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CHARACTERIZATION OF THE MICROBIAL ECOSYSTEM OF CEREAL FERMENTATIONS USING MOLECULAR BIOLOGICAL METHODS

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ZUSAMMENFASSUNG

CHARAKTERISIERUNG DES MIKROBIELLEN ÖKOSYSTEMS VON GETREIDEFERMENTATIONEN MIT HILFE VON MOLEKULARBIOLOGISCHEN METHODEN

Milchsäurebakterien (MSB) stellen den Hauptteil der mikrobiellen Flora bei der Fermentation pflanzlicher und tierischer Lebensmittel dar. Bei Getreidefermentationen ist der Gattung *Lactobacillus* eine tragende Rolle beizumessen. Sauerteigfermentationen lassen sich drei Typen (Typ I-III) zuordnen, die hauptsächlich die Art der Führung mit zahlreichen internen und externen Parametern widerspiegeln. Diese wiederum haben entscheidenden Einfluß auf die Zusammensetzung der Mikroflora. Typ I Teige sind Sauerteig-Starterpräparate, die fortlaufend oft über mehrere Jahre hinweg geführt werden. Durch wiederholtes Anfrischen werden die Organismen in einem stoffwechselaktiven Zustand gehalten. Die Hauptflora von Typ I Teigen besteht aus *Lactobacillus sanfranciscensis*, der meist mit der Hefe *Candida milleri* vergesellschaftet ist. Typ II Teige werden über einen längeren Zeitraum von bis zu fünf Tagen bei höheren Temperaturen (40°C) und Teigausbeuten geführt. Dies wiederum führt zu einer typischen Mikroflora, die häufig aus *L. pontis*, *L. panis*, *L. reuteri*, *L. fermentum* und homofermentativen Stämmen von *L. amylovorus* bestehen kann. Typ III Teige sind getrocknete, pulverförmige Sauerteige mit physiologisch aktiven Organismen, wie *L. plantarum*, *L. brevis* oder *Pediococcus pentosaceus,* die sich durch ihre Trocknungsresistenz auszeichnen.

Die klassische Identifizierung von MSB beruht auf einer Erfassung phänotypischer Merkmale, die dann zur Charakterisierung herangezogen werden. Standardmerkmale sind die Fähigkeit der Organismen bestimmte Kohlenhydrate zu verwerten, Substrate zu spalten oder ihr makro-, bzw. ihr mikroskopisches Erscheinungsbild. Gerade bei stark an ein Milieu angepaßten Organismen, wie es die MSB aus Sauerteig sind, führt diese Vorgehensweise nicht immer zu eindeutigen

Ergebnissen. Dazu kommt, dass Sauerteig-MSB eben durch ihre Anpassung an ihr Milieu auf Labormedien oft schwer kultivierbar sind, was eine schnelle Routineidentifizierung erschwert.

Um diese Problematik zu umgehen, wurden in dieser Arbeit Identifizierungsmethoden entwickelt, die auf den Genotyp von MSB gerichtet sind. Diese haben Nukleinsäuren als Zielmoleküle, wobei hier die 16S rRNA eine tragende Rolle spielt. Für einen schnellen Nachweis von Sauerteig-Laktobazillen wurde beruhend auf vorhandenen und selbst erzeugten 16S rRNA Daten ein PCR-Nachweis entwickelt, der eine schnelle und zuverlässige Identifizierung von *L. pontis*, *L. panis* und der in dieser Arbeit neu beschriebenen Spezies *L. frumenti* sowie eine Differenzierung von phylogenetisch verwandten Spezies und anderen Sauerteig-MSB erlaubt. Darüber hinaus wurde das PCR-System mit einer DNA-Extraktion direkt aus dem Sauerteig kombiniert, was einen noch schnelleren Nachweis ohne Kultivierungsschritt ermöglicht. Die Daten über das Vorhandensein von *L. pontis* und *L. frumenti* in einer Modellfermentation stimmen mit alternativen Untersuchungen überein. Die PCR-Nachweismethoden können als schnelles Identifizierungswerkzeug, sowie als zur Kontrolle und Analyse von Fermentationen eingesetzt werden.

Eine weitere PCR gestützte Technik, die in dieser Arbeit entwickelt wurde, ist die Erzeugung von "DNA-Fingerabdrücken" mittels RAPD-PCR. Sie spiegelt typische Polymorphismen auf der DNA der untersuchten Organismen wider. Diese Methode wurde angewandt, um das Verständnis zur mikrobiellen Ökologie in Sauerteigfermentationen zu verbessern. Dazu wurde die Flora industrieller Typ II Fermentationen untersucht, indem die Isolate aus unabhängigen Fermentationen aus einem längeren Zeitraum mittels RAPD-PCR zu Genotypen gruppiert wurden. Es konnte gezeigt werden, dass die Flora solcher unter nicht sterilen Bedingungen geführten Fermentationen sehr stabil ist. Die dominante Flora besteht aus 70% *L. amylovorus* und 30% *L. pontis*, *L. frumenti* und selten *L. reuteri*. Durch die Etablierung einer dem industriellen Prozess nachempfundenen Laborfermentation konnten wichtige Erkenntnisse zur Dynamik, sowie zum Verhalten bei Variation von

Prozessparametern, erhalten werden. Die Laborfermentation erwies sich als ähnlich stabil und von ihrer Florenzusammensetzung als vergleichbar zum industriellen Vorbild. Die prozentuale Zusammensetzung verschob sich mit zunehmender Fermentationszeit zur heterofermentativen Spezies *L. frumenti*, was durch physiologische Untersuchungen bestätigt wurde. Temperaturänderungen zwischen 34°C, 40°C und 46°C führten zu keiner nennenswerten Florenverschiebung, es konnte aber eine Temperatur für optimales Wachstum und Säureproduktion von 40°C bestimmt werden.

RAPD-PCR erwies sich neben der Charakterisierung von MSB auch für Hefen aus Sauerteigen geeignet. Dazu wurden aus traditionellen griechischen Sauerteigen 45 Hefestämme mit RAPD-PCR typisiert. Alle Isolate konnten drei Clustern zugeordnet werden. Durch Einbeziehung von Referenzstämmen konnten diese als *Saccharomyces cerevisiae*, *Yarrowia lipolytica* und *Pichia membranaefaciens* identifiziert werden.

Diese Ergebnisse konnten durch alternative Ansätze, wie FT-IR-Spektroskopie, SDS-PAGE und physiologische Charakterisierung untermauert werden. Während *S. cerevisiae* und *P. membranaefaciens* immer wieder in Sauerteigen angetroffen werden können, ist *Y. lipolytica* zuvor noch nicht in Sauerteigen beschrieben worden.

16S rDNA Sequenzierung und vergleichende Sequenzanalyse erwies sich als die zuverlässigste Methode zur Identifizierung von neuen Isolaten aus Sauerteigen. Zahlreiche Stämme aus einer Typ II Fermentation konnten nach der Einberechnung in einen phylogenetischen Baum keiner bekannten Spezies eindeutig zugeordnet werden. Die nächsten Nachbarn sind *L. vaginalis*, *L. oris*, *L. pontis*, *L. panis* und *L. reuteri*. Weitere Untersuchungen zu physiologischen Eigenschaften, chemotaxonomischen Merkmalen, sowie G + C-Gehalt und DNA-DNA Homologie, ergaben, dass es sich um eine eigenständige Spezies handelt, für die der Name *L. frumenti* vorgeschlagen wurde.

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GENERAL INTRODUCTION

In the beginning of mankind the pattern of living was mainly embossed by a nomadic way. Nutrition was ensured by collecting plant material and hunting of animals just in time. With the commencement of settling down, and the introduction of agriculture men reached independence. Perishable raw material had to be processed to obtain tenably food. Apart from cooking and drying and before the use of preservatives like salt or smoke, at least 3000 years ago the fermentation of vegetable and animal products was the main method to guarantee the shelf life of food. Since these products had preferable keeping properties, were edible, tasteful and moreover healthy the fermentation was carried out under empirically standardized conditions. It is assumed that fermentative conservation of food initially happened accidentally and without any prior knowledge about the underlying processes. Probably the first evidence of a selective inoculation can be dated back to 2500 BC to the Sumarians who induced the fermentation of milk (Fuller, 1992).

Another evidence for early microbial food preservations is the fermentation of cereals. In ancient times, cereals were consumed raw, or as porridge or gruel. As early about 7000-6000 B. C. humans baked their own bread (Lönner & Ahrné, 1995). Excavation in Switzerland established that sourdough bread was part of the typical diet over 5000 years ago (Währen, 1985).

