing gradient of urea and formamide. Migration stops when the DNA reaches its melting conditions, and the denatured DNA forms a band pattern according to the presence of different species, as variations in the DNA sequences cause different melting points. The band patterns are made visible by ethidium bromide staining. For identification of species, positions of bands can be compared with those of known species, or bands can be excised from the gel and sequenced (Muyzer and Smalla, 1998). An alternative is temperature denaturing gradient gel electrophoresis (TGGE), where a temperature gradient is used instead of urea and formamide. The result is a specific genetic fingerprint of the bacterial community in the analysed sample (Ercolini, 2004).

However, it has to be mentioned that only species that constitute 1 % or more of the total flora can be detected by PCR-DGGE. PCR bias can be caused by preferential amplification, leading to a lack of one or more species in the DGGE profile. Another problem can be co-migration of more than one different DNA sequences, which can be solved by using more than one primer pair for different regions of the 16S rDNA or by varying the DGGE conditions. Identification of excised DGGE bands by sequence analysis is often not reliable as DNA sequences suitable for DGGE can not be longer than 500bp. In addition, some species have a DGGE pattern with more than one band, caused by multiple copies of rRNA genes with sequence microheterogeneity. In addition to the living microorganisms, also DNA of dead cells can be detected (Muyzer und Smalla, 1998; Ercolini, 2004).

During the last ten years, DGGE has established as standard technique for a culture-independent investigation of the microbiota of various ecosystems, for example water from the river Seine (Cébron et al., 2004), human faeces (Walter et al., 2001), pozol (Ben Omar and Ampe, 2000), sausages (Cocolin et al., 2004), whisky (van Beek and Priest, 2002), mozzarella cheese (Cocolin et al., 2002; Ercolini et al., 2001; 2004), and soil (Martínez-Alonso et al., 2010). For sourdough, DGGE has also been adapted and used in numerous studies as it is a perfect tool for monitoring population dynamics over time (Meroth et al., 2003 a,b; 2004; Randazzo et al., 2005; Settanni et al., 2006; De Angelis et al., 2007; Garofalo

et al., 2008; Scheirlinck et al., 2008; 2009; Iacumin et al., 2009; Zannini et al., 2009; Minervini et al., 2010; Moroni et al., 2010; Valmorri et al., 2010).

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Chapter III

Adaptability of lactic acid bacteria and yeasts to sourdoughs prepared from cereals, pseudocereals and cassava and use of competitive strains as starters

Abstract

The adaptability of lactic acid bacteria (LAB) and yeasts to sourdoughs prepared from cereals, pseudocereals and cassava was investigated using PCR-DGGE and bacteriological culture combined with rRNA gene sequence analysis. Sourdoughs were prepared either from flours of the cereals wheat, rye, oat, barley, rice, maize, and millet, or from the pseudocereals amaranth, quinoa, and buckwheat, or from cassava, using a starter consisting of various species of LAB and yeasts. Doughs were propagated until a stable microbiota was established. The dominant LAB and yeast species were *Lactobacillus fermentum*, *Lactobacillus helveticus*, *Lactobacillus paralimentarius*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus spicheri*, *Issatchenkia orientalis* and *Saccharomyces cerevisiae*. The proportion of the species within the microbiota varied. *L. paralimentarius* dominated in the pseudocereal sourdoughs, *L. fermentum*, *L. plantarum* and *L. spicheri* in the cassava sourdough, and *L. fermentum*, *L. helveticus* and *L. pontis* in the cereal sourdoughs. *S. cerevisiae* constituted the dominating yeast, except for quinoa sourdough, where *I. orientalis* also reached similar counts, and buckwheat and oat sourdoughs, where no yeasts could be detected. To assess the usefulness of competitive LAB and yeasts as starters, the fermentations were repeated using flours from rice, maize, millet and the pseudocereals, and by starting the dough fermentation with selected dominant strains. At the end of fermentation, most of starter strains belonged to the dominating microbiota. For the rice, millet and quinoa sourdoughs the species composition was similar to that of the prior fermentation, whereas in the other sourdoughs, the composition differed.

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1. Introduction

Bread baking using sourdough is a common practice and has the advantage of improving the nutritional value and sensory qualities of breads, achieving the baking ability of doughs for rye bread production, and increasing the shelf life of breads by delaying the germination of bacterial and mould spores (Hammes et al., 2005). The microbiota of sourdoughs consists of specifically adapted lactic acid bacteria (LAB), mostly lactobacilli, as well as yeasts (Hammes and Gänzle, 1998). Its composition is affected by the endogenous ecological factors which in turn are determined by the flour and process (exogenous) factors. Although the microbiota of traditional wheat and rye sourdoughs has well been characterised, research on the sourdough microbiota underwent a renaissance in the last years, leading to an increase in description of new *Lactobacillus* species, e.g. *L. hammesii* (Valcheva et al., 2005), *L. rossiae* (Corsetti et al., 2005), *L. nantensis* (Valcheva et al., 2006), *L. siliginis* (Aslam et al., 2006), *L. secaliphilus* (Ehrmann et al., 2007), *L. namurensis* (Scheirlinck et al., 2007a) and *L. crustorum* (Scheirlinck et al., 2007b).

Recently, new consumer demands have emerged for food products with improved nutritional value or health benefit, posing new challenges also for the baking industry. Furthermore, baked goods from wheat and rye are problematic for an increasing amount of people suffering from celiac disease. Thus, there is a market for new novel bakery products produced by using alternative cereals like rice, maize, sorghum and millet, or pseudocereals such as buckwheat (*Fagopyrum esculentum* Mönch), amaranth (*Amaranthus caudatus* L.) and quinoa (*Chenopodium quinoa* Willd), and possibly even starchy roots such as cassava (*Manihot esculenta* Crantz). These plants do not contain gluten, the causative agent for celiac disease. Moreover, pseudocereals are rich in proteins, especially in essential amino acids such as lysine (Aufhammer, 2000), which is limited in wheat and rye flour. On the other hand, the use of such alternative flours is restricted due to their low baking quality, as well as the sensory quality of the baked products (Gallagher et al., 2003). Fermentation of such alternative flours may improve both the sensory and baking qualities. First sourdoughs from rice flour are already on the market, but are fermented by applying starters for wheat and rye sourdoughs (Meroth et al., 2004). Meroth et al. (2004) showed that during rice sourdough fermentation, substrate-specific LAB and yeast species establish which are different from the common microbiota of wheat and rye sourdoughs. In conclusion, there is a lack of knowledge on the adaptation and competition of LAB and yeasts in sourdough fermentation made from alternative cereals, pseudocereals or cassava, hindering the development of new starters.

Spontaneous fermentation of cereal substrates obtained especially from rice, maize, sorghum, millet and from cassava is applied all over the world, resulting in a huge variety of traditional products, e.g. Sudanese kiswa produced from sorghum, agbelima or fufu produced from fermented cassava doughs, pozol and kenkey produced from fermented maize. Studies on the characterisation of the microbiota of such traditional products revealed a great diversity of LAB and yeasts involved in the fermentation. Dominant LAB were shown to belong to the genera *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus*, and *Weissella*, dominant yeast genera are *Saccharomyces* and *Candida* (Amoa-Awua et al., 1996, 1997; Ben Omar and Ampe, 2000; Escalante et al., 2001; Hamad et al., 1992, 1997; Hayford and Jakobsen, 1999; Hayford and Jespersen, 1999; Hayford et al., 1999; Jespersen et al., 1994; Mante et al., 2003; Miambi et al., 2003; Obiri-Danso, 1994; Olsen et al., 1995). The most competitive species reported in the literature are *L. plantarum*, *L. fermentum*, *S. cerevisiae* and *I. orientalis*. The microbiota of such traditional fermentations has been studied widely but, to our opinion, not systematically. Often information about fermentation conditions is missing and species identification is often based on physiological criteria only, known to be insufficient for the identification of LAB. Moreover, no data are available about the microbiota of sourdoughs from buckwheat, amaranth, quinoa, oat and barley.

Denaturing gradient gel electrophoresis (DGGE) of PCR generated rRNA gene fragments has recently been shown to be a useful tool for rapid characterisation of the dominating fermentation biota at the species level. PCR-DGGE has successfully been applied not only to characterise the microbiota, but also to monitor the development of its composition during long-term fermentation. Examples of application are malt whisky fermentation (Van Beek and Priest, 2002), Mexican pozol (ben Omar and Ampe, 2000), rye sourdoughs (Meroth et al., 2003a, b), wheat sourdoughs (Randazzo et al., 2005), and rice sourdoughs (Meroth et al., 2004).

In this study, PCR-DGGE and bacteriological culture combined with RAPD-PCR and rRNA gene sequence analysis were used to characterise the adaptability of LAB and yeasts to sourdoughs made from flours of the cereals wheat, rye, oat, barley, rice, maize and millet, of the pseudocereals amaranth, quinoa and buckwheat, and of the starchy root cassava. Fermentations were inoculated with a starter mixture and continuously propagated until a stable microbiota was established. Dominating lactobacilli and yeasts were isolated from the sourdoughs and used as starter organisms in a sourdough fermentation to evaluate their competitiveness in the corresponding fermentation substrate.

2. Materials and Methods

2.1. Bacteria, yeasts and culture conditions

The following yeasts were used as reference RH in the DGGE analysis: *Debaryomyces hansenii* CBS 767^T, *Saccharomyces bayanus* CBS 380^T, *Saccharomyces uvarum* LTH H56, *Saccharomyces cerevisiae* CBS 1171^T, *Candida glabrata* DSM 6425, *Saccharomyces servazzii* CBS 4311^T, *Saccharomyces exiguus* CBS 379^T, *Candida humilis* CBS 6897^T, *Dekkera bruxellensis* CBS 74^T, and *Issatchenkia orientalis* CBS 5147^T. The following LAB were used as reference R1 in the DGGE analysis: *Weissella confusa* DSM 20196^T, *Lactobacillus johnsonii* DSM 10533^T, *Lactobacillus fermentum* DSM 20052^T, *Lactobacillus brevis* DSM 20054^T, *Lactobacillus crispatus* DSM 20584^T, *Lactobacillus acidophilus* DSM 20079^T, *Pediococcus pentosaceus* DSM 20336^T, *Lactobacillus farciminis* DSM 20184^T, *Lactobacillus panis* DSM 6035^T, *Pediococcus acidilactici* DSM 20284^T, *Lactobacillus pontis* DSM 8475^T, *Lactobacillus sanfranciscensis* DSM 20451^T, *Lactobacillus frumenti* DSM 13145^T, *Lactobacillus reuteri* DSM 20016^T, and *Lactobacillus paracasei* DSM 5622^T. For reference R2, the following strains were used: *Lactobacillus amylophilus* DSM 20533^T, *Lactobacillus plantarum* DSM 20174^T, *Lactobacillus paralimentarius* DSM 13238^T, *Lactobacillus amylovorus* DSM 20531^T, *Lactobacillus mindensis* LTH 5527, *Lactobacillus perolens* DSM 12744^T, *Lactobacillus buchneri* DSM 20057^T, *Lactobacillus spicheri* DSM 15429^T, *Lactobacillus fructivorans* DSM 20203^T, and *Lactobacillus ferintoshensis* DSM 15352^T (recently assigned to *L. parabuchneri* by Vancanneyt et al., 2005). Yeasts and LAB were routinely cultured in YG and MRS5 medium as described previously (Meroth et al., 2003a, b).

2.2. Sourdough fermentations and sampling

For fermentation I, eleven sourdough batches were prepared by using water and wholemeal flours of wheat, rye, oat, barley, rice, maize, millet, amaranth, quinoa, buckwheat, and cassava (dough yield 200). All batches were inoculated with 1% of baker's yeast and 10% of a starter mixture consisting in equal parts of the following starters: rye sourdough I, rye sourdough II, commercial rye full sour, rice starter, sorghum starter, teff starter, and cassava starter (see also Table 1). Rye sourdough I, a rye full sour was obtained from a local bakery and rye sourdough II was produced by inoculating rye dough with a commercial starter product. Before use, both were refreshed with wholemeal rye flour and fermented for 24 h at 30 °C. The rice, sorghum, teff, and cassava starter were self-made using LAB and yeasts isolated previously from traditional fermentations (Meroth et al., 2004; Hamad et al., 1992). For this,

doughs were prepared from flours, inoculated with overnight cultures of various LAB and yeasts and fermented for 24 h at 30 °C. Samples were taken from the different starters and baker's yeast and subjected to microbial counting and species determination by bacteriological culture and PCR-DGGE. Doughs of fermentation I were incubated at 30 °C and fermentation was continued by back-slopping every 24 h using 10% of the ripe sourdoughs until a stable microbiota was established. At each refreshment step, samples were taken from the ripe sourdoughs and the pH and total titratable acids (TTA) were determined. Samples were also subjected to microbial counting (at day 1, 2, 4, 6, 8, and 11). At the end of fermentation, samples were subjected to microbial counting and species determination by PCR-DGGE and bacteriological culture, combined with rRNA gene sequence analysis.

For fermentation II, lactobacilli and yeasts isolated from the dominating microbiota of fermentation I were used together with wholemeal flours of rice, maize, millet, buckwheat, amaranth, and quinoa. Doughs were prepared from water and flour (dough yield 200) and inoculated with strains grown overnight in YG or MRS5 medium to obtain final counts of 10^7 cfu/g dough. Fermentation was performed for 10 days as described for fermentation I. At each refreshment step, samples were taken from the ripe sourdoughs and the pH, TTA and microbial counts were determined. Samples were also subjected to species determination by PCR-DGGE (at day 0, 8 and 10). At the end of fermentation, samples were subjected to microbial counting and species determination by bacteriological culture and PCR-DGGE.

2.3. Analyses of samples

Cell counts of LAB and yeasts, pH, and TTA were determined as described previously (Meroth et al., 2003a, b). For species determination by bacteriological culture, dilutions of samples in saline-tryptone diluent (Meroth et al., 2003a) were plated on YGC agar (Meroth et al., 2003b) and MRS5 agar containing 0.1 g of cycloheximide/l (Meroth et al., 2003a), both supplemented with 25 mg bromocresol green/l for differentiation of colonies. Two to three colonies of each colony form and colour were picked from agar spread plates of the highest dilutions. The differentiation via colony forms in combination with the colony colours was found to be indicative in order to establish the species identification of the dominant microbiota (Meroth et al., 2003a,b, 2004). Shares of each LAB and yeast species in the sourdoughs were calculated by determining counts of each colony form as percentage of the total LAB or yeast count. Species identification of the isolates was performed as follows. DNA was isolated from pure cultures and used in RAPD-PCR as described below. One representative of each RAPD-type was subjected to sequencing of the first 550 bp of the

16 S rRNA gene (bacteria) or of D1/D2 region of the 28 S rRNA gene (yeast). For species determination by PCR-DGGE, total DNA was isolated from the samples as described below and subjected to PCR-DGGE.

2.4. DNA extraction

For the isolation of DNA of pure cultures, cells of 3 ml culture (A_{580} of 0.6-0.8) were harvested by centrifugation and resuspended in 1 ml of sterile phosphate-buffered saline (containing, per liter, 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 , and 0.24 g of KH_2PO_4 [pH 8.3]). A GenElute™ Bacterial Genomic DNA Kit (Sigma) was applied. Lysozyme (20 mg/ml; 185,000 U/mg) and mutanolysin (1000 U/ml) were used as lysing enzymes for LAB, a combination of 0.24 mg/ml of lysing enzymes from *Trichoderma harzianum* (Sigma) and 0.12 mg/ml of lyticase (Sigma) was used as lysing enzymes for yeasts. The lysis mixture was incubated at 37 °C for 60 min. The extraction of total DNA from dough samples was performed according to the method of Meroth et al. (2003a).

2.5. PCR-DGGE

For PCR-DGGE, primers L1GC (Meroth et al., 2003a) and HDA2 (Walter et al., 2000) were used for amplification of 16 S rDNA fragments of LAB in a Primus thermocycler (MWG-Biotech, Ebersberg, Germany). The reaction mixture (50 µl) contained 25 pmol of each primer, 0.2 mM of each deoxyribonucleotide triphosphate, reaction buffer (final concentrations, 10 mM Tris HCl [pH 8.3], 50 mM KCl, and 1.5 mM magnesium chloride, 0.1% Triton X-100), 2.5 U of *Taq* polymerase (Genaxxon, Biberach, Germany), 20 mM tetramethylammonium chloride (Sigma), 25 µg of bovine serum albumine (Sigma), and 1 µl of DNA solution. The amplification program was 95 °C for 2 min; 35 cycles of 94 °C for 30 s, 66 °C for 30 s, and 72 °C for 1 min; and 72 °C for 7 min. For amplification of the 28 S rDNA fragments of yeasts, primers U1 and U2 described by Sandhu et al. (1995) were used, but primer U1 was linked with the GC clamp described by Walter et al. (2001). The reaction mixture (50 µl) contained 25 pmol of each primer, 0.2 mM of each deoxyribonucleotide triphosphate, reaction buffer (final concentrations, 10 mM Tris HCl [pH 8.3], 50 mM KCl, and 1.5 mM magnesium chloride, 0.1% Triton X-100), 1.6 mmol MgCl_2 , 20 mM tetramethylammonium chloride, 2.5 U of *Taq* polymerase (Genaxxon), and 1 µl of DNA solution. The amplification program was as follows: 94 °C for 4 min; 35 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s; and 72 °C for 7 min. DGGE and excision of DNA fragments were performed as described previously (Walter et al., 2000). All

bands were cut from the gel and subjected to species determination via PCR and sequence analysis.