In modern times, fermentation as a mean of food preservation has been supplemented or partly replaced by other preservation methods in developed countries. However, fermentation remains a primary means of preservation in underdeveloped countries and is still important in developed countries, because of its low energy requirements and the unique organoleptic properties it imparts to the product (Daeschel *et al.*, 1987). The fermentation of plant material including cucumbers, cabbage, olives and namely the fermentation of cereal grains constitutes large volumes and diversity.

Microflora of Cereal Fermentations

Most plant materials will undergo a lactic acid fermentation if properly contained, as is the case for flour mixed with water. The microflora of a spontaneous cereal fermentation underlies a temporal succession. The surface of cereals and mature intact cereal grains is at the beginning entirely dominated by high numbers of gram negative, aerobic bacteria like *Enterobacteriaceae* (Krämer 1997). Most investigations have demonstrated that the number of lactic acid bacteria on plant material is very low (10 to 10^3 cells g^{-1}) or undetectable (Fenton, 1987). During storage, counts of these organisms decline and heterotrophic, saprophytic organisms appear, including lactic acid bacteria (LAB) like the genera *Pediococcus*, *Enterococcus*, *Leuconostoc* and *Weissella* (Spicher *et al.*, 1997).

These mesophilic organisms also form the microbial composition of flour, while both lactobacilli and pediococci were found among the lactic acid bacteria (Lönner & Ahrné, 1995). In the simplest way, fermentation of meal is started by the addition of water. Nutritional substrates are dissolved, diffuse into the brine and become available for the bacteria. Together with the reduced redox potential and commencing acidification of the substrate the microflora gradually alters to LAB during fermentation. The fact of a very low number of LAB on plant material as well as in unfermented meal (Hamad *et al.*, 1997) and the marked increase of LAB in fermentations has been partially explained by Müller & Seyfarth (1997) by a viable but non-cultivable state of epiphytic LAB on the surface of plant material. In general, a wide range of genera and species of LAB can be isolated from early stages of cereal fermentations. Homofermentative LAB generally associated with plant material like *Pediococcus* species, *Lactobacillus plantarum, L. casei, L. farciminis* are dominating diverse spontaneously fermented rye and wheat flour sourdoughs but also heterofermentative species like *L. brevis*, *L. buchneri*, and *L. fermentum* have been isolated.

The continuous propagation of cereal fermentations by back-slopping is leading to a stable sourdough microflora, characterized by a higher acid tolerance and a

metabolism well adapted to the cereal environment. In contrast to the mentioned spontaneous cereal fermentations the flora of continuously propagated sourdoughs is dominated by the genus *Lactobacillus* (Hammes & Vogel, 1997) and here mainly heterofermentative species (Stolz, 1995), which can be allotted to the species *L. sanfranciscensis* (Kline & Sugihara, 1971; Weiss & Schillinger, 1984; Böcker *et al.*, 1990), *L. fermentum*, *L. reuteri* (Vogel *et al.*, 1994; Stolz *et al.*, 1995; Hamad *et al.*, 1997), *L. pontis* (Vogel *et al.*, 1994; Müller *et al.*, 1999), *L. panis* (Wiese *et al.*, 1996). The dominance of heterofermentative LAB can be explained by their effective maltose metabolism, by their capability to use fructose as electron acceptor, and their glucose accumulation. The occurrence of homofermentative species in such established sourdoughs is rather uncommon. It is worth to mention that *L. amylovorus* constitutes a dominant element in the flora of sorghum sourdoughs (Hamad *et al.*, 1992) as well as in long term fermentations (Vogel *et al.*, 1996; Suwelack *et al.*, 1997; Müller *et al.*, 1999). Furthermore, strains of *L. johnsonii* and *L. crispatus* have been isolated from sourdoughs but there is no indication about their importance in sourdough (Böcker *et al.*, 1995).

The microbial ecology of sourdough fermentations is determined by various indigenous and exogenous parameters. Endogenous parameters are mainly comprising the chemical and microbiological quality of the meal, respectively. The possibility of an extern control stands behind the exogenous parameters. Fermentation temperature, dough yield, redox potential, fermentation time, manner of refreshment and last but not least the mother sponge are exhibiting strong influence on the microflora. Böcker *et al.* (1995) introduced a classification (type I-III) of industrial sourdough fermentations which takes into consideration the kind of propagation and the manner of preparation, resulting in typical bacterial communities. Type I doughs are characterized by continuous back-slopping with a lower dough yield. Most traditional sourdoughs can be classified as type I. The dominant organism isolated from these sourdoughs is *L. sanfranciscensis*. A typical commercial type I sourdough is the BRS (“Böcker Reinzucht Sauer”).

To fulfill the requirements of modern baking technology more efficient fermentation processes are emerging within the field of sourdough technology. Type II sourdoughs are produced by continuous propagation and an extended fermentation time. This relatively young type of sourdough fermentations originates from the demand for pumpable sourdoughs in industrial applications in bread factories, bakeries and producers of sourdough products. These demands have already been coped by the industry as it can be seen in patents (Menge 1977; Suwelack *et al.*, 1997). In contrast to type I doughs type II sourdoughs exhibit in contrast to type I doughs a higher dough yield and sometimes increased fermentation temperature. Microorganisms found in these sourdoughs are belonging to the species *L. pontis*, *L. panis*, *L. reuteri*, *L. fermentum* and *L. amylovorus* (Vogel *et al.*, 1999).

Type III sourdoughs can be regarded as artificially composed dried sourdoughs in that lactic acid starter bacteria have been selected with respect to their robustness for drying. They are added as souring enhancer to sourdoughs for the bread dough production. Isolates from these sourdoughs matching the desired properties can be allotted to the species *L. plantarum*, *L. brevis* and *Pc. pentosaceus* (Hochstrasser *et al.*, 1993; Böcker *et al.*, 1995).

Applying LAB in a freeze dried state is a further method to initiate sourdough fermentation. Bacterial isolates from, for example, a mature sourdough or other natural environments are selected and tested on their suitability as sourdough starters but also on their viability after drying. Commercial strains are for example *L. delbrueckii* L-22 and *L. brevis* L-62 from Chr. Hansen's Laboratories (Budolfsen-Hansen, 1989). In contrast to the type I sourdough starters these strains are not well adapted to the cereal environment and cannot compete with the indigenous flora, which makes a frequent inoculation necessary.

Taxonomy of Lactic Acid Bacteria

Lactic acid bacteria are a group of bacteria united by a constellation of morphological, metabolic, and physiological characteristics. The general description of the bacteria included in the LAB group is gram-positive, non-sporing, non-respiring cocci or rods, which produce lactic acid as the sole (homofermentative) or as a major (heterofermentative) end-product during the fermentation of carbohydrates. Over the years many attempts have been made for a comprehensive taxonomy of lactic acid bacteria.

About 100 years ago the term LAB was used synonymously with “milk-souring organisms” due to their occurrence in milk and the pioneer work of Lister (1873) isolating the first pure culture which was probably *Lactococcus lactis*.

In 1919 Orla Jensen presented a monograph which had great impact on bacterial systematic. He introduced some key characters including morphological, physiological properties, and optimum growth temperature. On the basic of these properties three sub genera of lactobacilli – *Streptobacterium*, *Betabacterium*, *Thermobacterium* – have been formed. Despite considerable changes in taxonomy his classification scheme remained accepted and remarkably unchanged.

While in former times only phenotypic characters could be examined and evaluated as “phylogenetic” markers, today's scientists have the means to study in detail macromolecules of the cell, believed to be more accurate in defining relationships and phylogenetic positions. The greatest advances have been achieved by studying the structure and sequence of different kinds of nucleic acids. Already in 1965 Zuckerkandl & Pauling introduced the powerful idea that molecules can be documents of evolutionary history or molecular chronometers and they postulated that the comparison of macromolecular sequences could be used to determine the full range of phylogenetic relationships, including bacteria. The development of efficient methods to determine the sequence of nucleic acids (Sanger, 1977) was a prerequisite for the construction of new evolutionary concepts. An important milestone was the extensive work of Woese and his colleagues a decade later over the primary and

secondary structure of 16S rRNA molecule. rRNA is the core of the ribosome holding the key to the mechanism of translation (Gutell *et al.*, 1994). Therefore, rRNA sequences are under strict evolutionary constraints, most likely due to the functional importance of the rRNA. However, within this nearly constant overall structure, molecular sequences in most regions of the molecule are continually evolving, corresponding to the evolutionary distance, and are a prerequisite for good evolutionary chronometers. For *E. coli* the 16S rRNA is composed by 1,542 nt split into 568 nt with conservative and 974 nt with variable character. The direct sequencing of rRNA by using the enzyme reverse transcriptase (Lane *et al.*, 1985) and finally the development of the Polymerase Chain Reaction (Saiki *et al.*, 1988) the number of ribosomal sequences steadily increased. They constitute the basis of modern microbial taxonomy. Comparative sequence analysis and phylogenetic calculations resulted in the construction of phylogenetic trees. For the small subunit (16S) rRNA expanded sequence databases for today well over 16,000 of such molecules have been catalogued in public databases.

Our current picture of the phylogeny of the Bacteria is derived almost entirely from analysis of only one gene and for example the forthcoming editions of Bergey's Manual of Systematic Bacteriology base their respective phylogenetic relationships among microorganisms upon the 16S rRNA tree. Therefore some additional considerations and evaluations are made on this. For instance, although the overall phylogenetic information content of the 23S rRNA molecule is greater than that of the 16S rRNA molecule, the number of currently available complete 23S rRNA sequences is rather poor in comparison to those of the 16S rRNA. Therefore, it can be assumed that the 16S rRNA approach to elucidating bacterial phylogeny remains the standard for this field.

The question if phylogenetic markers exist other than the 16S rRNA or respective genes and if the corroborate the rRNA-based relationship is treated in the following. A phylogenetic marker should fulfill as partially mentioned above several prerequisites, like wide distribution, functional constancy, genetic stability, and a

reasonable number of independently evolving positions or regions. Another desirable feature of such a marker is the possibility to generate a sequence database describing a wide spectrum of phylogenetically diverse organisms. Genome sequencing and comparative sequence analysis revealed that the major part of the genes is not ubiquitously present. Data sets comprising the major part of the bacterial phyla are available for the 23S rRNA, the rRNA polymerase, the elongation factor Tu, the F_1F_{10}ATPase b-subunit, the RecA protein, and the HSP60 heat shock protein. Phylogenetic trees based upon these markers support principally the phylogenetic relationships that appear in the 16S rRNA-based view of bacterial phylogeny. Differences may be due to the reduced information content and the resolution power of the protein markers, gene duplications leading to paralogous markers, lateral gene transfer or a too fast evolution. (Ludwig *et al.*, 1998). It can not be completely excluded that even stable markers such as rRNA genes are subjected to lateral gene transfer, but they are certainly less exchangeable than most other genes. Groisillier & Lonvaud-Funel (1999) carried out a study based confirming this theory for LAB. They compared 16S rRNA sequences with malolactic enzyme gene sequences (*mle*) and additionally with the amino acid sequence of several malolactic and malic enzymes. In contrast to the 16S rRNA derived tree where *Pediococcus* and *Lactobacillus* are intermixed they were separated in the *mle* tree. The phylogenetic tree of the amino sequences of malic and malolactic enzymes showed two groups one where all bacteria species are intermixed. In here to some extend the influence of the habitat is reflected. *L. salivarius* which is only found in human oral cavity and in the intestine of animals seems to evolve faster, *L. plantarum*, *L. brevis*, *L. rhamnosus* are found both in fermented food and in human samples and are therefore grouped together with *L. salivarius.* Other LAB exclusively isolated from fermented food are grouped separately. Moreover, the good agreement of rRNA derived trees to such from other macromolecules supports the conclusion that the 16S rRNA approach is a proper method for inferring phylogenetic relationship among bacteria.

Based on 16S rRNA data bacteria can be divided into 17 phyla. The gram positive bacteria form two lines of descent, one with a G + C (mol%) content with less than 50%, the so called *Clostridium* phylum and another with more than 50% the *Actinomyces* phylum (Schleifer *et al.*, 1995b). Phylogenetically LAB belong to the *Clostridium* branch of gram positive bacteria. Traditionally the genus *Bifidobacterium* was associated with the LAB but has been separated because of the G+C content greater than 55 mol% and have been therefore clustered to the *Actinomyces* branch. Nevertheless, the latter are also considered as LAB, because of their similar physiological and biochemical properties and the sharing of some common ecological niches such as the gastro-intestinal tract (Klein *et al.*, 1998). Often mentioned in regard with LAB is the genus *Sporolactobacillus*, which originally consisted of catalase-negative, spore-forming homofermentative strains (Yanagida *et al.*, 1997). Phylogenetically they are not related to LAB but rather to the genus *Bacillus*. Although there are applications of such strains like *S. cellulosolvens* or *S. inulinus* (Kanwar *et al.*, 1995; Abelyan & Abelyan, 1997) in the fermentative production of lactic acid they are not considered in the following. Other food relevant bacteria belonging to the *Actinomycetes* are *Propionibacterium* sp. and *Brevibacterium* sp..

Today, LAB with a certain importance in foods can be assigned to the genera *Carnobacterium, Vagococcus, Enterococcus, Aerococcus, Alloiococcus, Tetragenococcus, Lactococcus, Streptococcus, Weissella, Leuconostoc, Lactobacillus* and *Pediococcus* (Vandamme *et al.*, 1996). A detailed description of the latter genera with respect to their role in fermentative food production, human disease and spoilage of food a given in the review article of Stiles & Holzapfel (1997). Very recently the new genus *Paralactobacillus*, with a single species *P. selangorensis* was introduced (Leisner *et al.*, 2000). These strains have been isolated from chili bo a Malaysian food ingredient. By phenotypic as well as by genotypic methods it could be shown that this new taxon can be clearly separated from the *Lactobacillus casei-Pediococcus* group.

The most prominent LAB inevitably associated with fermentative food and feed production are *Lactobacillus* (*L*.), *Lactococcus* (*Lc*.), *Streptococcus* (*Str*.), *Leuconostoc* (*Leuc*.) and *Pediococcus* (*Pc*.).

In the non-dairy field of fermentative food production the genus *Lactobacillus* plays a dominant role (Vogel *et al.*, 1999) thus described here more in detail. As mentioned above first trails have been made by Orla Jensen (1919) to subdivide the physiologically heterogeneous genus *Lactobacillus*. Genetically lactobacilli are also exhibiting a great variety. The G + C-content varies between minimum 32% of *L. mali* and maximum 54% of *L. pontis* or *L. fermentum*. This span is twice as large as the proposed treshold value of 10% for genus identity (Schleifer & Stackebrandt, 1983). Furthermore, they are exceeding the 50 mol% proposed as threshold between the *Clostridium* and the *Actinomyces* branch.

A first comprehensive phylogenetic study of LAB including lactobacilli was carried out by Collins and co-workers (1991). Sequence data were determined by RT sequencing of 16S rRNA and aligned to several reference strains. Although it should not be attached to much importance to these data because RT sequences often contain sequence errors (Schleifer & Ludwig, 1995) a picture of the relatedness could be formed. All lactobacilli could be clustered into three groups. **Group I** (*L. delbrueckii*-group) encompasses mainly homofermentative species, but also facultatively heterofermentative species are included. **Group II** – the largest one – consists of over 30 lactobacilli, whereby no difference between homo- or heterofermentative metabolism can be recognized. Furthermore, underlining that morphology is a poor indicator for relatedness also *Pediococcus* species belong to this group. **Group III** (designated the *Leuc. paramesenteroides*-group) contained the genus *Leuconostoc* and some obligatory heterofermentative lactobacilli. Species of this group like *Leuc. paramesenteroides* and some atypical *Lactobacillus* sp. like *L. confusus, L. halotolerans, L. kandleri, L. minor* and *L. viridescens* have been reclassified and moreover a new species *W. hellenica* was described by Collins *et al.* (1993) to the new

genus *Weissella*. Recently the genus *Oenococcus* was proposed comprising *Oenococcus oeni* former *Leuc. oenos* from wine (Dicks *et al.*, 1995).

A more sophisticated classification scheme was presented by Schleifer & Ludwig (1995) using only fully (at least 90%) sequenced 16S rRNA and analyzed by more than one algorithm for the calculation of phylogenetic inference, namely distance matrix, maximum parsimony and maximum likelihood. Following grouping has been proposed:

***L. acidophilus*-group:** former *L. delbrueckii*-group (Collins *et al.*, 1991), named after *L. acidophilus*, because it is a more representative species than *L. delbrueckii* regarding the G+C-content

***L. salivarius*-group:** obligatory homofermentative and facultatively heterofermentative species, no consistent peptidoglycan type (*L. agilis, L. mali* and *L. ruminis* contain meso-diaminopimelic acid instead of lysine in their peptidoglycan)

***L. buchneri*-group:** obligatory heterofermentative lactobacilli, remarkable differences in their DNA composition

***L. reuteri*-group:** obligatory heterofermentative species, widespread range of G+C-content, derivation in the peptidoglycan with ornithine instead of lysine at *L. fermentum* and *L. vaginalis*.