2.6. RAPD-PCR

With DNA isolated from pure cultures, RAPD-PCR was performed as described by Müller et al. (2001) with modifications. Briefly, the reaction mixture (50 µl) contained 100 pmol of primer M13V (Biomers, Ulm, Germany), 0.4 mM of each deoxyribonucleotide triphosphate, 3.5 mM MgCl₂, reaction buffer (final concentrations, 10 mM Tris HCl [pH 8.3], 50 mM KCl, and 1.5 mM magnesium chloride, 0.1% Triton X-100), 1.5 U of *Taq* polymerase (Genaxxon), and 1 µl of DNA solution. The amplification program and electrophoretic separation was performed as described previously (Meroth et al., 2003a).

2.7. Sequence analysis and species determination

DNA sequences of PCR fragments obtained from purified DGGE bands and pure cultures were determined as described previously (Meroth et al., 2003a, b). When the 16 S rRNA gene sequencing failed (some isolates of *L. pontis*), the 16 S rRNA gene fragments were subcloned in *E. coli* before sequencing. To determine the closest relatives of 16 S or 28 S rRNA gene sequences, a search of the Arb database (Ludwig et al., 2004) and GenBank database by using the BLAST algorithm (Altschul et al., 1990) was conducted. A similarity of >97% to 16 S or 28 S rRNA gene sequences of type strains was used as the criterion for identification.

As the sequence analysis of the first 600 bp of the 16 S rRNA did not permit to determine the species belonging to the *L. plantarum* group, a multiplex PCR targeting the *recA* gene described by Torriani et al. (2001) was used with the following modifications. The reaction mixture contained 100 pmol of each of the 4 primers, 0.2 mmol of each dNTP, reaction buffer (final concentrations, 10 mM Tris HCl [pH 8.3], 50 mM KCl, and 1.5 mM magnesium chloride, 0.1% Triton X-100), and 2.5 U of *Taq* polymerase (Genaxxon). The amplification program was 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s; and 72 °C for 5 min. The PCR products were visualized in a 2% agarose gel with a 100 bp marker as length standard.

3. Results

3.1. Species determination of the starter mixture and baker's yeast

To provide a diversity of LAB and yeasts for selection of highly competitive strains, a mixture of 7 starters and baker's yeast were used to inoculate doughs of fermentation I

(Table 1). Characterisation of the various starters and baker's yeast by PCR-DGGE and bacteriological culture combined with RAPD-PCR and rRNA gene sequence analysis revealed species compiled in Table 1. In general, some of the species could only be detected by bacteriological culture but not by PCR-DGGE analysis, and vice versa. With regard to yeasts, all isolates of *S. cerevisiae* exhibited identical RAPD patterns, whereas the patterns of *I. orientalis* isolates of the two starters slightly differed. In addition to *I. orientalis* in the cassava starter, an unknown yeast and a yeast of the genus *Torulaspora* were detected, the latter could however not be allotted to a species based on physiological tests and 28 S rRNA gene sequencing.

In contrast to the yeasts, the diversity of LAB in the starters was rather high (Table 1). *L. plantarum* and/or *L. fermentum* were the dominating microbiota in nearly all starters. The RAPD types within a species mostly differed from starter to starter, but in rye sourdough I and the commercial rye full sour the same RAPD type of *L. plantarum* was found. For some species, more than one RAPD type was isolated from a starter, e.g. *L. plantarum* of the commercial rye full sour and *L. fermentum* of the cassava starter. For preparation of the rice starter, strains of *L. paracasei*, *L. paralimentarius*, *L. perolens*, and *L. spicheri* were used, which had previously been isolated from rice sourdough (Meroth et al., 2004). Beside *L. perolens*, *L. spicheri*, which was previously shown to be competitive in rice sourdoughs (Meroth et al., 2004), failed to compete in the fermentation and thus did not belong to the dominating *Lactobacillus* biota of the rice starter. The two lactobacilli and the yeast used to prepare the sorghum starter had been isolated by Hamad et al. (1992) from a Sudanese sorghum sourdough and had been identified by physiological and morphological criteria as *L. fermentum*, *L. amylovorus* and *C. krusei*. However, based on the 16 S rRNA gene sequence analysis we re-identified the *L. fermentum* and *L. amylovorus* species as *L. pontis* and *L. helveticus* species, respectively. Remarkably, *Acetobacter* spec. was found to predominate in the bacterial biota of the commercial rye full sour and could be isolated on MRS5 agar plates. Lactobacilli were only detected by PCR-DGGE analysis or in low counts on the 10^{-1} dilution agar plates.

3.2. Microbial counts, pH, and TTA of fermentation I

At day 1, the pH, TTA and cell counts did not reach the values of the ensuing refreshment steps (data not shown). From day 2, cell counts of 10^8 to 10^9 CFU/g were obtained for LAB and of 10^5 to 10^7 CFU/g for yeasts in all fermentation batches (Table 2). In doughs prepared from oat and buckwheat flour, the yeast counts rapidly declined and after 4 days of

fermentation fell below the detection limit. The pH and TTA value ranges of the ripe sourdoughs differed with respect to the fermentation substrate (Table 2). Doughs of buckwheat and cassava showed slightly increased pH values compared to the other sourdoughs. Furthermore, highest TTA values were obtained for doughs of the pseudocereals amaranth and quinoa, whereas the cassava sourdoughs showed markedly decreased values.

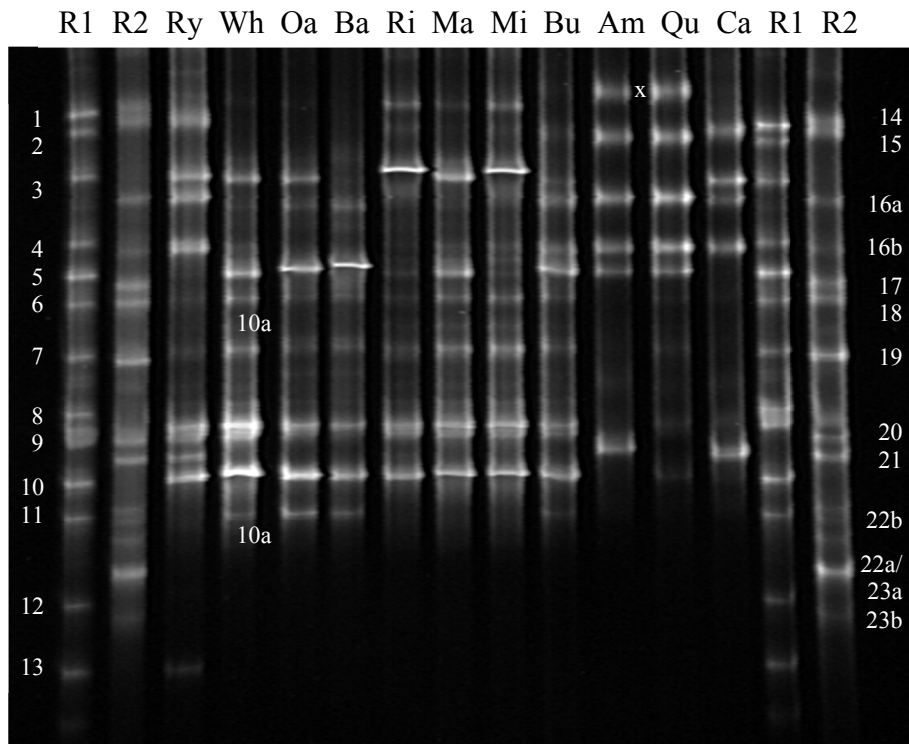


Fig. 1. DGGE profiles of PCR products obtained with primer pair L1GC-HDA2 and DNA isolated from sourdoughs at the last fermentation day.

Lane R1, reference strains: 1, *W. confusa*; 2, *L. johnsonii*; 3, *L. fermentum*; 4, *L. brevis*; 5, *L. crispatus*/*L. acidophilus*/*L. helveticus*; 6, *P. pentosaceus*; 7, *L. farciminis*; 8, *L. panis*; 9, *P. acidilactici*; 10, *L. sanfranciscensis*/*L. pontis*; 10a, unspecific bands of *L. pontis*; 11, *L. frumenti*; 12, *L. reuteri*; 13, *L. paracasei*. Lane R2, reference strains: 14, *L. amylophilus*; 15, *L. plantarum*; 16a/b, *L. paralimentarius*; 17, *L. amylovorus*; 18, *L. mindensis*; 19, *L. perolens*; 20, *L. buchneri*; 21, *L. spicheri*; 22a/b, *L. fructivorans*; 23a/b, *L. parabuchneri*, x, unknown. Lanes in between: Sourdoughs: Ry, rye; Wh, wheat; Oa, oat; Ba, barley; Ri, rice; Ma, maize; Mi, millet; Bu, buckwheat; Am, amaranth; Ca, cassava.

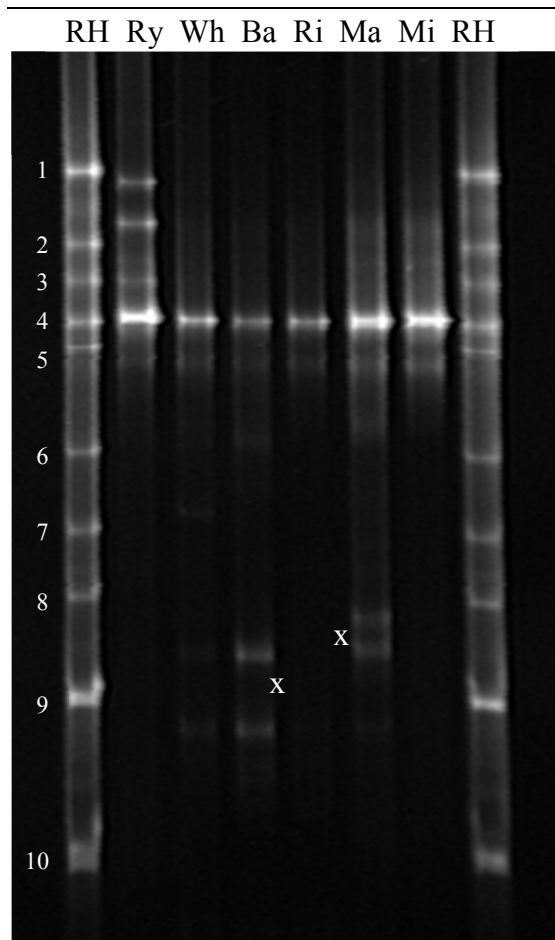


Fig. 2. DGGE profiles of PCR products obtained with primer pair U1GC-U2 and DNA isolated from sourdoughs at the last fermentation day.

Lane RH, reference strains: 1, *D. hansenii*; 2, *S. bayanus*; 3, *S. uvarum*; 4, *S. cerevisiae*; 5, *S. inusitatus*; 6, *S. servazii*; 7, *S. exiguus*; 8, *C. humilis*; 9, *Brettanomyces custersii*; 10, *I. orientalis*; x, contaminating molds. Lanes in between: Sourdoughs: Ry, rye; Wh, wheat; Ba, barley; Ri, rice; Ma, maize; Mi, millet. In the other fermentations, no yeasts were detected.

3.3. Competitive yeast and LAB species in sourdoughs of fermentation I

The composition of the yeast and LAB biota of the sourdoughs at the end of fermentation I is shown in Table 3, as detected by bacteriological culture combined with RAPD-PCR and rRNA gene sequence analyses as well as by PCR-DGGE analyses. The DGGE profiles of the LAB biota occurring in the sourdoughs are shown in Fig. 1, those of yeast biota in Fig. 2. With regard to yeasts, *S. cerevisiae* was found to dominate in most of the sourdoughs (Table 3). Only in the quinoa sourdough, *I. orientalis* was able to compete with *S. cerevisiae* and reached nearly identical counts. *I. orientalis* also occurred in the maize sourdough, however in this dough it constituted only a minor part (6%) of the yeast biota. Using PCR-DGGE analysis, only *S. cerevisiae* could be detected (Fig. 2) in the doughs, except for the

amaranth, quinoa and cassava sourdoughs. For these doughs, amplification of 28 S rRNA gene sequences from the extracted DNA failed.

In contrast to the high diversity of LAB observed in the starter mixture, only six species could be isolated from the sourdoughs at the end of fermentation I. Among these, *L. fermentum*, *L. helveticus*, *L. paralimentarius* and *L. pontis* constituted the most competitive species. *L. fermentum* occurred ubiquitously; however, only in five sourdoughs it accounted for more than 10% of the total LAB count. In contrast, *L. helveticus* and *L. pontis* did not predominate in all sourdoughs but when present in the dominating biota, they generally constituted more than 10% of the LAB species encountered. Remarkably, *L. paralimentarius* and *L. plantarum* constituted the most dominating species in the sourdoughs of the pseudocereals and cassava, respectively. The results obtained by bacteriological culture-dependent identifications were not always consistent with those of the PCR-DGGE analysis. For example, the LAB species often could not be detected by PCR-DGGE when the share among total LAB was 5% and lower. Furthermore, *L. spicheri* could mostly be detected by PCR-DGGE but not by bacteriological culture-dependent identification techniques.

3.4. Investigation of the origins of the predominant strains

By comparing the RAPD patterns of the isolates obtained from sourdoughs of fermentation I, it was possible to trace back the origin of organisms of the dominating LAB and yeast biota. *S. cerevisiae* occurred in one RAPD type only, which had been found in the baker's yeast and the starter rye sourdough I. In the quinoa sourdough, both RAPD types of *I. orientalis* originating from the cassava (type 1) and sorghum (type 2) starter were recovered. The RAPD type of *I. orientalis* in the maize sourdough was identical to that of the sorghum starter. Most *L. fermentum* isolates of the sourdoughs exhibited RAPD type 1, used to prepare the teff starter. However, in the cassava and rice fermentation another RAPD type (type 2) of *L. fermentum* occurred besides RAPD type 1, originating from the cassava starter. The isolates of *L. pontis* and *L. helveticus* exhibited one RAPD type each, which had been used to prepare the sorghum starter. The RAPD types of *L. paralimentarius* and *L. plantarum* were generally identical to the types of strains used to prepare the rice and teff starter, respectively. Here also, a second RAPD type (type 2) of *L. plantarum* was found in the buckwheat sourdough and in the cassava sourdough, originating from the commercial rye full sour and rye sourdough I, where isolates of *L. plantarum* had identical RAPD types.

3.5. Selection of competitive starter strains for fermentation of non-bread cereals and pseudocereals

The competitiveness of isolates from the non-bread cereal and pseudocereal sourdoughs was evaluated by a second fermentation, using flours of rice, maize, millet, buckwheat, amaranth and quinoa. For each sourdough batch, an individual starter mixture was composed by including only the competitive strains from fermentation I (Table 4). All isolates from fermentation I were used as starter in the rice, maize and millet sourdoughs. However, for the amaranth sourdough, *C. glabrata* was omitted due to its pathogenic potential. In the quinoa sourdough, only RAPD type 1 of *I. orientalis* was used. *L. fermentum* was omitted in the buckwheat and amaranth sourdoughs, as it occurred only in a low proportion (less than 2 among the total LAB biota in fermentation I. For *L. fermentum* in the rice sourdough, only RAPD type 1 of the teff starter was used. *L. plantarum* RAPD type 2 was used to start the buckwheat fermentation.

3.6. Investigation of the competitiveness of starter strains in fermentation II

Again, fermentation II was monitored by PCR-DGGE analysis and bacteriological culture combined with species identification and RAPD typing. Cell counts, pH and TTA values during fermentation II (data not shown) were comparable to the counts and values obtained for fermentation I. After 10 days of fermentation, the dominant LAB and yeast biota was investigated. Identical results were obtained for the PCR-DGGE analysis and bacteriological culture (data not shown). The data are summarized in Table 4. Most of the starter organisms belonged to the dominating microbiota at the end of fermentation II. However, strains of *L. pontis* and *L. helveticus* generally were not able to compete, except for the case of *L. helveticus* in the rice fermentation, and either disappeared completely from or constituted only a minor part of the dominating LAB biota. For rice, millet and quinoa sourdoughs, the composition of species was comparable to that obtained in fermentation I, except for yeasts in the quinoa sourdough. However, the proportions of LAB species in the amaranth, buckwheat and maize sourdough were quite different compared to fermentation I. In the amaranth sourdough, a *L. plantarum* RAPD type occurred exhibiting an identical RAPD pattern to the *L. plantarum* strains of the quinoa and rice sourdoughs.

Table 1. Characterization of the baker's yeast and various starters of the starter mixture used to inoculate sourdoughs of fermentation I

Baker's yeast or starter	Species detected		Cell counts (CFU/g)	
	LAB (% of total LAB)	Yeasts (% of total yeasts)	LAB	yeasts
Rye sourdough I	<i>L. pontis</i> (70) <i>L. brevis</i> (20) <i>L. plantarum</i> (10)	<i>S. cerevisiae</i> (100)	2.9×10^9	2.8×10^7
Rye sourdough II	<i>L. fermentum</i> (100)	<i>S. cerevisiae</i> ^a	1.2×10^9	$<10^3$
Commercial rye full sour	<i>L. plantarum</i> ^{b, c} (0.0001) <i>L. fermentum</i> ^{b, c} (0.0001) <i>L. paralimentarius</i> ^{b, c} (0.0001) <i>Acetobacter spec.</i> ^b (100) <i>L. sanfranciscensis</i> ^{a, d} <i>L. pontis</i> ^a <i>Lc.</i> ^c <i>lactis</i> ^a <i>L. acetotolerans</i> ^{a, d}	<i>C. humilis</i> ^a	5.0×10^7	$<10^2$
Rice starter	<i>L. paracasei</i> ^b (50) <i>L. paralimentarius</i> (50) <i>L. spicheri</i> (<1)	<i>P. anomala</i> ^b (100)	1.0×10^9	2.8×10^5
Sorghum starter	<i>L. helveticus</i> ^d (50) <i>L. pontis</i> (50)	<i>I. orientalis</i> (100)	1.6×10^9	1.9×10^7
Teff starter	<i>L. fermentum</i> (50) <i>L. plantarum</i> ^b (50)		5.0×10^8	$<10^3$
Cassava starter	<i>L. fermentum</i> (45) <i>L. plantarum</i> (45) <i>L. casei</i> group ^b (10)	<i>I. orientalis</i> ^b (70) <i>Torulaspora spec.</i> ^b (20) Unknown yeast ^b (10)	2.0×10^6	5.3×10^5
Baker's yeast	<i>L. plantarum</i> (70) <i>L. curvatus</i> group (<1) <i>L. brevis</i> ^b (10) <i>Lc.</i> ^c <i>lactis</i> ^b (10) <i>Le.</i> ^f <i>paramesenteroides</i> ^b (10) <i>L. fermentum</i> ^a	<i>S. cerevisiae</i> (100)	3.7×10^8	8.9×10^9

^a detected by PCR-DGGE only.^b detected by bacteriological culture only.^c detected on the 10^{-1} dilution agar plates as large colonies overgrowing the small and colourless colonies of *Acetobacter*.^d sequencing of DGGE bands revealed similarities of less than 97%.^e *Lactococcus*.^f *Leuconostoc*.