***L. plantarum*-group:** no consistent metabolic activity

For a consensus type of taxonomy Hammes & Vogel (1995) proposed in their review about the genus *Lactobacillus* an arrangement of species with respect to their fermentation pathway of pentoses and hexoses (Group A-C) in combination with their phylogenetic relationship (a = *L. delbrueckii*-group, b = *L. casei-Pediococcus* group, c = *Leuconostoc* group) according to the grouping of Collins *et al.* (1991) and the peptidoglycan type of the cell wall.

Identification of LAB

The identification with traditional methods are mainly based on physiological characters like the capability to ferment certain sugars, to produce gas or to exhibit certain enzyme activities. They are sufficient for a rough characterization but not for unequivocal identification purposes. Furthermore, these procedures are time-consuming and ambiguous (Pot *et al.*, 1993). Phenotypic responses can also be affected by environmental conditions (Schleifer *et al.*, 1995b), e.g. during the investigation of sourdough lactobacilli, certain wild-type strains fermented more carbon-sources than the corresponding type strain (Lönner *et al.*, 1990). Furthermore, it may be impossible that conventional methods do allow a differentiation between phylogenetically distinct species as stated by Hayford *et al.* (1999) for *L. reuteri* and *L. fermentum*, thus applying genotypic methods. For further phenotypic properties like cell or colony morphology similar observations can be made. On the other hand, an advantage of phenotypic tests is that they provide evidence of the functionality of strains. Therefore a great demand exists for fast and reliable application for identification purposes.

The Ribosomal Genes

As mentioned in the general part about taxonomy ribosomal sequences are reflecting the genotype of bacteria. With these especially 16S ribosomal sequences many possibilities were opened for basic as well as for applied research. The strategy of sequencing of rRNA including stretches of variable regions with subsequent comparative sequence analysis in already existing databases allows an unequivocal identification of LAB at the species level and at last a grouping into phylogenetic trees (Hamad *et al.*, 1997; Cocconcelli *et al.*, 1997; Kurzak *et al.*, 1998, Morea *et al.*, 1998; Roushdy *et al.*, 1998, Müller *et al.*, 1999). Although the species-specific region of the 16S rRNA is located in the V1 to the V3 region, identification is more accurate, if the whole gene is sequenced (Stackebrandt & Goebel, 1994).

This means that nearly 1.5 kb have to be sequenced. In some cases the sequence analysis of the region between 16S and 23S rRNA genes (intergenic spacer region = ITS) have a greater force of expression concerning the species-specifity, than 16S rRNA itself and even species like *L. plantarum*, *L. pentosus* and *L. pseudoplantarum* or *L. casei* and *L. rhamnosus* can be discriminated from each other (Tilsala-Timisjärvi & Alatossava, 1997; Berthier & Ehrlich, 1998, Tannock *et al.*, 1999).

By comparative sequence analyses of large numbers of rRNA, regions of different variability can be recognized. They serve as ideal targets for the detection and identification of bacteria from the genus down to the subspecies level (Vandamme *et al.*, 1996) with derived oligonucleotide probes or PCR primers.

Probes for the lactobacilli occurring in cereal fermentation have been itemized by Vogel *et al.* (1999). If the resolution on the 16S rRNA is no more sufficient the 23S rRNA has been chosen as target molecule (Betzl *et al.*, 1990; Ehrmann *et al.*, 1992; Hertel *et al.*, 1991). Despite the greater force of expression of the 23S rRNA sequence data they didn't win too much recognition due to the sequence length of 2.3 kbp in comparison to 1.5 kbp.

Since probes are applied in food microbiology different variations of hybridization techniques have been elaborated. If the aim of an experiment is to proof the identity of strains dot blot hybridization against blotted rRNA (Ampe *et al.*, 1999) or specifically amplified 16S rDNA for enhanced sensitivity is the appropriate tool (Klijn *et al.*, 1991).

To study mixed culture populations colony hybridization was developed. Colonies are grown on membranes placed on an agar plate or transferred from the plate to the membrane. After lysis the released nucleic acids can be detected and colonies are specifically quantified. This method has been successfully applied to differentiate dairy starters (lactococci) and contaminants (enterococci) (Betzl *et al.*, 1990) and for a specific enumeration of LAB in grape must and wine (Lonvaud-Funel *et al.*, 1991).

A simultaneous identification for LAB in fermented food without a previous cultivation step can be achieved by reverse dot blot hybridization (Ehrmann *et al.*, 1994). Tailed oligonucleotides are applied as capture probes on the membrane and in vitro amplified mixed culture rRNA is hybridized against.

A method with increasing impact for the understanding of microbial community structure in environmental microbiology is the in-situ hybridization. Cells can be detected without prior cultivation directly in the respective habitat. The application has widely been used to elucidate the microbial composition of ecosystems whose bacteria are difficult or not cultivable. The principle is the same as for other hybridization techniques with the difference that organisms stay intact, only rendered permeable for the probe targeting the rRNA. By coupling different fluorescent dyes to the probe a simultaneous detection at different taxonomic levels or of different species is possible. Investigations of diverse food relevant LAB genera has been carried out by Beimfohr and co-workers (1993).

A logical advancement from the application of probes in hybridization procedures is the development of specific PCR protocols. The main advantages in comparison to other phenotypical analyses is the increased sensitivity of amplified target regions and the reduced time need. Furthermore, the elaboration of multiplex PCR assays allows the simultaneous detection of more than one species or other genetically encoded properties in one reaction, respectively. Examples for a sensitive 16S rRNA based detection of in foods are the detection of beer spoiling LAB (Yasui *et al.*, 1997; Stewart *et al.*, 1996), *L. sanfranciscensis* (Zapparoli & Torriani, 1997) from sourdough and a specific multiplex PCR for the detection of *L. pontis*, *L. panis*, *L. cerealis* sp. nov. in sourdough samples (Müller *et al.*, 2000). The simultaneous identification of the aggregation-promoting factor (APF) and *L. gasseri* bearing this gene was enabled by a multiplex PCR assay elaborated by Lucchini *et al.* (1998).

DNA-Based Typing

The advances in molecular biology during the last decade has resulted in a large number of methods for the analysis and characterization of nucleic acids. In particular, since the introduction of the PCR (Saiki *et al.*, 1987) most of the nucleic acid based methods rely on the amplification of target sequences. In contrast to the described methods for identification by probes typing methods are based on the generation of fingerprints generated by electrophoretic separation of DNA fragments. Today the most important methods to distinguish bacteria at the (sub)specific level are Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), and Amplified Ribosomal DNA Restriction Analysis (ARDRA) including an enzymatic restriction besides a PCR step. Further methods based on nucleic acids are genomic DNA restriction analysis, plasmid profiling and ribotyping (Rodtong & Tannock, 1993). For the latter automated systems like the RiboPrinter® (Microbial Characterization System (Qualicon™, Wilmington, Detroit, USA) are available. The system includes DNA isolation, restriction with *Eco*R1, separation by gel electrophoresis directly linked to a membrane transfer, hybridization with an universal ribosomal probe, and the visualization and characterization of the patterns. Very recently Kontula *et al.* (2000) used this system for the identification of LAB isolates from human colon biopsies. Zhong *et al.* (1998) did a comprehensive investigation applying these methods for the differentiation of *Lactobacillus* species.

RAPD, first introduced by Williams *et al.* (1990), relies on the amplification of fragments with only a single short primer present. In order to allow annealing of the primer to the target DNA, the annealing step of the reaction is run under low stringency conditions. Together with a random, non-specific primer sequence species up to strain specific fingerprints are generated. This method has especially some merit when no sequence data are available for the genome in question. Dykes & van Holy (1994) have pointed out the need to utilize new and rapid techniques, such as RAPD, for strain typing among *Lactobacillus* sp. Since these days the RAPD-PCR technique

has been applied to many problems in bacterial microbiology mainly in the characterization of complex habitats or the differentiation of isolates.

A protocol for the typing of strains belonging to lactobacilli, enterococci and streptococci was developed by Cocconcelli and co-workers (1995). In further studies the same protocol was used to study population dynamics in whey fermentation (Cocconcelli *et al.*, 1997).

L. plantarum and *L. pentosus* are not distinguishable by their 16S rRNA sequence. Van Reenen & Dicks (1996) presented an RAPD-PCR analysis of the latter strains by which they could be differentiated. *L. plantarum* was also the subject of an investigation carried out by Johansson and co-workers (1995) examining the influence of DNA preparation on the pattern quality. They found out that RAPD was able to group strains according to their functionality as the ability to break down starch and the *in vitro* adherence to human intestinal cells.

L. helveticus is a LAB species well adapted to the dairy environment and involved in the production of cheese. In a work performed by Quiberoni *et al.* (1998) the typing potential of RAPD-PCR technique to investigate the genetic diversity among *L. helveticus* strains from whey was evaluated and compared with the phenotypical diversity of the strains, determined by technological parameters.

Some strains of *L. sakei* have the ability to produce ropy slime, which may spoil vacuum-packaged cooked meat products. Björkroth and co-workers (1996) applied RAPD as a method to discriminate between non-slime and slime producing strains. The genetic fingerprints of these strains have been compared with physiological properties.

Similar investigations about *L. helveticus* were carried out by Giraffa *et al.* (1998). By RAPD typing they could explain that strain heterogeneity was not only strain-dependent but could also be related to the source of isolates.

L. hilgardii and *L. brevis* constitute two phenotypically close species differing in their ability to ferment arabinose. Sohier *et al.* (1999) used the discriminatory power of RAPD-PCR to classify strains isolated from different wines. Similar investigations

availing RAPD-fingerprinting have been carried out by Torriani *et al.* (1996) to clarify the taxonomic position of *L. sakei* and *L. curvatus* strains.

It is important that LAB especially applied as starter cultures can be followed and recognized during fermentation. Several studies showed that this is possible by RAPD products which may serve as probes or may be sequenced to provide oligonucleotide probes and primers for specifically detecting groups of strains. As demonstrated by Erlandson & Batt (1998) probes derived from RAPD fragments can be applied in a colony hybridization assay for the specific detection of lactococci strains in mixed starter culture preparations. Hayford *et al.* (1999) used this approach for a specific differentiation between *L. fermentum* and *L. reuteri* in maize dough. A PCR application with primers derived from fragments was performed by Berthier & Ehrlich (1999) for the specific detection of *L. curvatus* and *L. sakei*.

Analogous problems as for bacteria arise for yeast taxonomy. Traditionally, yeasts are identified by morphological and physiological criteria or by the biochemical composition of the yeast cells. However, these methods are generally laborious and time consuming. The RAPD assay is a less time consuming tool and has also been shown suitable for the identification of food-borne yeast species (Baleiras Couta *et al.*, 1994, Baleiras Couta *et al.*, 1995; Laidlaw *et al.* 1996; Paramithiotis *et al.*, 2000).

AFLP (Amplified Fragment Length Polymorphism) was first introduced by Vos and co-workers (1995) for the analysis of plant genomes. As the RAPD technique this method is based on the detection of naturally occurring DNA polymorphisms. The underlying theory is that variations in banding patterns are a direct reflection of the genetic relationship between bacterial strains examined and therefore that these banding patterns can be considered as genomic fingerprints allowing numerical analyses for characterization and identification purposes (Janssen *et al.*, 1996). The AFLP concept basically consists of three steps: (i) digestion of total cellular DNA with two restriction enzymes, a rare cutter and a frequent cutter and the ligation of halfsite-secific adapters to the restriction sites of al fragments, (ii) the selective amplification of these fragments with two PCR primers that have corresponding adaptor- and

restriction site sequences as their target sites, (iii) the separation of PCR products by PAGE. The primers contain at their 3' end one or more bases, the selective ends. So only primers, which match perfectly will initiate DNA synthesis. The variation of the selective ends has therefore major influence of the complexity of the patterns and on the discriminatory power. The first AFLP studies in the field of prokaryotes dealt with the investigation of the diversity of clinical relevant strains (Valsangiacomo *et al.*, 1995; Huys *et al.*, 1996). In the field of LAB only a few approaches using the AFLP technique have been and up to now two published investigations are available. Gancheva and colleagues (Gancheva *et al.*, 1999) performed a polyphasic approach towards the identification of strains belonging to the *L. acidophilus* rRNA group (Schleifer & Ludwig, 1995). AFLP typing was compared with result of SDS-PAGE and RAPD typing. The discriminatory power of AFLP derived patterns was comparable with the others and even species like *L. gasseri* and *L. johnsonii* which could not be separated by SDS-PAGE could be clearly differentiated by AFLP. The clear allocation of the recently described new species *L. amylolyticus* and *L. iners* into new clusters demonstrated the applicability of AFLP to identify new taxons. Kunene and co-workers (Kunene *et al.*, 2000) applied AFLP to distinguish strains of *L. plantarum* and *Leuc. mesenteroides* from different sorghum doughs.

ARDRA is a technique combining the knowledge of ribosomal RNA sequences and their specific amplification (Amplified Ribosomal DNA) together with the characterization of strains by their restriction pattern (Restriction Analysis). This kind of RFLP was named ARDRA by Vaneechoutte *et al.* (1992) in a study which enabled the distinction of well characterized cultured type strains. The main advantage of this method is, that no sequence information about the amplified 16S rRNA is required. An advancement of this method is the characterization of pure culture rDNA for the analysis of natural microbial communities without cultivation (Weidner *et al.*, 1996). Total community genomic DNA is extracted without culturing the participating microorganisms. The presence of universally conserved sequences at the 5’ and 3’ ends allows the amplification of nearly complete 16S rRNA genes fragments of the

extracted DNA. The PCR product can than be cloned and the resulting 16S rRNA gene library can be screened by a variety of methods. Colony hybridization using specific probes may be used. As mentioned for the conventional RFLP inserts may be amplified and characterized by a restriction digest. Alternatively, single-lane sequencing can also be done to allow higher resolution screening (Ward *et al.*, 1990) followed by complete sequencing and identification as reported above.

While the analysis of cloned PCR products is almost exclusively applied in environmental biology, ARDRA of pure cultures can also be found in the investigation of LAB in foods (Giraffa *et al.*, 1998b).

DNA-DNA Hybridization

The properties of specific base pair formation between complementary or foreign strands together with the temperature or alkali dependent denaturation and reversible reassociation makes DNA-DNA hybridization a powerful and sensitive tool to assess genetic relatedness between organisms. While in the above described methods for identification and determination of species relatedness only stretches of DNA are applied as target regions, in DNA-DNA hybridization (DDH) studies the entire genome of two organisms is compared. Hence, the force of expression is markedly increased as compared to other methods. In the discussion whether 16S rRNA sequence determination or DDH is the appropriate tool for species delineation Stackebrandt & Goebel stated in their taxonomic note (1994) that 16S rRNA may not the appropriate method to replace DNA reassociation for the delineation of species and measurement of intraspecies relationship. The rationale for the DDH as standard origins from numerous studies, in which a high degree of correlation between DNA similarity and chemotaxonomic, genomic, serological and numerical phenetic similarity could be found. Nevertheless, 16S rRNA analysis is a most valuable addition to the polyphasic approach to bacterial classification, and for the species level it is extremely helpful in deciding whether DNA reassociation needs to be performed (Stackebrandt & Goebel, 1994).

Many different methods based on diverse physiochemical properties are available for measuring the reassociation of DNA from different strains. The degree of reassociation depends upon the similarity of the nucleotide sequence, thus allowing a quantification of the degree of relatedness, commonly expressed as percent homology.

There are at least five different hybridization methods available for taxonomic studies (Schleifer & Stackebrandt, 1983):

- DNA-filter method
- Competition method
- Hydroxyapatite method
- Spectrophotometric method
- Nuclease S_1 method

Chemotaxonomic Methods

Chemotaxonomy refers to the application of analytical methods to collect information on various chemical constituents of the cell to classify bacteria

Whole Cell Protein Patterns

The electrophoretic separation (SDS-PAGE) of extracted cell proteins results in patterns with a resolution between the species and sub-species level. It has proven to be extremely reliable for comparing and grouping large numbers of closely related strains. Furthermore, it may serve as an identification tool if large databases including reference are built up. It can be seen as a helpful tool integrated in a polyphasic approach for the characterization of bacteria. Tsakalidou *et al.* (1997) applied SDS-PAGE of whole cell proteins to *Weissella* strains which could not be allotted to known *Weissella* species. Pot *et al.* (1993) used SDS-PAGE to resolve a taxonomic problem concerning phylogenetically close related species of the heterogeneous *L. acidophilus* complex.

The Potential of Fermented Cereal-Based Products as Probiotics (Symbiotics)

Cereal-based foods are a major source of dietary energy and nutrients worldwide (Salovaara, 1998). Apart from non-fermented cereal foods like rice or pasta, fermented products are constituting the staple food in almost every civilization, reflected in a huge variety of different products. Especially in developing countries with their tropical climates lactic acid fermentation is a low cost method for enhancing food quality, safety and shelf-life (Cooke *et al.*, 1987). Processing of cereal foods is often completed with a final boiling or baking stage killing any lactic acid bacteria present in the cereal material. However, it is not mandatory to cook or bake the fermented cereals. Examples were fermentation follows heat treatment is “kishk” a product combination of fermented milk with boiled, dried and ground grains (Steinkraus *et al.*, 1983).