Table 2. Fermentation time, pH range, total titratable acids and cell counts of fermentation I

Sourdough fermentation batch	Fermentation time (days)	pH range ^a	TTA range ^a (° SH)	Cell count (CFU/g) ^a	
				LAB	yeasts
Wheat	15	3.7-3.8	22.3-28.7	1.3×10^9 - 3.0×10^9	8.4×10^6 - 1.8×10^7
Rye	15	3.7-3.8	22.4-28.4	1.1×10^9 - 4.6×10^9	7.0×10^6 - 1.4×10^7
Oat	14	3.6-3.8	23.9-28.7	1.4×10^9 - 3.0×10^9	3.2×10^4 - $<10^2$ ^b
Barley	14	3.5-3.7	20.7-25.7	9.6×10^8 - 2.0×10^9	1.1×10^7 - 1.9×10^7
Rice	13	3.6-3.7	16.7-22.2	6.2×10^8 - 1.8×10^9	7.2×10^6 - 5.1×10^7
Maize	13	3.5-3.8	17.0-23.6	8.7×10^8 - 2.2×10^9	1.9×10^5 - 9.1×10^6
Millet	13	3.6-3.8	16.8-21.4	7.3×10^8 - 1.7×10^9	1.5×10^6 - 1.7×10^7
Amaranth	13	3.7-3.8	26.4-30.4	1.8×10^9 - 4.6×10^9	5.5×10^5 - 2.8×10^6
Quinoa	12	3.8-3.9	35.3-38.6	1.3×10^9 - 2.7×10^9	1.1×10^6 - 4.6×10^6
Buckwheat	12	4.1-4.2	20.8-25.6	1.0×10^9 - 2.7×10^9	5.7×10^4 - $<10^2$ ^b
Cassava	12	4.1-4.2	10.3-11.5	2.6×10^8 - 1.4×10^9	2.0×10^6 - 1.4×10^7

^a detected at the end of fermentation in the ripe dough of each refreshment, beginning with day 2.

^b no longer detectable after 4 days of fermentation.

Table 3. Species composition of the LAB and yeast biota of the sourdoughs at the end of fermentation I

Species	Sourdough fermentation batch ^a										
	Wheat	Rye	Oat	Barley	Rice	Maize	Millet	Amaranth	Quinoa	Buckwheat	Cassava
LAB											
<i>L. fermentum</i>	D/K (30)	D/K (5)	D/K (2)	K (5)	D/K (50)	D/K (28)	D/K (60)	K (<2)	K (3,5)	K (<1)	D/K (25)
<i>L. paralimentarius</i>		D/K (10)	K (2)	K (5)		K (4)		D/K (>78)	D/K (>78)	D/K (97)	D/K (5)
<i>L. helveticus</i>	D/K (35)	K (30)	D/K (94)	D/K (40)	D/K (30)	D/K (40)	D/K (20)	D/K (20)	D/K (3,5)	D	
<i>L. plantarum</i>		D/K (10)			K (10)			D	D/K (<1)	D/K (2)	D/K (50)
<i>L. spicheri</i>		D						D			D/K (20)
<i>L. pontis</i>	D/K (35)	D/K (45)	D/K (2)	D/K (50)	D/K (10)	D/K (28)	D/K (20)		D/K (14)		
<i>L. paracasei</i>		D									
Yeasts											
<i>S. cerevisiae</i>	D/K (100)	D/K (100)		D/K (100)	D/K (100)	D/K (94)	D/K (100)	K (>94)	K (50)		K (100)
<i>I. orientalis</i>						K (6)			K (50)		
<i>C. glabrata</i>								K (<6)			

^a D, detected by PCR-DGGE; K, detected by biological culturing; Numbers in parentheses show the approximate share of the organism in % related to all yeasts/LAB.

Table 4. Starter organisms used in fermentation II and microorganisms isolated after 10 days of fermentation

Sourdough batch	Starter organisms	Isolated microorganisms (% of total LAB or yeast biota)
Rice	<i>L. fermentum</i> (RAPD type 1)	<i>L. fermentum</i> ^a (56)
	<i>L. helveticus</i>	<i>L. helveticus</i> ^a (32)
	<i>L. plantarum</i>	<i>L. plantarum</i> ^a (5)
	<i>L. pontis</i>	<i>L. pontis</i> ^a (7)
	<i>S. cerevisiae</i>	<i>S. cerevisiae</i> ^a (100)
Maize	<i>L. fermentum</i>	<i>L. fermentum</i> ^a (62)
	<i>L. paralimentarius</i>	<i>L. paralimentarius</i> ^a (36)
	<i>L. helveticus</i>	
	<i>L. pontis</i>	<i>L. pontis</i> ^a (4)
	<i>S. cerevisiae</i>	<i>S. cerevisiae</i> ^a (100)
	<i>I. orientalis</i> (RAPD type 2)	
Millet	<i>L. fermentum</i>	<i>L. fermentum</i> ^a (74)
	<i>L. helveticus</i>	<i>L. helveticus</i> ^a (15)
	<i>L. pontis</i>	<i>L. pontis</i> ^a (10)
	<i>S. cerevisiae</i>	<i>S. cerevisiae</i> ^a (100)
Amaranth	<i>L. paralimentarius</i>	<i>L. paralimentarius</i> ^a (10)
	<i>L. helveticus</i>	<i>L. helveticus</i> ^a (12)
		<i>L. plantarum</i> ^b (78)
	<i>S. cerevisiae</i>	<i>S. cerevisiae</i> ^a (100)
Quinoa	<i>L. fermentum</i>	<i>L. fermentum</i> ^a (3)
	<i>L. paralimentarius</i>	<i>L. paralimentarius</i> ^a (73)
	<i>L. helveticus</i>	
	<i>L. plantarum</i>	<i>L. plantarum</i> ^a (14)
	<i>L. pontis</i>	
	<i>S. cerevisiae</i>	<i>S. cerevisiae</i> ^a (99)
	<i>I. orientalis</i> (RAPD type 1)	<i>I. orientalis</i> ^a (1)
Buckwheat	<i>L. paralimentarius</i>	<i>L. paralimentarius</i> ^a (15)
	<i>L. plantarum</i> (RAPD type 2)	<i>L. plantarum</i> ^a (75)
	<i>L. pontis</i>	<i>L. pontis</i> ^a (10)

^a RAPD type was identical with that of the starter organism.

^b RAPD type as *L. plantarum* in the quinoa and rice batch.

4. Discussion

Our investigation of the adaptability of LAB and yeasts to sourdoughs made from flours of cereals, pseudocereals and cassava revealed that only few strains, belonging to the species *L. fermentum*, *L. helveticus*, *L. paralimentarius*, *L. plantarum*, *L. pontis*, *L. spicheri*, *I. orientalis* and *S. cerevisiae* are competitive. For the pseudocereals quinoa, amaranth and

buckwheat and for oat and barley, this is the first study on the adaptability of LAB and yeasts to such sourdough fermentations. Notably, in our study some of the strains, e.g. those belonging to the species of *L. fermentum* and *L. helveticus*, were found in nearly every substrate, whereas the competitiveness of other strains, e.g. those of *L. spicheri*, seemed to be restricted to few substrates only. Therefore, the question arises as to the ecological factors responsible for such a selection and to the properties contributing to the competitiveness of lactobacilli and yeasts in sourdoughs.

Numerous studies reported on the ecological factors affecting the microbiota of sourdoughs, but still only little is known about their effect on the competitiveness of LAB or yeasts in cereal fermentation (Hammes et al., 2005; Vogel, 1997). By considering that in our study some factors were kept constant, the competitiveness should depend on the type and quality of substrate, on the water activity, as well as on the microbial interactions of the starter and spontaneous biota during fermentation. But even with such a reduction of possible factors, it was not easy to understand our observations. For example, *L. spicheri* had previously been described as competitive LAB in rice sourdoughs (Meroth et al., 2004). However in this study, the species could not be found to dominate in rice sourdough but could be isolated from cassava sourdough and detected by PCR-DGGE in rye and amaranth sourdoughs. Thus, it seems that in addition to the type of substrate interactions among microorganisms or the substrate quality may also be of importance for the competitiveness of LAB in sourdoughs. Furthermore, we found that *L. paralimentarius* is highly competitive in pseudocereal sourdough fermentation, repressing the otherwise widely spread and most dominant species *L. fermentum*, *L. pontis* and *L. helveticus*. No published data on the microbiota of amaranth, quinoa and buckwheat sourdoughs are available to support our finding. One possible explanation for the competitiveness of *L. paralimentarius* could be the presence of substances like tannins in buckwheat and amaranth, rutin in buckwheat and saponins in quinoa and amaranth, for which antimicrobial activities have been demonstrated (Pepeljnjak et al., 2005; Scalbert, 1991; Soetan et al., 2006). A higher tolerance against such substances, maybe due to the presence of hydrolytic enzymes like tannase, as already reported for other LAB (Osawa et al., 2000), could be one aspect contributing to the competitiveness of *L. paralimentarius* in the pseudocereal sourdoughs. Furthermore, crude protein contents in pseudocereals are higher than in cereals, and amino acid contents also differ, especially lysine and threonine contents are higher in pseudocereals (Aufhammer, 2000). Further studies are needed to get a deeper insight into competitiveness of *L. paralimentarius* in pseudocereal fermentation.

In all cereal sourdoughs as well as in the amaranth sourdough, *L. helveticus* was found to be highly competitive, mostly in oat sourdough. This was surprising, as *L. helveticus* is not a typical sourdough LAB, and so far was only isolated from Sudanese sorghum sourdough (Hamad et al., 1992, 1997). The species is commonly used in starter cultures for production of long-ripened cheese (Giraffa et al., 2000) and can be isolated from various fermented milk products (Depouilly et al., 2004; Obodai and Dodd, 2006). *L. helveticus* belongs to the ‘thermophilic’ lactobacilli (Kandler and Weiss, 1986), explaining its presence in the Sudanese sorghum sourdough, fermented at 37 °C, but not its high competitiveness in our sourdoughs fermented at 30 °C. Strains of this species might be interesting candidates for the development of sourdough starters due to properties like the production of exopolysaccharides (Torino et al., 2005), which can delay the staling of bread (Korakli et al., 2003), and proteolytic activities (Oberg et al., 2002), which can provide amino acids, serving as precursors for important bread aroma compounds (Czerny et al., 2005).

Currently, more and more genome sequences of *Lactobacillus* species are being published. *L. plantarum*, being an extremely successful LAB species in plant fermentations, is known to have one of the largest genomes (about 3.3 Mb) among the lactobacilli (Claesson et al., 2006, 2007). Boekhorst et al. (2004) reported that compared to other lactobacilli *L. plantarum* harbours numerous genes coding for enzymes involved in sugar metabolism or biosynthesis of amino acids, nucleotides, fatty acids and cofactors. For example, *L. plantarum* has the potential to synthesize all amino acids except leucine, isoleucine, and valine, whereas other *Lactobacillus* species are unable to synthesize most amino acids (Claesson et al., 2006). This might be one reason why *L. plantarum* is found in environments, where amino acids and peptides are not as readily available, such as on plants or plant-derived materials. However, despite its huge genetic potential, *L. plantarum* was not a very competitive LAB in our study, in contrast to *L. helveticus*, having a much smaller genome (2.1 Mb; Callanan et al., 2008).

It was not possible to identify the *Lactobacillus* species of all isolates using 16 S rRNA gene sequence analysis. *L. paralimentarius* (Cai et al., 1999) and *L. kimchii* (Yoon et al., 2000) have very similar 16 S rRNA gene sequences, and therefore could not be discriminated from each other. de Vuyst et al. (2002) questioned the separate species status, as they found DNA-DNA binding values of 68% between the type strains. Assuming that the two species will sooner or later be unified, we decided to allocate the isolates to the older species *L. paralimentarius*. In addition, it was also not possible to distinguish *L. helveticus* and *L. gallinarum*, as the first 600 bp of the 16 S rRNA gene sequence are rather similar, and even physiological tests gave no significant results (data not shown). However, RAPD-PCR

profiles showed clear differences between the type strains of *L. helveticus* and *L. gallinarum* (data not shown). Thus, based on the similarity of RAPD-PCR profiles, all the isolates could be characterised as belonging to the species *L. helveticus*.

In this study, strains of *L. fermentum*, *L. helveticus*, *L. paralimentarius*, *L. plantarum*, *L. pontis*, and *S. cerevisiae* were most competitive in cereal, non-bread cereal, pseudocereal and cassava sourdoughs. More research will be required to better understand the factors affecting competitiveness of bacterial and yeast strains in these environments. Based on our results, we conclude that the capability to adapt to a specific substrate is highly strain specific and depends strongly on the composition of the substrate itself. Even small changes of substrate quality and process factors will have effects on the microbiota. However, some LAB and yeasts are able to adapt to many different substrates and occur more often, and in higher cell counts, than others. The reasons for this are not yet known, but an understanding of these complex interactions would offer new possibilities to control cereal food fermentations.

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Chapter IV

Impact of ecological factors on the stability of microbial associations in sourdough fermentation

Abstract

The limits for the stability of the microbial association 1 (*Lactobacillus sanfranciscensis* and *Candida humilis*) and association 2 (*Lactobacillus reuteri*, *Lactobacillus johnsonii* and *Issatchenkia orientalis*) during sourdough fermentation were evaluated by investigating the effects of the ecological factors substrate, refreshment time, temperature, amount of backslopping and competing species in different combinations on their growth. Sourdoughs were fermented in 28 batches under different conditions using the associations and possible competing strains as starters. The dominating microbiota was characterized by bacteriological culture, rRNA gene sequence analysis and RAPD-PCR. Association 1 was found to be competitive in doughs with rye and wheat flour at temperatures between 20 and 30 °C, refreshment times of 12 and 24 h, amounts of backslopping dough from 5 to 20 % and against all competing lactic acid bacteria and yeasts. The processing parameters for the competitiveness of the association 2 were temperatures of 35-40 °C, refreshment times of 12-24 h and the substrates rye bran, wheat and rye flour, but not in every case. *Issatchenkia orientalis* could only grow when enough oxygen was available. Its cell counts fell rapidly under the limit of detection when using high amounts of doughs (small ratio of surface to volume) and refreshment times of 12 h. In conclusion, the results demonstrated that the two associations were remarkably stable under most of the investigated process conditions.

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1. Introduction

Sourdough is an intermediate product and contains metabolically active microorganisms (Hammes et al., 2005; Vogel and Ehrmann, 2008; De Vuyst et al., 2009). The growth of the microorganisms is affected by numerous endogenous and exogenous ecological factors, e.g. the type, comminution grade and (enzymatic and chemical) composition of flour, the process parameters like temperature, dough yield, redox potential, refreshment time and number of propagation steps, and the interaction between the microorganisms (Hammes et al., 1996, 2005). All these factors as well as their interaction among themselves contribute to the development of a specific microbiota (Meroth et al., 2003a,b; Vogelmann et al., 2009). The investigation of the composition of the specific microbiota was subject of numerous studies, revealing a great diversity of lactic acid bacteria (LAB), mostly *Lactobacillus* (*L.*) species, and yeasts (Hammes et al., 2005; Vogel and Ehrmann, 2008; De Vuyst et al., 2009). Furthermore, the specific ecological niches in sourdoughs are often occupied by numerous various species and/or strains which may form stable associations between LAB strains or LAB and yeasts (De Vuyst et al., 2009). However, there is only limited knowledge about the ecological factors contributing to the stability and competitiveness of such associations.