This strategy of combining the benefits of cereal dietary fibers, which may affect as so called prebiotic substances the intestinal microecology (Tannock 1990), and viable counts of lactic acid bacteria could act as an equivalent of lactic fermented dairy products. Here the application of so called probiotic starter cultures often in combination with prebiotics (fructo-oligosaccharides, xylo-oligosaccharides) leading to synbiotics already has been fully established. Most probiotic strains applied in the dairy field have been originally derived from the host’s intestine because of the suggestion that their colonization is improved by host-specific adherence properties (Tannock 1990). Following this strategy Molin *et al.* (1992) studied the influence of fermented oatmeal soup using intestinal lactobacilli on human’s health. A fermented oat product which matches these conceptions has been realized in Finland, where it is already marketed (Salovaara & Kurka, 1991). Alternatively an approach where the indigenous fermentation flora acts as “probiotic” could be imaginable (Müller *et al.*, 1998).

In both cases the knowledge of potential probiotic properties of the fermentation flora has to be improved. Amongst the characterization of strains, stability during processing, resistance against gastric juice, the adherence properties to intestinal cells

as a prerequisite for the host's colonization are of major interest (Havenaar *et al.*, 1992). To study bacterial adherence to intestinal cells and the problems which arise with in vivo investigations led to the development of in vitro model systems. The basic approach is based on the isolation of cell lines that have properties of various cell types which occur in the intestinal epithelium. One of the cell lines used extensively in studies of bacterial adherence is the Caco-2 cell line derived from a human colon carcinoma. Main feature of this cell lines why they are as suitable for this kind of studies is their spontaneous enterocyte-like differentiation of the brush border microvilli. Furthermore they provide an excellent system not only for studying the adherence but also how these bacteria may interact with pathogenic bacteria that compete within the same ecosystem (Greene & Klaenhammer, 1994). The alternative approach for an investigation of the behavior of LAB in the intestinal environment is to take in vivo colon biopsies.

Motivations and Objectives of the Study

Cereal fermentations represent a complex ecosystem. The microflora of such fermentations is, except at the beginning of the fermentation, dominated by lactic acid bacteria sometimes associated with yeasts. In contrast to the dairy field, where fermentations can be carried out with pasteurized materials and inoculated with defined starter organisms, cereal constitute a non sterile substrate with a rather heterogeneous composed flora. If a cereal fermentation was propagated over a longer time, the respective microflora is well adapted to this environment, resulting in a unique and stable composition of different species and strains. Traditionally, quantitative bacterial composition was determined by counting colony forming units with a subsequent identification by their physiological and morphological characteristics. The determination of such phenotypic properties is quite time consuming and not reliable. This is especially true for sourdough LAB exhibiting different properties in comparison to the corresponding type strains as a result of the already mentioned adaptation to their special environment. Therefore, more sophisticated strategies are required in food microbiology. In most cases they are targeting the genotype of bacteria revealed as the method of choice.

The overall aim of this work has been to establish more rapid and objective methods to identify, detect and characterize LAB from cereal fermentations and get insights in the ecology of cereal fermentations, the organisms involved, their behavior and conclude on process improvement and new fields of application.

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CHAPTER I

MONITORING THE GROWTH OF *LACTOBACILLUS* SPECIES DURING A RYE FLOUR FERMENTATION

Summary

The natural microbial community conducting an industrial sourdough fermentation was investigated by molecular biological methods using the following strategy: strains were isolated and subjected to RAPD (randomly-amplified polymorphic DNA) PCR. After computer-supported pattern analysis and clustering of the strains the 16S rDNA of members of each distinct cluster were partially (530 bp) or completely (1570 bp) sequenced and identified by comparative sequence analysis. The predominant strains of this fermentation could be allotted to the species *Lactobacillus amylovorus*, *Lactobacillus pontis* and a species, which phylogenetically takes an intermediate position between *L. pontis* and *L. panis.* Sporadically, strains were identified as *L. reuteri*. In a second step the effect of external factors was investigated under the controlled conditions of a lab-scale process. Fermentations were carried out at 34°C, 40°C and 46°C. The development of the flora was consistent in independent fermentations as proved by RAPD typing of randomly-picked colonies. The microbial community in these fermentations was identical to those found in an industrial scale. The qualitative composition of the flora was not affected by the temperature. *L. amylovorus* was the dominant species. With increasing fermentation time, a shift toward the predominance of heterofermentative lactobacilli was observed. This finding was underlined by metabolic studies and stoichiometric calculations of the metabolic pathways. With increasing temperature the percentage of homofermentative organisms was reduced. Furthermore, the growth rate and the metabolic activity increased, followed by an immediate decrease of the growth rate at 46°C and lower terminal values of lactate, acetate and ethanol, respectively.

Introduction

The application of mixed starter cultures has a long tradition in bread making. Before the pre-industrial era, artisanal bakeries obtained their sourdough starters from other bakeries or from a household. In both of these cases, the sourdough originated from a spontaneous sourdough fermentation. Through continuous propagation, adding flour and water at regular intervals, the sourdoughs have been kept metabolically active, enhancing the desired fermentation (Daeschel *et al.,* 1987). As a result of the selective pressure exerted by the environmental conditions non-aseptic cereal fermentations are characterized by a rather stable association of lactobacilli.

Sourdoughs produced by long term fermentations were recently designated as type I (Böcker *et al.,* 1995). They are mainly dominated both in rye and wheat flour doughs by the species *Lactobacillus sanfranciscensis* often associated with the yeast *Candida milleri* (Sugihara *et al.,* 1970, Spicher *et al.,* 1980, Böcker *et al.,* 1990). In addition to these traditional fermentation processes a huge variety of modified fermentations have been developed. To fulfill the requirements of modern baking technology, more efficient fermentation processes are being developed within the field of sourdough technology.

Sourdoughs produced by continuous propagation and a prolonged fermentation time recently were designated as type II (Böcker *et al.* 1995). This relatively young type of sourdough fermentation originates from the demand for pumpable sourdoughs in industrial applications in bread factories, bakeries and producers of sourdough products. In contrast to type I doughs, type II sourdoughs exhibit a higher dough yield and sometimes an increased fermentation temperature. The microflora differs from that of type I doughs. Strains often belong to the species *L. pontis, L. reuteri L. johnsonii* (Böcker *et al.,* 1995) or *L. panis* (Wiese *et al.,* 1996). In most of the cases the fermented broth is applied in liquid or dried form as a dough-souring supplement and aroma carrier.

The classical approach to bacterial taxonomy solely based on morphological and physiological features does not reflect the phylogenetic relationship of bacteria. The application of such methods, e.g. fermentation patterns, often leads to a misidentification of the strains and an insufficient description of the natural community (Vogel *et al.*, 1994). Even the inclusion of the cell wall composition, cellular fatty acids and other characteristics into the characterization scheme results in ambiguity, as these can be influenced by environmental parameters. Therefore, the application of more sophisticated methods is needed in food microbiology and the fermentation industry for a thorough and reliable description of the microflora.

The application of molecular genetic techniques to determine the relatedness of food-associated lactic acid bacteria (LAB) has resulted in significant changes in their taxonomic classification (Stiles & Holzapfel, 1997). Currently, most of the genotypic identification and detection techniques are focused on the rRNA molecule. Because the character of the 16S rRNA molecule ranges from conserved to highly variable, identification at different levels is possible. The methods for the identification of bacterial isolates include the sequencing of variable 16S rDNA regions and the comparative sequence analysis with already existing sequences organized in databases like the ARB database (Ludwig & Strunk, 1995). Polymerase chain reaction (PCR) has become a powerful tool in various environmental, clinical and food microbiological applications since its invention (Saiki *et al.*, 1987).

Arbitrary amplification of polymorphic DNA sequences has increasingly been reported as a method for the genetic characterization of micro-organisms, and there are many variations of this technique (Tyler *et al.*, 1997). Arbitrarily-primed PCR, RAPD analysis, and DNA amplification fingerprinting are the main variants (Caetano-Anollés *et al.*, 1991, Williams *et al.*, 1990, Welsh *et al.*, 1990). The underlying theory is the same: single primers with arbitrary sequence are used to target an unspecified genomic sequence in order to generate a genetic profile. The amplified fragments are resolved by gel electrophoresis, resulting in band patterns. These fingerprints are specific for

individual organisms leading to a discrimination among bacterial isolates, whereby no prior knowledge of genetic information is required. A refined technique for the determination at the strain level in combination with oligonucleotide hybridization techniques is presented by Erlandson & Batt (1997).

Our objective was not only to characterize the bacterial microflora of an industrial type II rye flour sourdough but to follow its composition and metabolic changes throughout a fermentation. To study the influence of fermentation parameters a lab-scale fermentation was installed.