Böcker et al. (1995) defined three types of sourdough (type I to III) based on the processing conditions and/or technology used for production, with a specific microbiota occurring in each type. It has been reported that the fermentation temperature is essential for the stability of a microbiota in sourdoughs (Brandt et al., 2004; Gänzle et al., 1998; Meroth et al., 2003a,b; Messens et al., 2002). For the growth of LAB, also the pH plays an important role. Gänzle et al. (1998) and Brandt et al. (2004) showed that *Lactobacillus sanfranciscensis* cannot grow below pH 3.8 or 4.0, respectively, whereas *Candida* (*C.*) *humilis* was not influenced by the pH at all (Valmorri et al., 2008). Furthermore, the ionic strength and concentration of salts, the organic acids and the buffer capacity have effects on microbial growth (Brandt et al., 2004; Gänzle et al., 1998; Passos et al., 1994; Spicher, 1961; Simonson et al., 2003; Rohrlach, 1960). The amount of backslopping dough defines the initial pH and in this way influences the growth rates of LAB (Brandt et al., 2004). Moreover, interactions between LAB and yeasts are an important aspect for the stability of the sourdough microbiota (Gobbetti, 1998; Brandt et al., 2004). Especially, interactions between *L. sanfranciscensis* and *Candida humilis* have been studied (Gobbetti et al., 1994a,b, Brandt et al., 2004, Gänzle et al., 1998). Brandt et al. (2004) were the first who studied the influence of temperature, pH, inoculum size and NaCl on growth of the association of *L. sanfranciscensis* and *C. humilis* under practical

conditions, meaning in sourdough and not in laboratory medium. However, little information is available about the influence of substrates, refreshment times and competing species on this association and the interactions of process parameters. Meroth et al. (2003a,b) found this association to be competitive together with *Lactobacillus mindensis* in a rye sourdough fermented at 25 °C with 12 h refreshment time and 10% backslopping dough. A fermentation temperature of 30 °C, 24 h refreshment time and 37.5% backslopping dough lead to a completely different microbiota. The association *Lactobacillus reuteri*, *Lactobacillus johnsonii* and *Issatchenkia (I.) orientalis* was competitive in rye bran sourdough fermented over 16 days by 10% backslopping every 48 h at 40 °C, process factors which are typical for type II sourdoughs (Meroth et al., 2003a,b). However, further information about the stability of this association is not available, as *L. johnsonii* has not often been described as typical sourdough LAB.

In this study, the limits for the stability and robustness of two microbial associations in sourdoughs, *L. sanfranciscensis*-*C. humilis* (association 1) and *L. reuteri*-*L. johnsonii*-*I. orientalis* (association 2) with regard to the effects of flour type, refreshment time, temperature, amount of backslopping and competing species were investigated under practical conditions. Fermentation batches were inoculated with these associations as starters and continuously propagated under different conditions. The dominating LAB/yeast microbiota was monitored by identifying bacterial and yeast strains using RAPD-PCR and 16S/28S rRNA sequence analysis.

2. Materials and methods

2.1. Bacteria, yeasts and culture conditions

The following strains were used as starter: *C. humilis* LTH 6102 (formerly LTH H198), *Saccharomyces cerevisiae* LTH 6679, *I. orientalis* LTH 6127, *Lactobacillus fermentum* LTH 6676, *Lactobacillus helveticus* LTH 6678, *Lactobacillus pontis* LTH 6677, *L. sanfranciscensis* LTH 2581, *L. reuteri* LTH 5531 and *L. johnsonii* LTH 5533. Yeasts and LAB were routinely cultured in YG and MRS5 medium, respectively, as described previously (Meroth et al., 2003a,b).

2.2. Sourdough fermentations, sampling and microbial characterization

In total, five trials (I to V), each consisting of different batches, were performed. The compositions of batches as well as fermentation conditions are depicted in Table 1. The

dough yield was generally 200, except for rye bran, where the yield was 400. Sterile containers (volume of 1 L) were filled with 400 g dough, except for trial II, where the amount was 200 g. Doughs were prepared from flours, inoculated with overnight cultures of the starter LAB and yeasts to obtain a final density of 10^7 cfu/g dough. Doughs were fermented under the conditions indicated in Table 1 by backslopping every 12 or 24 h until a stable microbiota was obtained. This was assessed by counting separately all different colony forms. At each refreshment step, after homogenization samples were taken from the ripe sourdoughs and the pH and total titratable acids (TTA) were determined. In addition, samples were subjected to microbial counting at each or each second refreshment step. At the end of fermentation, samples were subjected to pH and TTA determination, microbial counting and species determination by DNA isolation, RAPD-PCR and 16S/28S rRNA gene sequence analysis. Cell counts of LAB and yeasts, pH, and TTA were determined as described previously (Meroth et al., 2003a,b). Sourdough samples were diluted in saline-tryptone diluent (Meroth et al., 2003a) and plated on YGC agar (Meroth et al., 2003b), containing 0.1 g of chloramphenicol/liter, and MRS5 agar containing 0.1 g of cycloheximide/liter (Meroth et al., 2003a), both supplemented with 25 mg/l bromocresol green for easier differentiation of colonies. In trial I, 5 colonies of each colony form were picked from agar plates on which the two highest dilutions were plated. As the species determination led to identical results for all five isolates of each colony form, the method of visual picking was found to be appropriate and for further experiments only two isolates were picked per colony form. Colonies were purified, cells were grown in liquid YG (yeasts) or MRS5 (LAB) medium, harvested by centrifugation and stored in 30% glycerine at -85°C . DNA was isolated from pure cultures as described below and used for RAPD-PCR. RAPD patterns of isolates were compared with those of starter strains and supposed to be the same strain when the patterns were identical. If patterns could not be allotted to a starter, one representative of each RAPD-type was subjected to sequencing of the first 550 bp of the 16S rRNA gene (bacteria) or of D1/D2 region of the 28S rRNA gene (yeast).

2.3. DNA extraction

For DNA isolation, cells of 3 ml culture (OD_{580} of 0.6-0.8) were harvested by centrifugation and washed with 1 ml of sterile phosphate-buffered saline (containing, per liter, 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 , and 0.24 g of KH_2PO_4 [pH 8.3]). The pellet was resuspended in 130 μl of lysis buffer, containing 6.7% sucrose, 50 mM Tris HCl [pH 8.0], 1 mM EDTA, 100 μg of RNase per ml and for LAB 20 mg of lysozyme per ml. For yeasts,

lysozyme was replaced by a combination of 0.24 mg/ml of lysing enzymes from *Trichoderma harzianum* (Sigma) and 0.12 mg/ml of lyticase (Sigma). After incubation for 1.5 h at 37 °C, 20 µl of proteinase K solution (15 mg/ml) and 10 µl of sodium dodecyl sulfate (20%) were added, and the mixture was incubated for 30 min at 60 °C or until the suspension became clear. Then, 200 µl of phenol (65 °C; pH 7) was added and mixed, and the mixture was incubated for 6 min at 65 °C. After cooling, 220 µl of Tris HCl (10 mM; pH 8.0) and equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1) were added. After centrifugation of 5 min at 16.000g, the phenolic phase was removed. The watery phase was washed once more with phenol-chloroform-isoamyl alcohol and twice with chloroform. DNA was harvested by ethanol precipitation and dissolved in 100 µl of Tris HCl (10 mM; pH 8.0).

2.4. RAPD-PCR

RAPD-PCR was performed as described by Müller et al. (2001) with the following modifications. The reaction mixture (50 µl) contained 100 pmol of primer M13V (Biomers, Ulm, Germany), 0.4 mM of each deoxyribonucleotide triphosphate, 3.5 mM MgCl₂, reaction buffer (final concentrations, 10 mM Tris HCl [pH 8.3], 50 mM KCl, and 1.5 mM magnesium chloride, 0.1% Triton X-100), 1.5 U of *Taq* polymerase (Genaxxon), and 1 µl of DNA solution. The amplification program and electrophoretic separation was performed as described previously (Meroth et al., 2003a). RAPD patterns were compared and analyzed using the BioNumerics software (Applied Maths). Distance trees were calculated using pearson correlation and UPGMA.

2.5. Sequence analysis and species determination

DNA sequences of PCR fragments obtained from pure cultures were determined as described previously (Meroth et al., 2003a,b), but with a capillary sequencer. To determine the closest relatives of 16S or 28S rRNA gene sequences, a search of the Arb database (Ludwig et al., 2004) and GenBank database by using the BLAST algorithm (Altschul et al., 1990) was conducted. A similarity of >97% to 16S or 28S rRNA gene sequences of type strains was used as the criterion for identification.

Table 1

Composition of batches and fermentation conditions of the sourdough fermentations.

Trial/batch no.	Composition		Fermentation conditions			
	Flour	Starter culture ^a	Temperature (°C)	Refreshment share (%)	Refreshment time (h)	Refreshment steps ^b
Trial I						
1	Rye T1150	LS, CH	30	10	24	6
2	Rye T1150	LR, LJ, IO	37	10	24	6
3	Wheat T550	LS, CH	30	10	24	6
4	Wheat T550	LR, LJ, IO	37	10	24	6
5	Rye bran	LS, CH	30	10	24	6
6	Rye bran	LR, LJ, IO	37	10	24	6
Trial II						
7	Rye whole-meal	LR, LJ, IO	35	10	12	6
8	Rye whole-meal	LR, LJ, IO	40	10	12	6
9	Rye whole-meal	LR, LJ, IO	35	10	24	6
10	Rye whole-meal	LR, LJ, IO	40	10	24	6
Trial III						
11	Rye T1150	LS, CH	25	10	12	7
12	Rye T1150	LS, CH	25	10	24	7
13	Rye T1150	LS, CH	30	10	12	7
14	Rye T1150	LS, CH	30	10	24	7
15	Rye T1150	LS, CH	30	5	12	7
16	Rye T1150	LS, CH	30	20	12	7
Trial IV						
17	Rye T1150	LS, LF, CH, SC	30	10	24	6
18	Rye T1150	LS, LP, CH, SC	30	10	24	6
19	Rye T1150	LS, LH, CH, SC	30	10	24	6
20	Rye T1150	LS, LH, CH, SC	30	10	12	7
21	Rye T1150	LS, LF, LP, LH, CH, SC	30	10	24	6
22	Rye T1150	LS, LF, LP, LH, CH, SC	30	10	12	7
Trial V						
23	Rye T1150	LS, LF, CH, SC	20	10	24	6
24	Rye T1150	LS, LF, CH, SC	20	10	12	7
25	Rye T1150	LS, LR, LJ, IO, CH	30	10	24	6
26	Rye T1150	LS, LR, LJ, IO, CH	30	10	12	7
27	Rye T1150	LS, LR, LJ, IO, CH	35	10	24	6
28	Rye T1150	LS, LR, LJ, IO, CH	35	10	12	7

^a CH, *C. humilis* LTH 6102; SC, *S. cerevisiae* LTH 6679; IO, *I. orientalis* LTH 6127; LF, *L. fermentum* LTH 6676; LH, *L. helveticus* LTH 6678; LP, *L. pontis* LTH 6677; LS, *L. sanfranciscensis* LTH 2581; LR, *L. reuteri* LTH 5531; LJ, *L. johnsonii* LTH 5533.

^b No. of steps required to obtain a stable microbiota.

Table 2

Ranges of pH, TTA and cell counts of LAB and yeast strains of the different sourdough fermentation batches.

Batch	pH ^a	TTA ^a (° SH)	Cell counts ^a (CFU/g) of ^b					
Trial I			LS	LR	LJ	CH	IO	
1	3.8-3.7	18.4-19.4	1.4x10 ⁹ -5.5x10 ⁸			2.0x10 ⁷ -6.5x10 ⁶		
2	3.5-3.4	22.4-23.3		5.0x10 ⁹ -3.6x10 ⁸	3.7x10 ⁹ -1.5x10 ⁹		1.6x10 ⁷ -3.7x10 ⁶	
3	3.8-3.7	12.4-12.8	1.5x10 ⁹ -7.5x10 ⁸			4.3x10 ⁷ -1.4x10 ⁷		
4	3.4	16.5-16.9		2.8x10 ⁹ -3.5x10 ⁸	1.4x10 ⁹ -8.5x10 ⁸		7.9x10 ⁶ -5.7x10 ⁵	
5	4.3-3.9	15.8-20.6	2.8x10 ⁹ -4.4x10 ⁸			1.1x10 ⁸ -1.9x10 ⁷		
6	3.8	20.1-21.3		3.0x10 ⁹ -1.5x10 ⁸	5.2x10 ⁹ -2.6x10 ⁹		2.1x10 ⁸ -1.2x10 ⁸	
Trial II				LR	LJ		IO	
7	3.8-3.7	21.1-23.6		8.0x10 ⁸ -1.6x10 ⁸	8.6x10 ⁸ -4.9x10 ⁸		1.2x10 ⁷ -1.4x10 ⁶	
8	3.7	23.7-24.7		9.6x10 ⁸ -4.2x10 ⁸	1.9x10 ⁹ -1.2x10 ⁹		1.2x10 ⁷ -5.0x10 ⁵	
9	3.7-3.6	24.9-27.0		1.2x10 ⁹ -6.7x10 ⁸	1.1x10 ⁹ -6.8x10 ⁸		3.9x10 ⁷ -6.1x10 ⁶	
10	3.7	25.4-26.6		1.6x10 ⁹ -6.9x10 ⁸	1.8x10 ⁹ -6.6x10 ⁸		7.2x10 ⁷ -1.0x10 ⁶	
Trial III			LS			CH		
11	3.9	14.3-14.8	2.8x10 ⁹ -1.8x10 ⁹			9.8x10 ⁷ -5.0x10 ⁷		
12	3.9	16.9-17.3	2.8x10 ⁹ -1.2x10 ⁹			1.1x10 ⁸ -5.0x10 ⁷		
13	3.9	15.6-16.1	1.8x10 ⁹ -1.2x10 ⁹			7.4x10 ⁷ -3.5x10 ⁷		
14	3.8	17.8-18.8	2.5x10 ⁹ -9.5x10 ⁸			7.2x10 ⁷ -2.7x10 ⁷		
15	3.9-3.8	15.1-15.7	3.0x10 ⁹ -1.8x10 ⁹			6.6x10 ⁷ -4.2x10 ⁷		
16	3.9	15.5-16.7	1.7x10 ⁹ -1.1x10 ⁹			7.8x10 ⁷ -3.9x10 ⁷		
Trial IV			LS	LF	LP	LH	CH	SC
17	3.7	19.8-20.0	2.0x10 ⁹ -1.1x10 ⁹	2.9x10 ⁹ -1.1x10 ⁹			1.5x10 ⁷ -9.3x10 ⁶	1.9x10 ⁷ -6.4x10 ⁶
18	3.7-3.6	18.7-19.2	3.9x10 ⁹ -1.3x10 ⁹		1.6x10 ⁸ -6.0x10 ⁶		1.1x10 ⁷ -2.1x10 ⁷	3.4x10 ⁷ -1.3x10 ⁷
19	3.6	18.8-19.4	3.3x10 ⁹ -8.7x10 ⁸			3.0x10 ⁶ -<10 ⁶	1.6x10 ⁷ -8.8x10 ⁶	2.7x10 ⁷ -1.2x10 ⁷
20	3.7	16.5-17.0	3.9x10 ⁹ -1.3x10 ⁹			7.0x10 ⁶ -<10 ⁶	4.4x10 ⁷ -3.5x10 ⁷	2.2x10 ⁷ -8.0x10 ⁵
21	3.7	19.5-20.1	2.3x10 ⁹ -1.1x10 ⁹	2.9x10 ⁹ -1.6x10 ⁹	1.4x10 ⁸ -1.0x10 ⁷	<10 ⁷	1.7x10 ⁷ -4.8x10 ⁶	1.4x10 ⁷ -7.8x10 ⁶
22	3.8-3.7	15.4-16.2	1.8x10 ⁹ -1.4x10 ⁹	2.6x10 ⁹ -1.3x10 ⁹	6.0x10 ⁷ -<10 ⁷	<10 ⁷	3.7x10 ⁷ -2.7x10 ⁷	1.5x10 ⁷ -6.0x10 ⁵
Trial V			LS	LF	LR	LJ	CH	SC or IO ^c
23	3.8-3.7	15.3-15.8	4.0x10 ⁹ -1.9x10 ⁹	1.2x10 ⁸ -<10 ⁶			8.3x10 ⁷ -6.0x10 ⁷	9.3x10 ⁶ -5.0x10 ⁵
24	4.4-3.9	8.8-11.8	4.5x10 ⁹ -2.5x10 ⁹	8.0x10 ⁷ -<10 ⁶			6.1 x10 ⁷ -3.7x10 ⁷	5.9x10 ⁶ -1x10 ⁵
25	3.7-3.6	18.2-19.2	3.2x10 ⁹ -1.4x10 ⁹		3.5x10 ⁸ -<10 ⁷	1.0x10 ⁸ -<10 ⁷	3.1x10 ⁷ -1.7x10 ⁷	2.5x10 ⁷ -5.7x10 ⁵
26	3.8-3.6	16.9-17.1	3.8x10 ⁹ -2.6x10 ⁹		2.6x10 ⁸ -3.0x10 ⁷	2.5x10 ⁷ -<10 ⁷	4.8x10 ⁷ -3.0x10 ⁷	3.5x10 ⁵ -<10 ²
27	3.5-3.4	21.8-22.8	<10 ⁶ -<10 ⁵		3.5x10 ⁹ -1.7x10 ⁹	8.0x10 ⁸ -2.3x10 ⁹	<10 ⁴	3.5x10 ⁷ -7.3x10 ⁶
28	3.6-3.5	18.7-19.3	1.6x10 ⁸ -<10 ²		4.0x10 ⁹ -7.6x10 ⁸	1.7x10 ⁹ -1.5x10 ⁸	2.5x10 ⁵ -<10 ²	3.8x10 ⁶ -2.0x10 ⁵

^a detected in the ripe dough at the end of the fermentation step, beginning with the second refreshment step.

^b CH, *C. humilis* LTH 6102; SC, *S. cerevisiae* LTH 6679; IO, *I. orientalis* LTH 6127; LF, *L. fermentum* LTH 6676; LH, *L. helveticus* LTH 6678; LP, *L. pontis* LTH 6677; LS, *L. sanfranciscensis* LTH 2581; LR, *L. reuteri* LTH 5531; LJ, *L. johnsonii* LTH 5533.

^c batch 23 and 24: SC; batches 25 to 28: IO.