Materials and Methods

Strains, Media and Cultivation Conditions

All strains were numbered according to the strain collection of the Technische Mikrobiologie Weihenstephan (TMW). Reference and type strains were included in the RAPD analysis: *L. panis* TMW 1.684 (DSM 6035^T), *L. panis* TMW 1.649 (DSM 6036), *L. reuteri* TMW 1.693 (DSM 20016^T), *L. oris* TMW 1.16 (DSM 4864^T), *L. pontis* TMW 1.84 (ATCC 51518^T) *L. pontis* TMW 1.85 (ATCC 51519), *L. pontis* TMW 1.597 (LTH 3572).

The isolated strains were grown on a modified MRS (mMRS) medium (Vogel *et al.*, 1994). All reagents were ordered from Merck (Darmstadt, Germany) except meat extract (Oxoid, Wesel, Germany) and agar-agar (Difco, Detroit, USA). Ninety percent of the water was replaced by a rye flour extract. For the preparation of 1 l of rye flour extract, 40 g of commercial rye flour together with 2 g of malted wheat meal and 0,8 g of trypsine (1:250, 1300 BAEE U/ mg solid, Sigma, Deisenhofen, Germany) were incubated at 50°C for 24 h in a shaking incubator. The solids were separated by centrifugation (5000 x g for 15 min).

Strains from sourdough samples were incubated in 10 ml screw cap tubes at 40°C. Solid media were incubated under an atmosphere consisting of 90% N_2 / 10% CO_2 (v/v). Reference strains were incubated at temperatures indicated by the strain collection.

Fermentation and Sampling

Samples of the industrial process were taken daily over the whole fermentation period of 5 days. Additionally, pH-value and total titrable acids (TTA) were determined on an aliquot of 10 g sourdough blended with 100 ml distilled water. The TTA is expressed as the amount of 0.1 M NaOH (ml) to reach a final pH of 8.5 in a titration. The total amount of LAB was determined by counting colony forming units (cfu) on mMRS.

For the lab-scale fermentation, 600 g of commercial rye flour were mixed with 2400 ml of temperated tap water, 900 µl of amyloglucosidase (Novo, Denmark) and 90 g of 48-hour-old starter material of the industrial process in a Biostat-fermenter (Braun, Melsungen, Germany). All fermentations were run under temperature control.

HPLC analysis

The metabolites lactate, acetate and ethanol were determined and quantified by HPLC. A Merck (Darmstadt, Germany) OAKC column was used at 70°C for the separation. The fluid phase was 5 mM H_2SO_4, the flow rate was 0.4 ml min^{-1}. For detecting the peaks a refractometric detector was used.

DNA isolation

DNA was extracted from pure cultures using a modified accelerated enzymatic lysis method after Lewington (Lewington *et al.*, 1987). After lysis was completed cell debris was incubated for 10 min with chloroform-isoamyl alcohol (24:1, v:v) without prior separation of the cell debris. RNase and proteinase treatments were not carried out.

RAPD-PCR

The colonies subjected to RAPD-PCR were picked randomly. Before the isolation they were marked on the bottom of the petri-dish so as not to be influenced by colony morphology. At least 30 colonies were selected. RAPD-PCR was carried out with the oligonucleotide primer M13V. The primer sequence was 5‘ - GTT TTC CCA GTC ACG AC - 3‘. All RAPD-PCR reactions were performed in TopYield™ Strips (Nunc, Denmark) with oil overlay (50µl) and TECAN sealings (Tecan, Kreilsheim, Germany). The conditions for PCR amplification were as follows:

One µl of genomic DNA, 5 µl of 10 x reaction buffer, 5 mM $MgCl_2$, 200 nM each of the four deoxynucleotides, 1,5 U Taq polymerase (all components from Amersham Pharmacia Biotech, Freiburg, Germany) and 20 pmol of primer M13V (Interactiva, Ulm, Germany). The PCR reactions were carried out on a Hybaid Omni Gene thermocycler (MWG-Biotech, Ebersberg, Germany). The cycling program was: (96°C/3 min; 35°C/5 min; 75°C/5 min) 3cycles; (96°C/1 min; 55°C/2 min; 75°C/3 min) 32 cycles.

Electrophoresis of RAPD-PCR products

All PCR products (10 µl DNA+10µl water+5µl AGS running buffer) were electrophoretically separated on a 1.5% TBE agarose gel (0,5xTBE) in a MWG-Biotech chamber (20 cm x 25 cm) for three hours at constant 170 V. As size marker encompassing the whole range of fragments was loaded at 1 µg per lane of the BioSizer™ (AGS, Heidelberg, Germany). After 15 min staining with ethidium bromide, patterns were digitally saved by the E.A.S.Y. system (Herolab, Griesheim, Germany).

Cluster analysis

The patterns were evaluated by the Gel Compar 4.1 package (Applied Math, Kortrijk, Belgium). As clustering algorithm, the Unweighted Pair Group Method using arithmetic averages (UPGMA), was applied. Similarities between RAPD patterns were calculated using the Pearson product-moment correlation coefficient (r x 100).

16S rDNA amplification and sequencing.

PCR-mediated amplification of the 16S rDNA was carried out in strips (Brandt, Wertheim, Germany) on a Gradient Master Thermocycler (Eppendorff, Hamburg, Germany). The amplification conditions were as follows: 1 µl of genomic DNA, 10 µl 10 x reaction buffer, 200 nM each of the four deoxynucleotides, 1,5 U Taq polymerase, 20 pmol primer each (616V, 630R), $H_2O_{bidest.}$ to a final volume of 100 µl. The PCR program used was: (94°C/2 min) 1 x, (94°C/45 sec-52°C/1 min-72°C/30sec) 30 x, (94°C/1 min-72°C/4 min) 1 x. PCR products were purified by QIAquick PCR purification kit (Quiagen, Hilden, Germany) and were eluted with 60 µl Tris (10 mM, pH 7). For a routine identification the primer 610R was used leading to a sequence of 520 bp. Sequence determination of the 16S rRNA gene regions were performed as described previously (Weizenegger *et al.,* 1991)

DNA sequences were determined by the chain-termination method (Sanger *et al.*, 1977) using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Life Science, Buckinghamshire, England) and electrophoresed on an ALFexpress sequencer (Amersham Pharmacia Biotech, Freiburg, Germany) or alternatively using the ABI Prism™ Dye Terminator Cycle Sequencing Kit (Perkin Elmer) on an ABI 373 stretch sequencing system by a commercial service (SequiServe, Vaterstetten, Germany).

Phylogenetic Analysis.

The first step of an identification was an on-line investigation using the computational service of the Ribosomal Database Project (http://www.cme.msu.edu/RDP/analyses.html). A precise phylogenetic position was obtained by alignment and parsimony analysis using the ARB database package (Ludwig and Strunk 1996).

Results

Microbiological and Analytical Data of an Industrial Rye Sourdough Process

The total cell count was determined and expressed in colony forming units (cfu) of an industrial rye flour fermentation over the whole period of 5 days. Additional characterization was achieved by pH, TTA and temperature measurement of the fermentation broth **(Figure 1)**.

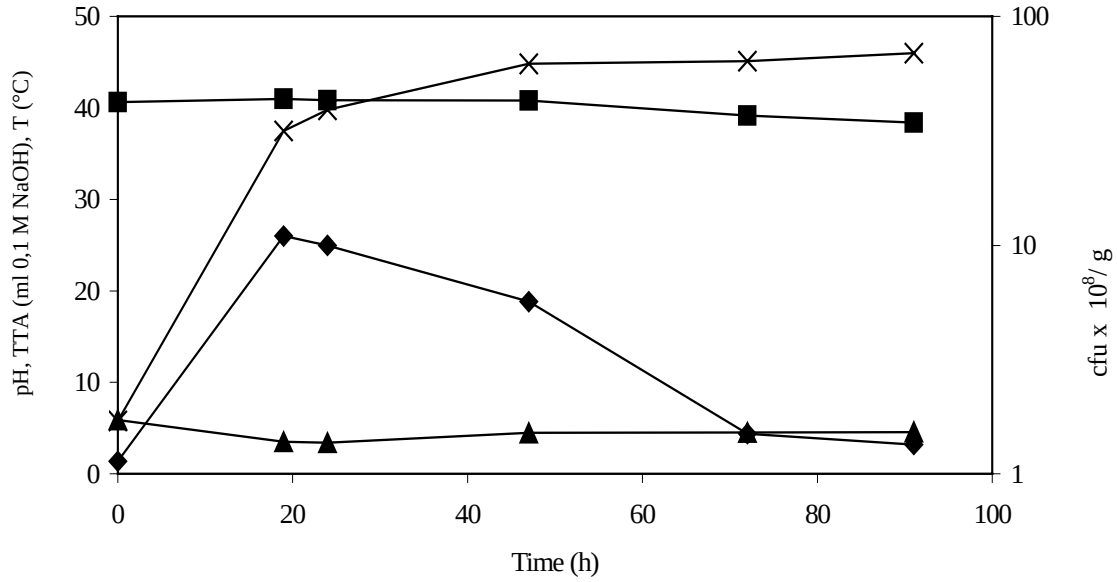

Figure 1. Plots of growth (◆) expressed by colony forming units (cfu), pH (▲), TTA (X) measured by the amount of 0.1 M NaOH to titrate a pH of 8.5, and temperature (■) during an industrial type II rye flour fermentation.