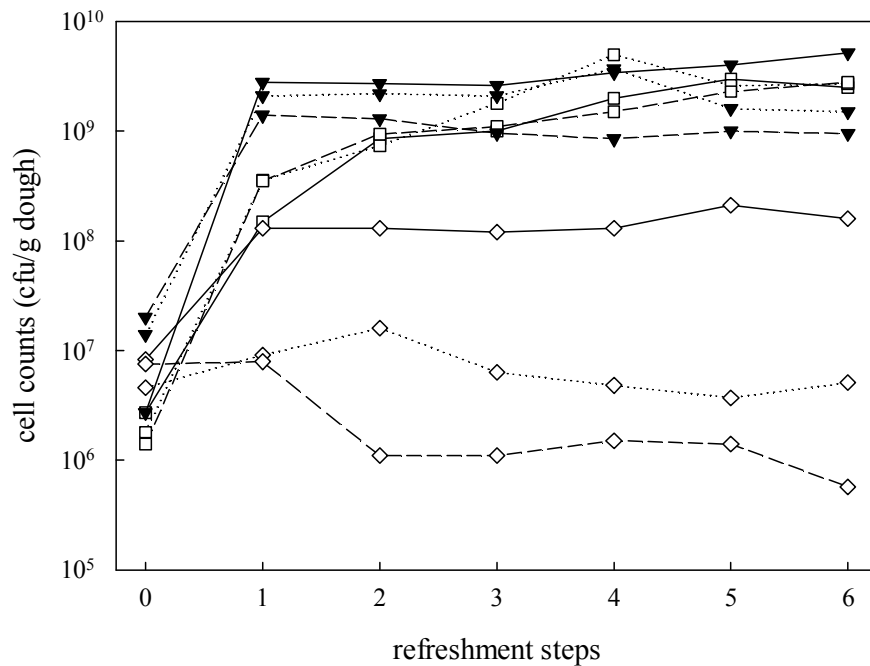


Fig. 1. Course of cell counts of LAB and yeast strains in batches 2, 4 and 6 of trial I started by inoculation of association 2 containing (\square) *L. reuteri*, (\blacktriangledown) *L. johnsonii* and (\diamond) *I. orientalis*. Continuous lines: rye bran; dotted lines: rye flour; dashed lines: wheat flour.

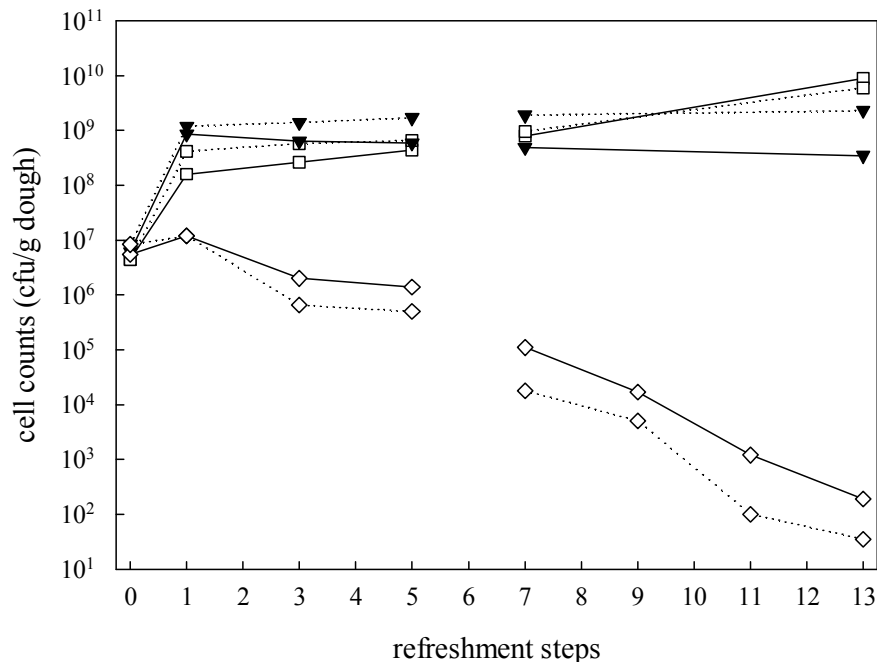


Fig. 2. Course of cell counts of LAB and yeast strains in batches 7 and 8 of trial II started by inoculation of association 2 containing (\square) *L. reuteri*, (\blacktriangledown) *L. johnsonii* and (\diamond) *I. orientalis*. After refreshment step 6, fermentations were continued with an increased amount (400 g) of sourdough until step 13. Continuous lines: batch 7; dotted lines: batch 8.

3. Results

To investigate the effect of substrate, temperature, fermentation time, percentage of backslopping and competing organisms on the stability of LAB and yeast associations that have previously been described to be competitive in rye sourdoughs (Meroth et al., 2003a,b; Böcker, 1993), five sourdough fermentation trials, each consisting of different batches (in total 28 batches) were carried out. The batches differed with regard to the flour type, starter culture combination and fermentation conditions (Table 1). The performance of different LAB and yeasts within the associations *L. sanfranciscensis*-*C. humilis* (association 1) and *L. reuteri*-*L. johnsonii*-*I. orientalis* (association 2) was characterized by monitoring the predominant LAB/yeast biota at the strain level. The ranges of pH and TTA values as well as the cell counts of LAB and yeast strains of all fermentation batches are compiled in Table 2.

In trial I, the effect of the substrate on the stability of the two microbial associations were examined by fermenting sourdoughs made with rye flour type 1150, wheat flour type 550 and rye bran (Table 1). Batches inoculated with *L. sanfranciscensis* and *C. humilis* were fermented at their mutual temperature optimum of 30 °C (Brandt et al., 2004), the others at 37 °C. Depending on the substrate and association, the pH decreased and TTA increased to final values given in Table 2 within the first refreshment step, except for batch 5 which required 4 steps (data not shown). In general, the sourdoughs made of wheat flour showed the lowest TTA values and cell counts. The cell counts (around 10^9 cfu/g) of *L. reuteri* and *L. johnsonii* in batches 2, 4 and 6 remained stable during fermentation (Fig. 1, Table 2). *I. orientalis* reached cell counts of $>10^8$ cfu/g in rye bran, but slightly declined over time in rye and wheat sourdoughs to values of about 10^6 cfu/g. This yeast always grew visibly on the dough surface, forming a more or less hard, white surface. Independent of the substrate used, association 1 was stable in all batches (1, 3 and 5), reaching cell counts of about 10^9 cfu/g for *L. sanfranciscensis* and 10^7 cfu/g for *C. humilis* (Table 2).

In trial II, the effect of temperature (35 or 40 °C) and refreshment times (12 or 24 h) on the stability of association 2 in rye whole meal sourdoughs was investigated. TTA values at 40 °C were slightly higher than at 35 °C. In all batches, cell counts of *L. reuteri* and *L. johnsonii* accounted for around 10^9 cfu/g and did not show marked differences between the batches with different temperatures or refreshment times (Fig. 2, Table 2). However, *I. orientalis* preferred lower temperatures, as cell counts at 40 °C were 0.5-1 orders of magnitude lower than at 35 °C (batch 8 and 10) and 37 °C (trial I, batch 2). As *I. orientalis* tended to grow on the

surface of sourdoughs, its growth behaviour with less access to oxygen was investigated by decreasing the ratio of surface to volume of the sourdough. For this purpose, fermentations in batches 7 and 8 were continued after refreshment step 6 with an increased amount (400 g) of sourdough until step 13 (Fig. 2). The cell counts of LAB strains remained stable at around 10^9 cfu/g, but the counts of *I. orientalis* declined rapidly. At refreshment step 13, the counts were about 1.9×10^2 for batch 7 and 3.5×10^1 for batch 8.

Trial III was conducted in order to examine the effect of temperature, refreshment time and amount of backslopping material (refreshment share) on the stability of association 1. Although the pH values remained between 3.8 and 3.9, the TTA values increased with increasing temperature, refreshment share and refreshment time (Table 2). For the cell counts, there were only small differences between the batches with the different process parameters. At a fermentation temperature of 25 °C (batches 11 and 12), the cell counts of *C. humilis* showed slightly increased values, the highest at the refreshment time of 24 h (batch 12). The cell counts of *L. sanfranciscensis* were similar in all batches. The amount of backslopping dough did not show any effect.

In trial IV, the association 1 was co-fermented with several other LAB and yeasts which were found previously to be competitive in wheat, rye and other sourdoughs (Vogelmann et al., 2009), in order to examine whether the association is still stable in presence of other adapted species. Process parameters varied in refreshment times only (Table 1). Competing strains belonged to the species *L. fermentum*, *L. pontis*, *L. helveticus* and *S. cerevisiae*, added singly or together to association 1. The sourdough with *L. fermentum* (batch 17) and the sourdough with all competing species (batch 21) had higher TTA values than the other sourdoughs in trial IV and the corresponding batch 14 in trial III. Batches with 12 h refreshment time (20 and 22) had much lower TTA values than those with 24 h (Table 2). *L. fermentum* and *S. cerevisiae* were found to be highly competitive. The cell counts were equal to those of *L. sanfranciscensis* and *C. humilis*, except for batch 22 with a refreshment time of 12 h, where *S. cerevisiae* slowly declined to cell counts of 6.0×10^5 cfu/g (Fig. 3). *L. pontis* also declined in all three batches, it even fell under the limit of detection in batch 22 after step 3. *L. helveticus* fell under the detection limit after step 2 in batch 19, after step 3 in batch 20, after step 1 in batch 21, but fastest in batch 22 (Fig. 3).

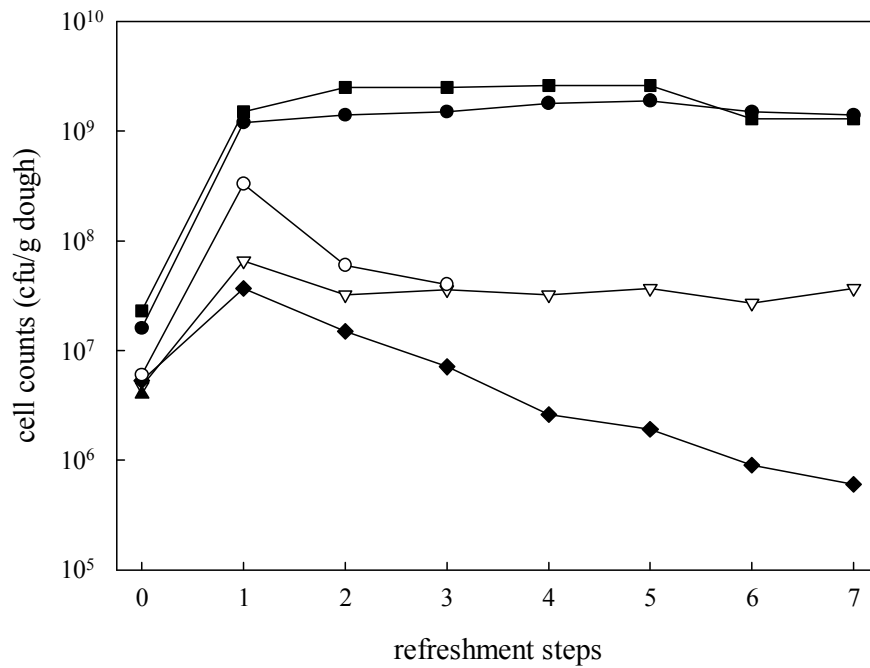


Fig. 3. Course of cell counts of LAB and yeast strains in batch 22 of trial IV started by inoculation of strains of *L. sanfranciscensis* (●), *L. fermentum* (■), *L. pontis* (○), *L. helveticus* (▲), *C. humilis* (▽), and *S. cerevisiae* (◆).

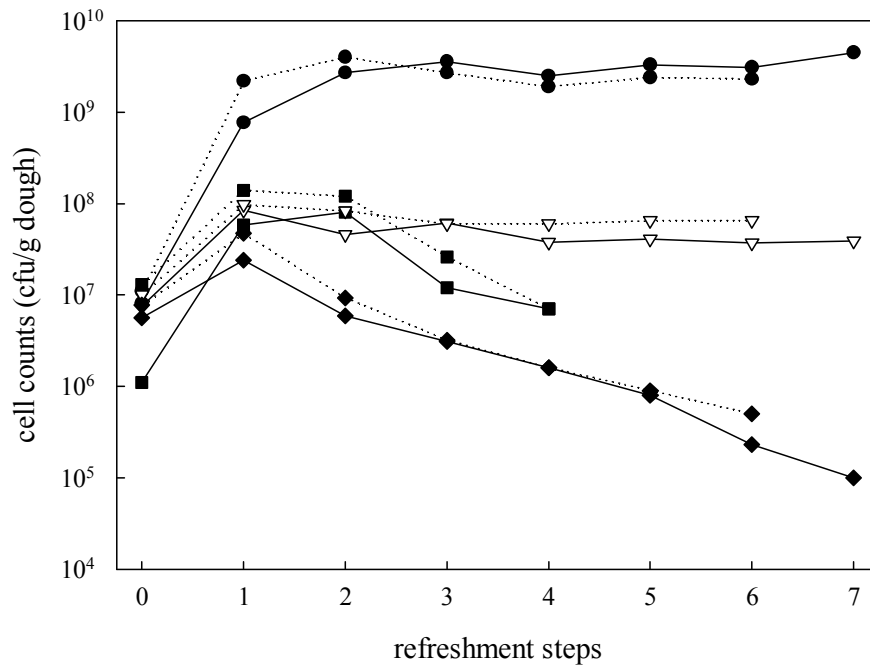


Fig. 4. Course of cell counts of LAB and yeast strains in batches 23 (dotted lines) and 24 (continuous lines) of trial V started by inoculation with strains of *L. sanfranciscensis* (●), *L. fermentum* (■), *C. humilis* (▽) and *S. cerevisiae* (◆).

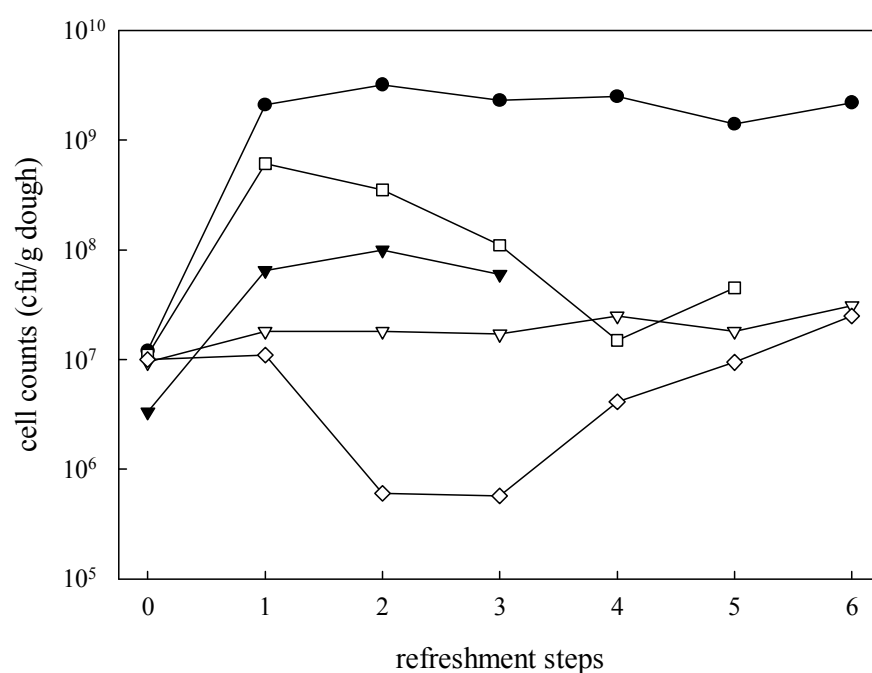


Fig. 5. Course of cell counts of LAB and yeast strains in batch 25 of trial V started by inoculation of strains of *L. sanfranciscensis* (●), *L. reuteri* (□), *L. johnsonii* (▼), *C. humilis* (▽) and *I. orientalis* (◇).

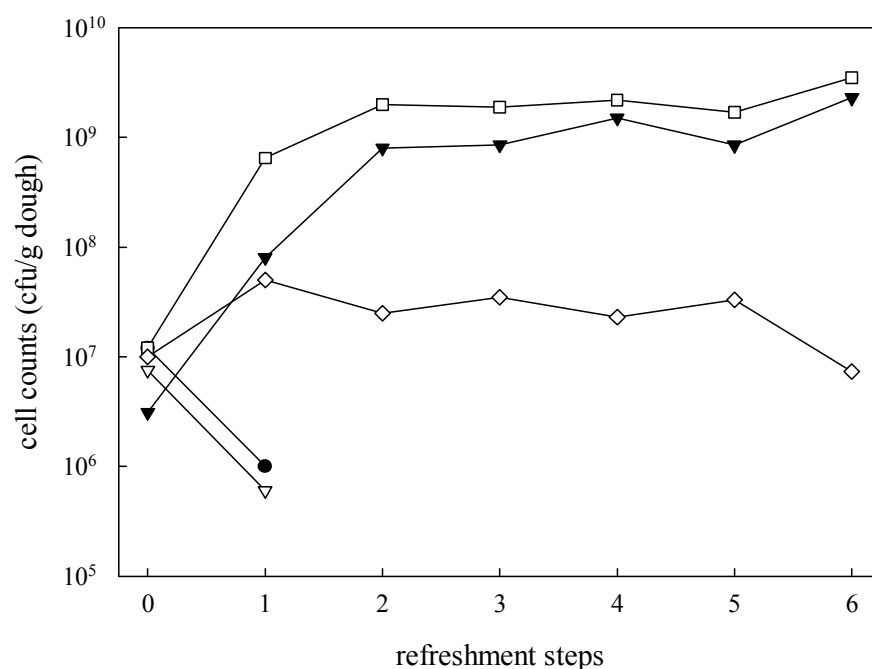


Fig. 6. Course of cell counts of LAB and yeast strains in batch 27 of trial V started by inoculation of strains of *L. sanfranciscensis* (●), *L. reuteri* (□), *L. johnsonii* (▼), *C. humilis* (▽) and *I. orientalis* (◇).

In trial V, the stability of association 1 at lower temperature (20 °C) in the presence of competing strains of *L. fermentum* and *S. cerevisiae* was investigated (batches 23 and 24). *L. sanfranciscensis* and *C. humilis* were found to be highly competitive, as the cell counts of the competing strains declined rapidly (Table 2, Fig. 4). *L. fermentum* was detectable until the 4th refreshment step, cell counts of *S. cerevisiae* were finally at a level of 10⁵ cfu/g. The pH values of batch 23 were comparable to the values obtained in batches 17 and 21, but the TTA values laid far below, for batch 24 even lower (Table 2). Moreover, the two associations were fermented together at 30 °C and 35 °C with a refreshment period of 12 and 24 h, to examine their growth behaviour in combination. In batch 25, fermented at 30°C with 24 h refreshment period, *L. reuteri* and *L. johnsonii* fell under the limit of detection after step 5 and 3, respectively, but *I. orientalis* could compete with *C. humilis* after an adaptation period of three steps (Fig. 5). With 12 h refreshment time (batch 26), cell counts were similar, but *I. orientalis* fell under the limit of detection after step 3, counts of *L. johnsonii* after step 4, and counts of *L. reuteri* were just above the detection limit of 10⁷ cfu/g dough. At 35 °C, the association 2 dominated the microbiota at least after step 2, but *I. orientalis* declined slowly in batch 28. *L. sanfranciscensis* and *C. humilis* rapidly fell under the limit of detection, in batch 28 after step 5 and 2, respectively, in batch 27 after step 1 (Fig. 6).

4. Discussion

In this study, the limits for ecological factors on the stability of two associations of LAB and yeasts in sourdoughs were monitored by applying various combinations of the factors substrate, refreshment time, temperature, amount of backslopping and competing species under practical conditions. The results clearly demonstrated that the two associations were remarkably stable under most of the investigated process conditions. For the association *L. sanfranciscensis*-*C. humilis*, this stability is consistent with the fact that the strains used in this study are part of the microbiota of a commercial mother sponge which has been propagated for more than 60 years (Böcker et al., 1990). Furthermore, Meroth et al. (2003a,b), Gänzle et al. (1998), Brandt and Hammes (2001), Brandt et al. (2004) and others also showed that the association *L. sanfranciscensis*-*C. humilis* is very stable and shows a symbiotic-like character.

For growth of *L. sanfranciscensis* LTH 2581, Gänzle et al. (1998) and Brandt et al. (2004) reported a temperature optimum of 32 °C; for *C. humilis* LTH H198 of 27 °C (Gänzle et al., 1998) and 28 °C (Brandt et al., 2004), respectively. At 35 °C, *C. humilis* cannot grow (Brandt

and Hammes (2001), Brandt et al., 2004; Gänzle et al., 1998; Yarrow, 1978). This is in agreement with our finding that growth of the association *L. sanfranciscensis*-*C. humilis* was optimal at 25 °C and 30 °C and was stable between 20 °C and 30 °C. On the other hand, both strains were found to be uncompetitive at 35 °C (batches 27 and 28). Although Brandt and Hammes (2001) and Brandt et al. (2004) reported a lower growth rate for *L. sanfranciscensis* LTH 2581 at 35 °C, maximum cell counts were not affected by higher temperatures in single culture with *C. humilis*. In contrast, our results showed that *L. sanfranciscensis* is not competitive at 35 °C when other LAB species are present. Although cell counts of *L. sanfranciscensis* and pH values in batches 1, 3, and 5 were comparable, TTA values in the dough with rye bran (batch 5) were nearly twice as high as in the wheat dough. This is associated with the high buffering capacity of rye bran, which among others is depending on the high content of phytic acid (Rohrlich, 1960). As *L. sanfranciscensis* does not multiply at pH values below 3.8 (Brandt et al., 2004; Gänzle et al., 1998), pH values of our fermentations were found to be at this level. In contrast, growth of *C. humilis* is not affected by external pH (Brandt et al., 2004; Gänzle et al., 1998; Valmorri et al., 2008). In trial III, TTA values were higher in the batches with 24 h refreshment time, which correlates with the observation of Brandt (2001), Brandt et al. (2004), and Böcker (1993) that growth of *L. sanfranciscensis* stops after about 12 h of fermentation, when the pH value of 3.8 is obtained, but production of acids still continues.

The association *L. sanfranciscensis*-*C. humilis* could prevail against all inserted competing species, except in batches 27 and 28, in which the fermentation temperature was above their optimal growth temperature. Among the competing LAB species, *L. fermentum* could only compete at 30 °C but not at 20 °C. This is in agreement with the finding that *L. fermentum* is commonly found in type II sourdoughs (Vogel et al., 1999) and thus grows preferably at higher temperatures. Doughs with *L. fermentum* generally exhibited higher TTA values, thus this species seems to be responsible for the increased acid production, compared to *L. sanfranciscensis*. Interestingly, *S. cerevisiae* could compete with *C. humilis* in all batches, but had a clear disadvantage in the batches at 20 °C (batch 23 and 24) and/or with refreshment time of 12 h (batch 20, 22 and 24). This is congruent with the finding that when fermenting *C. humilis* LTH H198 together with *S. cerevisiae* LTH H238 (which was once isolated from a dry baker's yeast) and *L. sanfranciscensis* LTH 2581 in a rye sourdough fermentation at 25 °C with 12 h refreshment time, *S. cerevisiae* disappears within 48 h (Julia Rosenheim, unpublished results). With regard to the latter, this may be explained by differences in the

growth rate (unpublished data). In an analogue experiment fermenting each yeast with *L. sanfranciscensis* LTH 2581 at 30 °C, *C. humilis* showed a markedly higher growth rate with $\mu_{\max} = 0.3$ than *S. cerevisiae* with $\mu_{\max} = 0.2$ (Julia Rosenheim, unpublished results). One reason for this may be the smaller cell size of *C. humilis* (4-5 μm) compared to *S. cerevisiae* (8 μm). Thus, it is tempting to speculate that *C. humilis* is favoured in sourdoughs with rather short refreshment times due to the small cell size enabling the high growth rates. This assumption is supported by the findings of Nout and Creemers-Molenaar (1987), coming to the same supposition. However, further studies are needed to confirm the assumption that the cell size markedly affects the growth rate.

The association *L. reuteri*-*L. johnsonii*-*I. orientalis* was previously described to be highly competitive in rye bran sourdough fermentation, conducted for 16 days at 40 °C with refreshment time of 48 h (Meroth et al., 2003a,b). In our study, this association was found to be stable in a wide range of fermentations, performed at temperatures from 35 to 40 °C, with all substrates, with refreshment times of 12 and 24 h and with competing microorganisms. In the fermentations, the cell counts for LAB and yeasts were comparable to those reported by Meroth et al. (2003a,b). In addition, both LAB species have previously been described to be present in a long-term type II sourdough (Böcker et al., 1995). Furthermore, *L. reuteri* has often been characterized as common in type II sourdoughs (Vogel et al., 1999; Hammes and Gänzle, 1998; De Vuyst et al., 2009). Interestingly, Gänzle and Vogel (2003) reported that some strains of *L. reuteri* produce reutericyclin, which suppresses the growth of *L. sanfranciscensis* LTH 2581. This may not be the case with our *L. reuteri* strain LTH 5531, as cell counts of *L. sanfranciscensis* were not decreased in presence of *L. reuteri* in batches 25 and 26. Moreover, for *L. reuteri* LTH 5531, Dal Bello et al. (2005) investigated the specific gene expression during type II sourdough fermentation using *in vivo* expression technology. The authors observed induction of genes involved in stress response, especially genes of the arginine deiminase pathway. This pathway is discussed to be involved in the response of lactobacilli to acid stress (De Angelis and Gobbetti, 2004) and thus may contribute to the competitiveness of strain LTH 5531 in sourdough fermentation.

The yeast *I. orientalis* was found to be highly competitive in most of the batches. It grew visibly on the dough surface, where best access to oxygen is given. In addition, the cell counts of *I. orientalis* were highest in the rye bran doughs and lowest in wheat doughs. This may be due to more aerobic conditions in the rye bran dough, as this dough was fluffier and less

dense. This is consistent with the finding that a decrease of the ratio of surface to volume of the sourdough results in a rapid decline of the *I. orientalis* counts (batch 7 and 8, Fig. 2), even without any other competing yeasts. Furthermore, cell counts of this yeast also decreased in batch 28 (12 h refreshment time, 35 °C) as well as in batch 26 (12 h refreshment time, 30 °C), where it decreased under the level of detection. This confirms what has already been indicated in trial I: *I. orientalis* is competitive between 30 and 40 °C in presence of oxygen and in sourdoughs with long refreshment times, but not under anaerobic conditions in long-term sourdoughs with refreshment times less than 24 h. The competitiveness of *I. orientalis* in cereal fermentations has frequently been reported, especially for maize and sorghum sourdoughs in tropical countries (Hamad et al., 1992; Hayford and Jakobsen, 1999; Jespersen et al., 1994). These cereals do not contain gluten, for which reason the doughs made thereof are also less dense; fermentation temperatures are above 30 °C, and propagation times are mostly above 24 h. All these facts are congruent with our findings, which in this way have not been reported before.

Our studies revealed various ecological factors and ranges of these factors for the stability and competitiveness of the two microbial associations *L. sanfranciscensis*-*C. humilis* and *L. reuteri*-*L. johnsonii*-*I. orientalis* in sourdough fermentation. They contribute to a better understanding of the interplay of LAB and yeasts in sourdough fermentations and thus permit to facilitate the control of fermentation in order to keep quality of the produced sourdoughs. Nevertheless, the competitiveness of LAB and yeasts in sourdough is strain and not species specific, as recently shown for *L. sanfranciscensis* (Siragusa et al., 2009) and *Lactobacillus plantarum* strains (Minervini et al., 2010) in wheat sourdough. Further research is required to elucidate which parameters are responsible for the competitiveness of a LAB or yeast strain and/or species under the numerous ecological conditions prevailing in the various sourdough fermentations.

Acknowledgements

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Chapter V

***Cryptococcus thermophilus* sp. nov., isolated from cassava sourdough**

Summary

A novel anamorphic yeast, strain LTH 6662^T, was isolated from cassava sourdough. The isolate supposedly originated from cassava flour or was a contaminant thereof. Sequencing of the D1/D2 domain of the 26S rRNA gene indicated that strain LTH 6662^T represents a novel species. Its closest relatives were members of the *Cryptococcus humicola* complex. The novel strain had several physiological characteristics that differed from those of related species: the ability to assimilate raffinose and cadaverine, the inability to assimilate soluble starch, xylitol, galactitol, butane-2,3-diol, sodium nitrite and lysine, the ability to grow without vitamins and at 42 °C, and the inability to produce starch-like substances. Its major ubiquinone was Q-10. In addition, buds were formed on small neck-like structures. In liquid medium, green or blue fluorescent substances were produced. The name *Cryptococcus thermophilus* sp. nov. is proposed, with LTH 6662^T (= CBS 10687^T = DSM 19443^T) as type strain.

The GenBank/EMBL/DDBJ accession number for the 26S rRNA gene D1/D2 domain sequence of *Cryptococcus thermophilus* LTH 6662^T is AM746982.

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Introduction

The genus *Cryptococcus* (C.) Vuillemin includes a great variety of asexually reproducing species which are heterogeneous in their morphological and physiological characteristics. Despite common properties such as the absence of fermentative ability, the assimilation of D-glucuronate, synthesis of starch and utilization of *myo*-inositol, the presence of xylose in cell hydrolysates, as well as positive urease and diazonium blue B reactions, *Cryptococcus* species differ in cell shape, colony form and colour, formation of hyphae or pseudohyphae, major ubiquinone type, nutritional capabilities and habitat (Fonseca *et al.*, 2011). They occur ubiquitously, especially on plant material and soil, and have also been reported as human pathogens (Ahearn, 1998; Barnett *et al.*, 2000).

The taxonomy of the basidiomycetous yeasts is still in discussion. The current status is presented by Boekhout *et al.* (2011) in the most recent edition of 'The Yeasts, a Taxonomic Study'. In the subphylum Agaricomycotina, the class Tremellomycetes consists of the four orders: Cystofilobasidiales, Tremellales, Trichosporonales, and Filobasidiales. Hibbett *et al.* (2007) proposed a different classification system with the Trichosporonales being part of the Tremellales. This point still needs to be clarified (Boekhout *et al.*, 2011). The genus *Cryptococcus* is polyphyletic; thus, species are represented throughout all four (or three) orders. The following *Cryptococcus* species belong to the Trichosporonales clade: *C. humicola*, *C. musci*, *C. longus*, *C. pseudolongus*, *C. ramirezgomezianus*, *C. fragicola*, *C. curvatus*, *C. haglerorum*, *C. arboriformis*, and *C. daszewskae* (Boekhout *et al.*, 2011). Recently, Prillinger *et al.* (2007) proposed the new genus *Asterotremella*, comprising the species of the *Cryptococcus humicola* complex (Takashima *et al.*, 2001), which differ from other closely related species of the genus *Cryptococcus* by having Q-9 as major ubiquinone. However, there is an earlier name, *Vanrija* (Okoli *et al.*, 2007), which has priority over *Asterotremella*. As the nomenclature of these yeasts is still not clear, original nomenclature proposed by Takashima *et al.* (2001), as also used by Boekhout *et al.* (2011), Fonseca *et al.* (2011) and Sugita (2011) was used.

Methods

The microbiota of a cassava sourdough, which was made from cassava flour and water inoculated with lactic acid bacteria and fermented for 24 h at 30 °C, was investigated previously using bacteriological culture techniques (Vogelmann *et al.*, 2009). The ripe sourdough was serially diluted in saline-tryptone-diluent and aliquots of the dilutions were plated on YGC agar which contained (l⁻¹) 5 g yeast extract, 20 g glucose, 0.1 g

chloramphenicol and 25 mg bromocresol green. Colonies with different morphologies were picked and species were determined by sequence analysis of the D1/D2 region of the 26S rRNA gene. Identification of the yeast microbiota revealed the presence of two yeasts belonging to the *Torulaspora delbrueckii/globosa/pretoriensis* group and to the species *Issatchenkia orientalis*, as well as an unknown basidiomycetous yeast (strain LTH 6662^T). As no yeasts were inoculated into the starter preparation, the yeasts either originated from the cassava flour or were contaminants.

DNA was isolated using a GenElute™ Bacterial Genomic DNA Kit (Sigma) according to the supplier's instructions with the following modification. A combination of lysing enzymes from *Trichoderma harzianum* (0.24 mg ml⁻¹) and lyticase (0.12 mg ml⁻¹) were used as lysing enzyme. Partial amplification of the 26S rRNA gene (799 bp of the D1/D2 region) was carried out using the primers P1 and P2 described by Sandhu *et al.* (1995). PCR was conducted as described previously (Meroth *et al.*, 2003). Sequencing was performed using a capillary sequencer (Beckman Coulter). The partial 26S rRNA gene sequences were compared with corresponding sequences present in GenBank. Phylogenetic trees were computed with PAUP version 4.0 using maximum-parsimony analysis (heuristic search, stepwise addition, random addition sequence, nearest neighbour interchange, 100 maximum trees). Bootstrap analysis was based on 100 replicates.

The yeast strain LTH 6662^T was characterized morphologically and physiologically using standard methods with some modifications (Barnett *et al.*, 2000). Assimilation of carbon and nitrogen compounds was examined in liquid medium using a rotary shaker at 25 °C. Filamentous growth was investigated on agar plates and on sterile microscope slides (without a coverslip) that were coated with either cornmeal agar, yeast morphology agar or potato glucose agar according to the methods of Barnett *et al.* (2000). Urease activity was tested with urea agar (Christensen, 1946). All physiological and biochemical tests were performed at least twice and compared with a negative control (incubated medium without inoculation). Analysis of respiratory quinones was carried out by the Identification Service and Dr. B. J. Tindall of the DSMZ, Braunschweig, Germany.

Results and Discussion

Sequence analysis of the D1/D2 region of the 26S rRNA gene of LTH 6662^T revealed that *C. musci* was the closest relative with 25 nt substitutions (95% similarity). The remaining

members of the *C. humicola* complex (*C. ramirezgomezianus*, *C. humicola*, *C. longus* and *C. pseudolongus*) contained 26 to 35 nt substitutions. Similarities to species of the genus *Trichosporon* were 94 % or below. As shown in the phylogenetic tree (Fig. 1), the novel species was positioned within the *C. humicola* complex, but outside of the respective clade. As already shown by Takashima *et al.* (2001) based on 18S rRNA gene sequences, and by Prillinger *et al.* (2007), the type strains of *C. musci*, *C. humicola*, *C. longus*, *C. pseudolongus* and *C. ramirezgomezianus* form a cluster, named the *C. humicola* complex, within the order *Trichosporonales*. The tree presented in this study, which was based on the D1/D2 domain of the 26S rRNA gene sequences, revealed the same grouping. According to DNA sequence analysis, strain LTH 6662^T is, however, not closely related (i.e. it has more than 25 nt substitutions) to the species included in the *C. humicola* complex.

More and more studies address the problems of the current yeast classification system, especially the conflict between traditional phenotypic classification and modern molecular phylogeny. Okoli *et al.* (2007) addressed this problem for the order *Trichosporonales*. This order comprises several genera that are phylogenetically related, but their allocation to these different genera was based primarily on phenotypic characteristics, rather than on molecular phylogenetic relationships. Species of the genus *Cryptococcus*, for example, are scattered over three different orders, and often are phylogenetically unrelated. The difference to the genus *Bullera* is defined as the absence of ballistoconidia, but this is not a stable characteristic. Okoli *et al.* (2007) proposed a new yeast classification system based on phylogenetic differences without the differentiation between anamorphic and teleomorphic species. Boekhout *et al.* (2011), Fonseca *et al.* (2011) and Takashima *et al.* (2009) also recommended a new reliable taxonomy that is based on molecular phylogenetic studies.

Based on comparisons of physiological characteristics, LTH 6662^T is easily distinguishable from its relatives, e.g. by assimilation of some carbon and nitrogen compounds, inability to produce starch-like substances, growth without vitamins, maximum growth temperature (see Table 1), and morphological characteristics such as cell shape and budding. The isolate formed buds on short neck-like structures (Fig. 2a), which is not a common characteristic for *Cryptococcus* species, and produced abundantly branched pseudomycelium on plates of cornmeal agar and potato glucose agar (Fig. 2b), but did not produce arthroconidia. The maximum growth temperature was 42°C on solid medium, but in liquid medium growth was very weak at 42°C. Growth temperatures over 40°C are uncommon for *Cryptococcus* species

(Fonseca *et al.*, 2011, our own literature research). Only *Filobasidiella neoformans*, the teleomorphic form of *Cryptococcus neoformans*, is reported to grow slowly at 40°C (Kwon-Chung, 2011). The recently described thermotolerant species *C. tepidarius* is able to grow at 47°C (Takashima *et al.*, 2009). A further special characteristic of LTH 6662^T is the formation of green or blue fluorescent substances in liquid medium. Quinone analysis revealed the exclusive presence of the ubiquinone Q-10 in strain LTH 6662^T. Sugita & Nakase (1998) and Takashima *et al.* (2001) discussed the major ubiquinone type as an important taxonomic criterion within the Trichosporonales. Species of the genus *Trichosporon* have Q-9 or Q-10 as major ubiquinone (Sugita & Nakase, 1998) and the ubiquinone type correlates with the clustering in the phylogenetic tree of the 26S rRNA gene partial sequences. *Trichosporon* species in the ‘*gracile*’, ‘*brassicae*’, ‘*porosum*’ and ‘*ovoides*’ clades have Q-9, while those in the ‘*cutaneum*’ clade have the Q-10 ubiquinone type (Sugita & Nakase, 1998; Sugita, 2011). According to Prillinger *et al.* (2007), the *C. humicola* complex is separated from related species of the genus *Cryptococcus* by having Q-9 as major ubiquinone. *C. fragicola*, *C. haglerorum*, *C. daszewskae* and *C. curvatus* all have Q-10 as major ubiquinone.

In summary, strain LTH 6662^T clearly differed in some important characteristics from members of the *C. humicola* complex (Takashima *et al.*, 2001; Prillinger *et al.*, 2007), including containing ubiquinone Q-10 instead of Q-9, no production of extracellular amyloid substances, no need for thiamine for growth, growth at 42°C, and absence of formation of true hyphae. Taking into consideration its differences to its closest relatives, strain LTH 6662^T should be considered phylogenetically distinct from the species included in the *C. humicola* complex.

Therefore, the genotypic and phenotypic comparisons described above clearly indicate that strain LTH 6662^T represents a novel species, for which the name *Cryptococcus thermophilus* sp. nov. is proposed.

Table 1. Physiological characteristics that differentiate *Cryptococcus contaminans* sp. nov. from members of related species

Strains: 1, *Cryptococcus thermophilus* sp. nov. LTH 6662^T; 2, *Cryptococcus fragicola* CBS 8898^T; 3, *Cryptococcus humicola* CBS 571^T; 4, *Cryptococcus longus* CBS 5920^T; 5, *Cryptococcus pseudolongus* CBS 8297^T; 6, *Cryptococcus musci* CBS 8899^T; 7, *Cryptococcus ramirezgomezianus* CBS 2839^T (data of strains 2 and 4-7 from Takashima *et al.*, 2001). +, Positive; - negative; S, slow; L, latent; W, weak; LW, latent and weak; V, variable.

Characteristic	1	2	3	4	5	6	7
Assimilation of carbon compounds							
Raffinose	+	+	-/S*	L	-	-	L
Soluble starch	-	+	-*	+	+	+	+
Xylitol	-	L	+*†	+	+	+	+
Galactitol	-	+	+*	+	+	+	+
Butane-2,3-diol	-	LW	LW/W/L†	+	+	+	-
Assimilation of nitrogen compounds							
Sodium nitrite	-	-	V*†	-	-	+	-
Cadaverine	+	-	+*	+	+	+	+
L-lysine	-	+	V‡/+*	+	+	+	+
Production of starch-like substances	-	+	V*	+	+	+	+
Growth without vitamins	+	-	-*	-	-	-	-
Major ubiquinone	Q-10	Q-10	Q-9†	Q-9	Q-9	Q-9	Q-9
Maximum growth temperature (°C)	42	30-31	35*	31-32	32-33	33-34	33-34

*Data from Fonseca *et al.* (2011).

†Data from Takashima *et al.* (2001).

‡Data from Barnett *et al.* (2000).

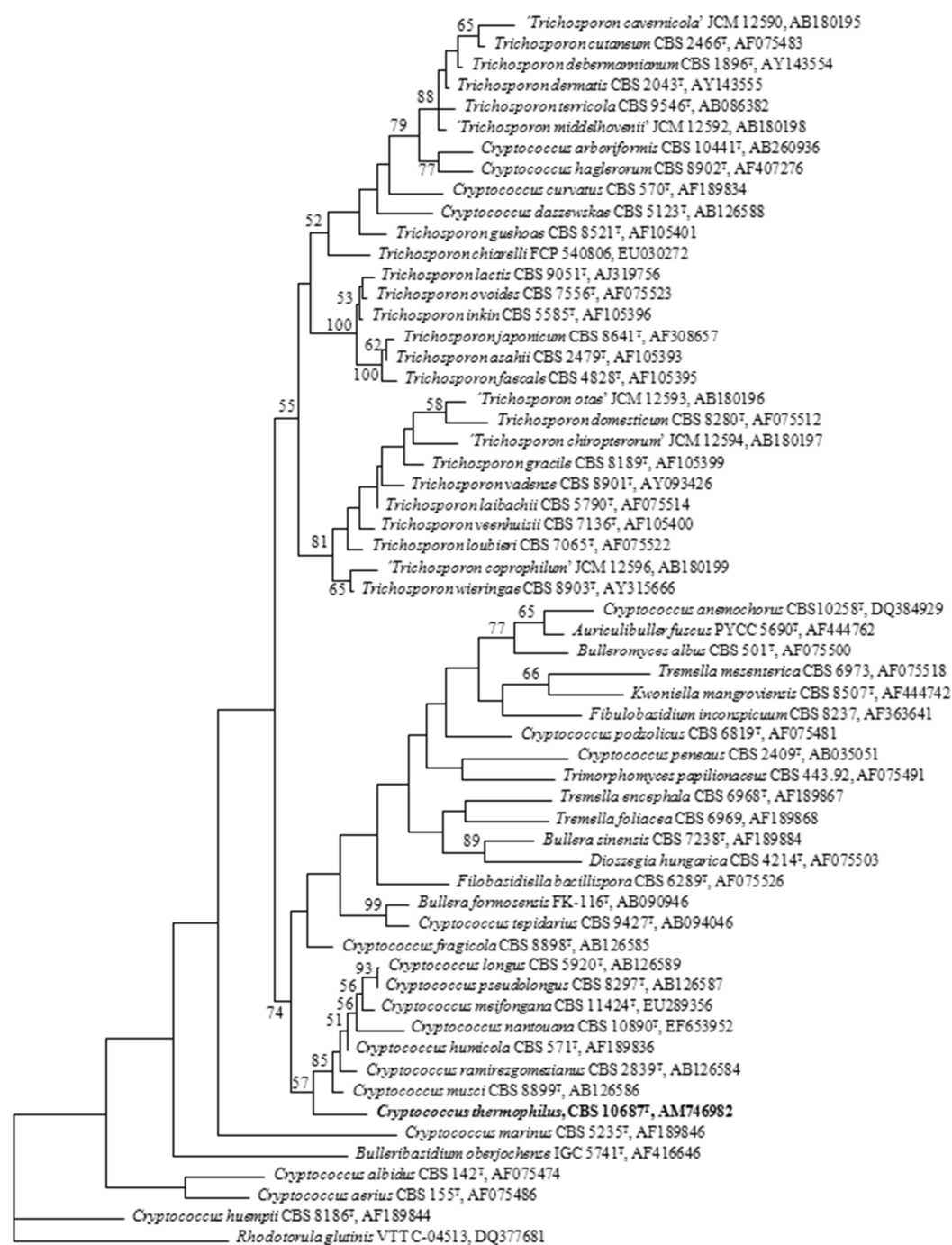


Figure 1. Phylogenetic tree including *Cryptococcus thermophilus* sp. nov. and related species, obtained with maximum-parsimony analysis (heuristic search, random addition, nearest neighbour interchange, 100 maximum trees) of the D1/D2 region of the 26S rRNA gene. Branch lengths are proportional to the number of nucleotide differences and the numbers given on the branches are the bootstrap values (>50%) of 100 replicates. The sequence from *Rhodotorula glutinis* VTT C-04513 was used as outgroup. GenBank accession numbers are indicated after species designations. Species names in quotation marks are those listed in the phylogenetic tree in the new edition of “The Yeasts” (Chapter 100 of Boekhout *et al.*, 2011), but are not validly published.

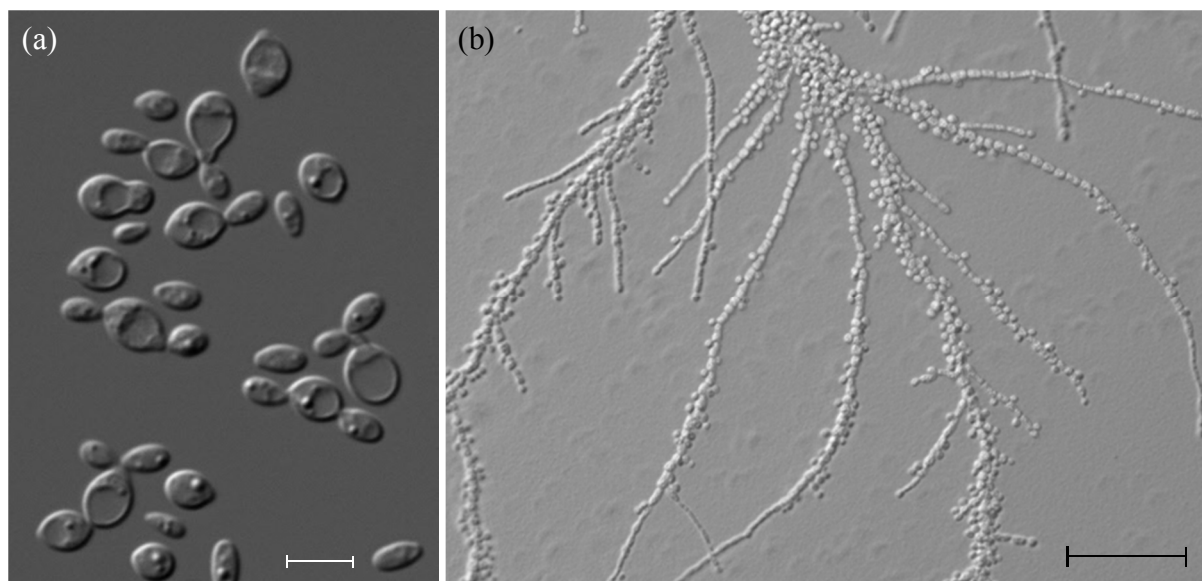


Figure 2. Vegetative cells grown in YG broth for 4 days (a; bar 5 μm) and filaments grown on potato glucose agar for 8 days at 25 $^{\circ}\text{C}$ (b; bar 50 μm).

Latin diagnosis of *Cryptococcus thermophilus* Vogelmann, Chaves and Hertel sp. nov.

In medio liquido extracta levidinis et glucosi (YG) post 3 dies ad 25 $^{\circ}\text{C}$, cellulae sunt ovoidae aut ellipsoidae, 2-4 x 3-5 μm , singulae aut in catenis cellularum gemmarumque ad quatuor, per germinationem multipolarem via petioli breve. Sedimentum formatus. In agar YG post 3 dies ad 25 $^{\circ}\text{C}$, albida cremae et butyrosa et margine glabra. In agar farinae Zea mays et agar extracto malti et levidinis post dies ad 25 $^{\circ}\text{C}$, pseudomycelium formatur. Fermentatio nulla. D-Glucosum, D-galactosum, L-sorbosum (exigue), sucrosum, maltosum, cellobiosum, trehalosum, lactosum, raffinose, melibiosum, melezitose, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, methyl- α -D-glucosidum, salicinum, glycerolum, erythritolum, D-mannitolum, myo-inositolum, D-glucitolum, ribitolum, ethanolum, propano-1,2-diolum, D-glucono-1,5-lactonum, acidum citricum (exigue), acidum lacticum (exigue), acidum succinicum, acidum galacturonicum, acidum D-glucuronicum, D-gluconatum et acetyl-D -glucosaminum assimilantur. Non assimilantur inulinum, amyllum solubile, xylitolum, galactitolum, methanolum et butano-2,3-diolum. Aethylaminum, cadaverinum et glucosaminum assimilantur, neque kalii nitratum, natrii nitritum et L-lysinum. Vitamina externa ad crescentiam non necessaria. 40 $^{\circ}\text{C}$ crescit, exigue 42 $^{\circ}\text{C}$, neque 45 $^{\circ}\text{C}$. Crescit in agar extracto fermenti 10 % NaCl/5 % glucosum. Non crescit agar extracto fermenti confecto 50 partes glucose per centrum. Materia amyloidea non formatur. Crescit in medio 0.01 % cycloheximido addito, neque 0.1 %. Reactiones Diazonium caeruleum B et ureasii positivae. Ubiquinonum: Q-10.

Typus: isolatus ex fermentum Manihot esculentum Crantz, LTH 6662^T, *depositus in collectione zymotica* Centraalbureau voor Schimmelcultures (CBS 10687^T) *et* German Collection of Microorganisms and Cell Cultures (DSM 19443^T).

Description of *Cryptococcus thermophilus* Vogelmann, Chaves and Hertel sp. nov.

Cryptococcus thermophilus (ther.mo'phi.lus. Gr. adj. *thermos* hot; Gr. adj. *philos* loving; N.L. masc. adj. *thermophilus* heat loving).

After 3 days at 25 °C in liquid YG medium, cells are ovoid to ellipsoidal (2-4 x 3-5 µm) and occur singly or in parent-bud chains of up to 4 cells. Buds are produced multilaterally on short neck-like structures. Sediment is formed. In liquid medium, yeast growth causes bright green or blue fluorescence of the broth. On YG agar after 3 days at 25 °C, colonies have a butyrous texture and are white-coloured and shiny in appearance with entire margins. After 4 weeks, colonies are yellowish with a corrugated surface and a fringe of pseudomycelium reaching into the agar. On yeast morphology agar, potato glucose agar and cornmeal agar after 3 days at 25 °C, masses of ovate budding cells are formed. Long, thin, branched pseudomycelium grows into the agar. Chains of ovate cells are formed terminally and laterally on hyphae. Fermentation ability is negative. The following carbon compounds are assimilated: D-glucose, D-galactose, L-sorbose (weak), sucrose, maltose, cellobiose, trehalose, lactose, raffinose, melezitose, melibiose, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, methyl α-D-glucoside, salicin, glycerol, erythritol, D-mannitol, *myo*-inositol, D-glucitol, ribitol, ethanol, propane-1,2-diol, D-glucono-1,5-lactone, citric acid (weak), DL-lactic acid (weak), succinic acid, D-galacturonic acid, D-glucuronic acid, D-gluconate, and acetylglucosamine. No growth occurs on inulin, soluble starch, xylitol, galactitol, methanol, or butane-2,3-diol. The nitrogen compounds ethylamine, cadaverine and D-glucosamine are assimilated, but not potassium nitrate, sodium nitrite and L-lysine. Growth in vitamin-free medium is positive. Growth occurs at 40 °C, weakly at 42 °C but not at 45 °C. Growth on yeast extract agar with 10 % NaCl/5 % glucose is positive. Growth on 50 % glucose/0.5 % yeast extract is negative. Starch-like compounds are not produced. Growth is positive in 0.01%, but negative in 0.1 % cycloheximide. Urease activity and Diazonium Blue B reactions are positive. The ubiquinone present is Q-10.

The type strain is LTH 6662^T (= DSM 19443^T = CBS 10687^T), which was isolated from cassava sourdough in Stuttgart, Germany.

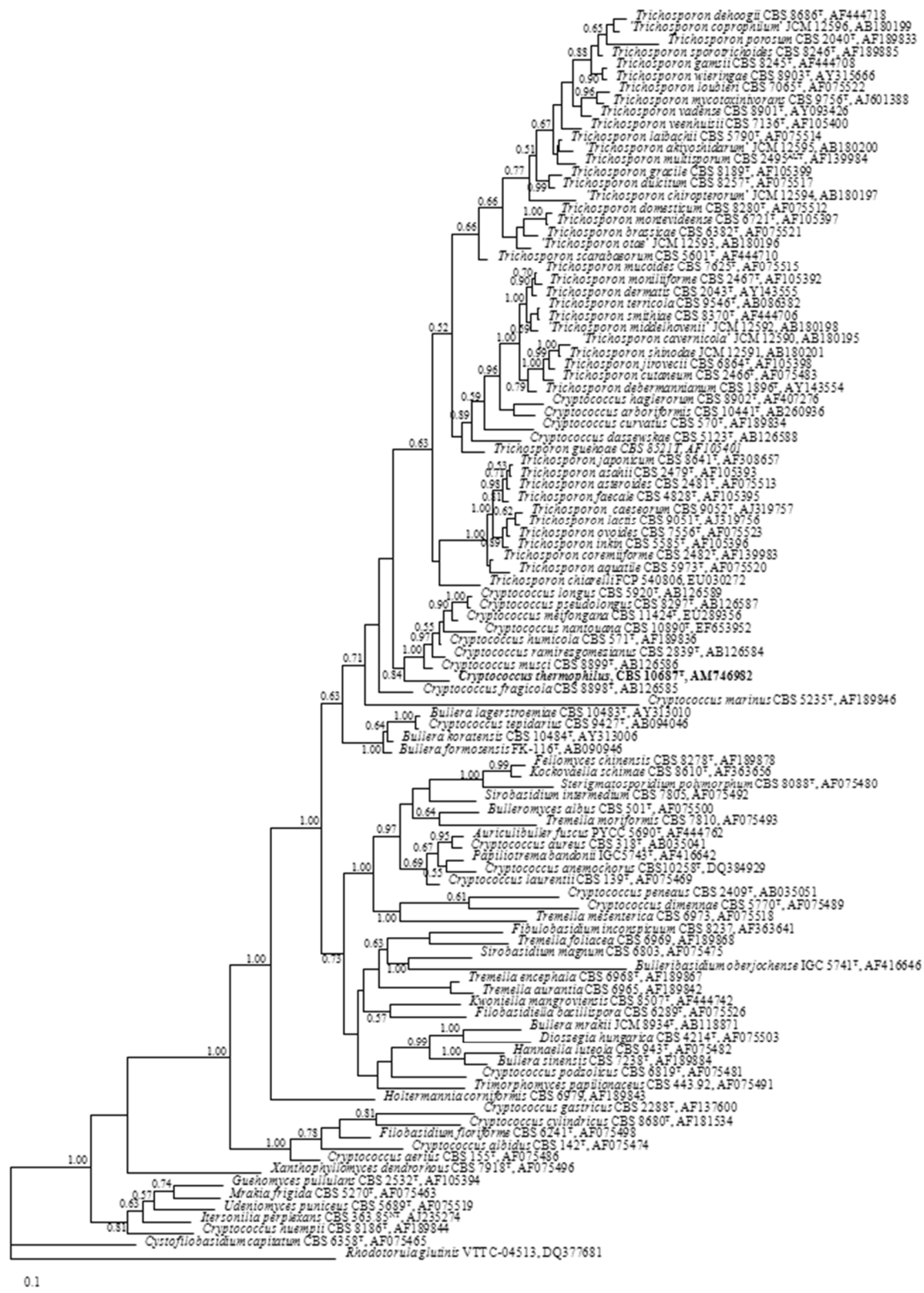
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Supplementary figure. Phylogenetic tree including *Cryptococcus thermophilus* sp. nov. and related species, obtained Bayesian Markov Chain Monte Carlo analysis of the D1/D2 region of the 26S rRNA gene. *Rhodotorula glutinis* was used as outgroup. Probability values (> 50%) associated with each node are shown. GenBank accession numbers are indicated following behind species designation. Species names in quotation marks are those listed in the phylogenetic tree in new edition of “The yeasts” (Chapter 100 of Boekhout *et al.*, 2011), but are not validly published.

Chapter VI

Summary

The microbiota of a ripe sourdough consists of lactic acid bacteria (LAB), especially of the genus *Lactobacillus*, and yeasts. Their composition is influenced by the interplay of species or strains, the kind of substrate as well as the process parameters temperature, dough yield, redox potential, refreshment time, and number of propagation steps (Hammes and Gänzle, 1997). As taste and quality of sourdough breads are mainly influenced by the fermentation microbiota, intense research has been focused on determination of sourdough associated species and search for new starter cultures. In recent years, economic competition pressure and new consumer demands have led to steady research for new cereal products, especially with health benefit or for people suffering from celiac disease. For these reasons, alternative cereals like oat and barley (both toxic for celiac disease patients) as well as the celiac disease compatible cereals rice and maize, sorghum and millets, the pseudocereals amaranth, quinoa and buckwheat as well as cassava got into the focus of interest. However, information about the microbiota of sourdoughs fermented with buckwheat, amaranth, quinoa, oat or barley is not available except for the following recent studies: a study about the microbiota of amaranth sourdoughs by Sterr et al. (2009), a study about barley sourdough by Zannini et al. (2009), a study about oat sourdoughs by Huettner et al. (2010) and a study about buckwheat and teff sourdoughs by Moroni et al. (2011). The microbiota of sourdoughs from the other mentioned cereals as well as cassava was multiply characterised but not systematically. Fermentation conditions were partly not clearly defined, and identification of species was often based on physiological criteria only, known to be insufficient for the exact classification of LAB. Thus, in this thesis, the influence of the process parameters substrate, temperature, refreshment time, amount of backslopping dough as well as the interplay between the different species or strains were examined and potential starter strains were selected.

In Chapter III, the effect of the substrate on the sourdough microbiota was examined and suitable starter cultures for fermentation of non-bread cereals and pseudocereals were selected. Eleven different flours from wheat, rye, oat, barley, millet, rice, maize, amaranth, quinoa, buckwheat and cassava were inoculated with a starter mixture containing numerous LAB and yeasts. Sourdoughs were fermented at 30 °C and refreshed every 24 hours until the microbiota was stable. Species were identified by PCR-DGGE as well as bacteriological

culture and RAPD-PCR, followed by 16S/26S rRNA sequence analysis. In these fermentations, the dominant yeast was *Saccharomyces cerevisiae*; *Issatchenkia (I.) orientalis* was only competitive in the quinoa and the maize sourdough. No yeasts were found in the buckwheat and the oat sourdough. The dominant LAB species were *Lactobacillus (L.) paralimentarius* in the pseudocereal sourdoughs, *L. fermentum*, *L. helveticus* and *L. pontis* in the cereal sourdoughs, and *L. fermentum*, *L. plantarum* and *L. spicheri* in the cassava sourdough. Competitive LAB and yeasts were inserted as starters for a further fermentation using new flours from rice, maize, millet and the pseudocereals. After ten days of fermentation, most of the starter strains were still dominant, but *L. pontis* and *L. helveticus* could not compete with the other species. It is remarkable that from the numerous starter strains which all were adapted to or isolated from sourdoughs, only a few were competitive in these fermentations; but if, then in most cases in a lot of different flours.

In Chapter IV, the effects of the exogenous process parameters substrate, refreshment time, temperature, amount of backslopping dough as well as competing species on the two microbial associations *L. sanfranciscensis* – *Candida (C.) humilis* and *L. reuteri* – *L. johnsonii* – *I. orientalis* were examined. Both associations had previously been found to be competitive in sourdough (Kline and Sugihara, 1971a; Nout and Creemers-Molenaar, 1987; Gobetti et al., 1994a; Garofalo et al., 2008; Böcker et al., 1990; Meroth et al., 2003a). 28 sourdough batches were fermented under defined conditions until the microbiota was stable. Dominant LAB and yeasts were characterized by bacteriological culture, RAPD-PCR and 16S/26S rRNA gene sequence analysis. The process parameters for the association *L. sanfranciscensis* – *C. humilis* could be defined as follows: rye bran, rye flour or wheat flour as substrate, temperatures between 20 and 30 °C, refreshment times of 12 to 24 hours and amounts of backslopping dough from 5 to 20 %. In addition, the association was predominating against all competing lactic acid bacteria and yeasts. The association *L. reuteri* – *L. johnsonii* – *I. orientalis* was competitive at temperatures of 35 to 40 °C, refreshment times of 12 to 24 hours and the substrates rye bran, wheat flour and rye flour, but only with sufficient oxygen supply. Cell counts of *I. orientalis* fell rapidly under the detection limit when using high amounts of doughs (small ratio of surface to volume) and refreshment times of 12 hours.

The fermentations depicted in Chapter III and IV give new information about the influence of process parameters on the sourdough microbiota. The studies show that the sourdough microbiota is markedly influenced by the process parameters and kind and quality of substrate. The competitiveness of a single LAB or yeast is strain specific. Interactions

between microorganisms also play an important role. However, for the search for suitable starter strains, it would be beneficial to know the reasons, why a single LAB or yeast strain is better adapted to specific process parameters or substrates than others.

One of the starter sourdoughs used for fermentation I described in Chapter III was a sourdough made from cassava flour, inoculated with several LAB. No yeast had been inserted, but several yeasts were isolated from the ripe sourdough, which are supposed to originate from the cassava flour. An unknown yeast species constituted 10 % of the isolated yeasts which is described as novel species *Cryptococcus thermophilus* sp. nov. in Chapter V. This yeast is characterized by budding on small neck-like structures, no fermentative ability, growth at 42 °C and without vitamins, a major ubiquinone of Q-10, as well as the production of green or blue fluorescent substances in the growth medium. It is distinct from related species by the ability to assimilate raffinose and cadaverine, the inability to assimilate soluble starch, xylitol, galactitol, butane-2,3-diol, sodium nitrite and lysine, and the inability to produce starch-like substances. The closest relatives are the yeasts belonging to the *Cryptococcus humicola* complex.

Zusammenfassung

Die Mikroflora eines reifen Sauerteiges besteht aus Milchsäurebakterien, insbesondere des Genus *Lactobacillus*, und Hefen. Ihre Zusammensetzung wird beeinflusst durch die Wechselwirkungen zwischen den einzelnen Spezies bzw. Stämmen, die Art des Substrats sowie die Prozessparameter Temperatur, Teigausbeute, Redoxpotential, Führungsdauer und Zahl der Propagationsschritte (Hammes und Gänzle, 1997). Da Geschmack und Qualität von Sauerteigbrot maßgeblich durch die Fermentationsflora beeinflusst werden, konzentriert sich die Forschung auf die Identifizierung sauerteigassoziierten Spezies und die Suche nach neuen Starterkulturen. In den letzten Jahren führten wirtschaftlicher Konkurrenzdruck und neue Verbraucherwünsche zur stetigen Entwicklung neuer Zerealienprodukte, insbesondere mit gesundheitlichem Nutzen oder für Menschen, die unter Zöliakie leiden. Aus diesen Gründen erlangten alternative Getreidearten wie Hafer und Gerste (beide zöliakietoxisch) sowie die zöliakieverträglichen Getreidearten Reis und Mais, Hirsearten, die Pseudozerealien Amaranth, Quinoa und Buchweizen und auch Maniok Interesse. Informationen über die Mikroflora von Sauerteigen aus Buchweizen, Amaranth, Quinoa, Hafer oder Gerste sind allerdings nicht erhältlich außer den folgenden kürzlich erschienenen Studien: eine Studie über die Mikroflora von Amaranthsauerteigen von Sterr et al. (2009), eine Studie über Gerstensauerteig von Zannini et al. (2009), eine Studie über Hafersauerteige von Huettnner et al. (2010) und eine Studie über Buchweizen- und Teffsauerteige von Moroni et al. (2011). Die Sauerteigmikroflora der übrigen oben erwähnten Zerealien sowie Maniok wurde vielfach charakterisiert, jedoch nicht systematisch. Teilweise wurden die Fermentationsbedingungen nicht klar aufgeführt, und die Identifikation der Spezies basierte oft nur auf physiologischen Kriterien, was bekanntermaßen für die exakte Bestimmung von Milchsäurebakterien nicht ausreicht. In dieser Arbeit wurden daher die Einflüsse der Prozessparameter Substrat, Temperatur, Führungsdauer, Menge des Anstellguts sowie Wechselwirkungen zwischen den verschiedenen Spezies bzw. Stämmen auf die Sauerteigmikroflora untersucht und potentielle Starterstämme selektiert.

In Kapitel III wurden die Auswirkungen des Substrats auf die Sauerteigmikroflora untersucht sowie geeignete Starterkulturen für die Fermentation von Nichtbrotgetreidearten und Pseudozerealien selektiert. Elf verschiedene Mehle aus Weizen, Roggen, Hafer, Gerste, Millet, Reis, Mais, Amaranth, Quinoa, Buchweizen und Maniok wurden mit einer Startermischung inokuliert, die eine Vielzahl von Milchsäurebakterien und Hefen enthielt. Die Sauerteige wurden bei 30 °C fermentiert und alle 24 Stunden neu angefrischt, bis die

Mikroflora stabil war. Spezies wurden mittels PCR-DGGE sowie mit bakteriologischer Kulturtechnik und RAPD-PCR gefolgt von 16S/26S rRNA-Sequenzanalyse identifiziert. In diesen Fermentationen war *Saccharomyces cerevisiae* die dominierende Hefe; *Issatchenkia (I.) orientalis* war nur im Quinoa- und im Maissauerteig wettbewerbsfähig. Im Buchweizen- und im Hafersauerteig wurden keine Hefen gefunden. Die dominanten Milchsäurebakterienspezies waren *Lactobacillus (L.) paralimentarius* in den Pseudozerealiensauerteigen, *L. fermentum*, *L. helveticus* und *L. pontis* in den Zerealiensauerteigen sowie *L. fermentum*, *L. plantarum* und *L. spicheri* im Manioksauerteig. Wettbewerbsstarke Milchsäurebakterien und Hefen wurden als Starter für eine weitere Fermentation eingesetzt, wobei neue Mehle aus Reis, Mais, Millet und den Pseudozerealien verwendet wurden. Nach zehntägiger Fermentation waren die meisten der Starterstämme noch dominant, jedoch konnten *L. pontis* und *L. helveticus* nicht mit den anderen Spezies mithalten. Es ist bemerkenswert, dass von den zahlreichen Starterstämmen, die alle an Sauerteig adaptiert waren oder aus Sauerteigen isoliert wurden, nur einige wenige in diesen Fermentationen wettbewerbsstark waren; aber wenn, dann meist in vielen verschiedenen Mehlen.

In Kapitel IV wurden die Auswirkungen der exogenen Prozessparameter Substrat, Führungsdauer, Temperatur, Menge des Anstellguts sowie konkurrierender Spezies auf die beiden mikrobiellen Gemeinschaften *L. sanfranciscensis* – *Candida (C.) humilis* und *L. reuteri* – *L. johnsonii* – *I. orientalis* untersucht. Beide Gemeinschaften wurden im Vorfeld als wettbewerbsfähig im Sauerteig befunden (Kline und Sugihara, 1971a; Nout und Creemers-Molenaar, 1987; Gobbetti et al., 1994a; Garofalo et al., 2008; Böcker et al., 1990; Meroth et al., 2003a). 28 Sauerteigansätze wurden unter definierten Bedingungen fermentiert, bis die Mikroflora stabil war. Dominante Milchsäurebakterien und Hefen wurden mit bakteriologischer Kulturtechnik, RAPD-PCR und 16S/26S rRNA-Gen-Sequenzanalyse identifiziert. Die Prozessparameter für die Gemeinschaft *L. sanfranciscensis* – *C. humilis* konnten folgendermaßen definiert werden: Roggenkleie, Roggen- oder Weizenmehl als Substrat, Temperaturen zwischen 20 und 30 °C, eine Führungsdauer von 12 bis 24 Stunden und eine Anstellgutmenge von 5 bis 20 %. Zudem war die Gemeinschaft vorherrschend gegen alle konkurrierenden Milchsäurebakterien und Hefen. Die Gemeinschaft *L. reuteri* – *L. johnsonii* – *I. orientalis* war wettbewerbsfähig bei Temperaturen zwischen 35 und 40 °C, Führungszeiten von 12 bis 24 Stunden und mit den Substraten Roggenkleie, Roggen- oder Weizenmehl, jedoch nur bei ausreichender Sauerstoffversorgung. Die Zellzahlen von *I. orientalis* fielen bei großen Teigmengen (geringes Verhältnis von Oberfläche zu Volumen) und Führungszeiten von 12 Stunden rasch unter die Nachweisgrenze.

Die Fermentationen aus Kapitel III und IV geben neue Informationen über die Auswirkungen von Prozessparametern auf die Sauerteigmikroflora. Die Studien zeigen, dass die Sauerteigmikroflora durch die Prozessparameter und die Art und Qualität des Substrats ausgesprochen beeinflusst wird. Die Wettbewerbsfähigkeit eines einzelnen Milchsäurebakteriums oder einer Hefe ist stammspezifisch. Auch Wechselwirkungen zwischen Mikroorganismen spielen eine bedeutende Rolle. Jedoch wäre es für die Suche nach passenden Starterstämmen von Vorteil, die Gründe zu kennen, warum ein einzelner Milchsäurebakterien- oder Hefestamm besser an spezifische Prozessparameter oder Substrate angepasst ist als andere.

Einer der Startersauerteige für die Fermentation I in Kapitel III war ein Sauerteig aus Maniokmehl, inokuliert mit verschiedenen Milchsäurebakterien. Es wurde keine Hefe eingesetzt, jedoch wurden mehrere Hefen aus dem reifen Sauerteig isoliert, welche wahrscheinlich aus dem Maniokmehl stammen. 10 % der isolierten Hefen stellte eine unbekannte Hefespezies dar, welche als neue Spezies *Cryptococcus thermophilus* sp. nov. in Kapitel V beschrieben wird. Diese Hefe ist charakterisiert durch Knospung an kurzen halsartigen Fortsätzen, Wachstum bei 42 °C und ohne Vitamine, Q-10 als Hauptubichinon sowie die Produktion von grün oder blau fluoreszierenden Substanzen im Wachstumsmedium. Sie unterscheidet sich von verwandten Spezies durch die Fähigkeit zur Assimilation von Raffinose und Cadaverin, die Unfähigkeit zur Assimilation löslicher Stärke, Xylitol, Galactitol, Butan-2,3-diol, Natriumnitrit und Lysin und die Unfähigkeit zur Bildung stärkeähnlicher Substanzen. Die nächsten Verwandten sind die Hefen des *Cryptococcus humicola*-Komplexes.

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