Samples taken from the first day directly after inoculation had a total bacterial count of 1.38 x 10^8 cfu g^{-1} on mMRS agar. The initial pH value was 5.8, the TTA was 5.8 and the temperature 42.2°C. After 24 h the maximum bacterial count of 2.6 x 10^9 was reached. 5 hours later the minimum pH value of 3.4 was measured. The maximum TTA of 46 was reached at the fifth day, whereby the bacterial count dropped down to 3.2 x 10^8 cfu g^{-1}. Temperature was over 40°C till the end of the second day, subsequently it dropped down to 34.3°C at the end of the fermentation.

Identification of Colonies from Mixed Culture Plates

To obtain a first overview of the fermentation flora, 36 strains collected over the whole fermentation period were subjected for RAPD fingerprinting together with reference strains usually expected in such fermentations. **Figure 2** depicts a digitized picture of the generated banding patterns after cluster analysis.

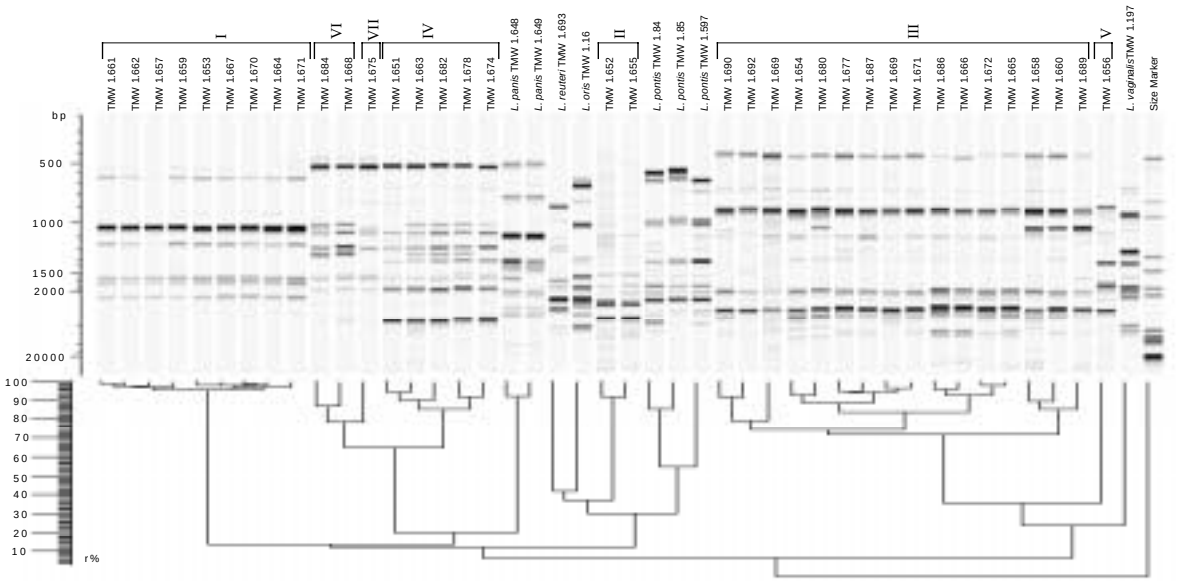

Figure 2. Clustering of RAPD patterns generated by PCR with M13V primer. Each cluster is numbered with roman numerals. I: *L. amylovorus*, II: *L. species*, III: *L. species*, IV: *L. pontis*, V: *L. reuteri*, VI: *L. pontis*, VII: *L. species*. Reference strains are indicated directly

Applying the UPGMA algorithm with the Pearson correlation coefficient (r), seven clusters with different inter-species similarity were obtained. The patterns consisted of three to six main fragments sized between 3.5 kb and approximately 0.5 kb. The highest conformity was exhibited by cluster I with a correlation coefficient of 96%, followed by cluster II with 90% and cluster III and IV with 70%. The remaining clusters were formed by single strains (V and VII) and two strains (II). The differences between the clusters were between 70% and 90%. None of the reference strains showed resemblance with the patterns of the fermentation organisms. For a reliable identification a minimum of 520 bp from the 5‘ terminus of at least one representative

of each cluster was determined. Strains of cluster I comprise the obligate homofermentative species *L. amylovorus*. The two isolates of cluster II could not be allotted to a certain species. Together with the strains of cluster III and VII they took an intermediate position between their closest phylogenetic neighbors *L. pontis*, and *L. vaginalis* and exhibit a heterofermentative metabolism. For more detailed information the complete 16S rDNA was sequenced of the strains *L. species* TMW 1.655 and *L. species* TMW 1.666. To each of the phylogenetically related species differences of 30 bp were determined. In the following the strains are named *L. species* together with the roman number of the cluster. Cluster IV and VI comprise the heterofermentative species *L. pontis* with a correlation of 64%. The single species of cluster V was identified as *L. reuteri* also a heterofermentative *Lactobacillus*.

The distribution of strains isolated at the different stages of the fermentation is summarized in **Table 1**.

Table 1. Counts of different strains occurring in an industrial fermentation at different times after RAPD fingerprinting

Species (cluster)	Sample (h)				
	0	19	47	72	91
L. amylovorus (I)	1	4	4	n.d.	n.d.
L. species (II)	2	n.d.	n.d.	n.d.	n.d.
L. species (VII)	n.d.	n.d.	1	n.d.	n.d.
L. species (III)	1	3	6	3	6
L. pontis (IV)	1	1	1	3	1
L. reuteri (V)	n.d.	1	n.d.	n.d.	n.d.

n. d. = not detected

The rate of the dominant species at the total flora was determined on a sourdough sample, which was fermented for 48 h, by RAPD-PCR of all colonies (46) from a 10^{-8} dilution. The flora consisted of 63% of *L. amylovorus*, 15% of *L. pontis*, 15% of *L. species* III and 7% of *L. reuteri*. The investigations of samples two years after these isolations showed similar results concerning strain composition and even the patterns were comparable.

Establishment of a Lab-Scale Type II Fermentation: Starter Preparation.

To study the influence of external fermentation parameters, a lab-scale fermentation that simulated the industrial process was built. The starter preparation was produced in a 48-hour-fermentation, which paralleled the industrial process. At the end of each cycle the broth was used for inoculating the main process, which lasted for 5 days. To obtain a stable composition of the starter material, the 48-hour-process was repeated following consecutive re-inoculations. The composition and the stability were easily checked for the first five consecutive fermentation cycles by RAPD-PCR, and revealed to be very reproducible, also between different PCR reactions and independent from DNA isolation procedure **(Figure 3)**.

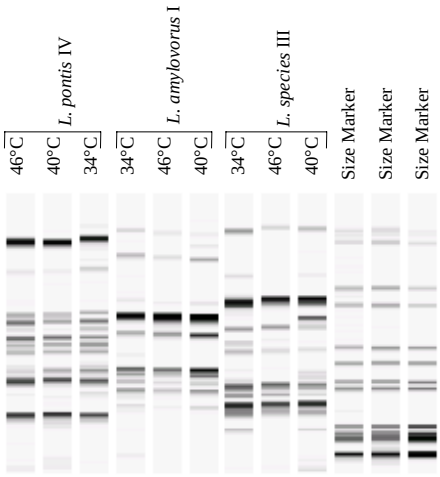

Figure 3. Comparison of RAPD patterns from strains belonging to fermentations with different temperatures (34°C, 40°C, 46°C). For each fermentation DNA isolation and PCR reaction has been carried out separately. The roman numbers are referring to the RAPD clusters in Figure 2.

Patterns of the ‘lab-scale strains’ were assorted by the computer-supported pattern search function to already existing clusters. Therefore, a repeated identification by sequencing was no longer necessary. After the first refreshment, a composition similar to the industrial starter was reached. The dominant species was *L. amylovorus* I with

72% of the total bacterial count, followed by *L. species* III with 15% and *L. pontis* IV with 13%. The other strains from the industrial fermentation could not be isolated.

Lab-scale Type II Fermentation: Monitoring the Main Fermentation.

The flora of the inoculum and the mature sourdough were compared. While the starter showed the same percental bacterial composition as described, the mature (150 h) sourdough consisted of 100% *L. species* III. To evaluate the growth dynamics of the strains during the main fermentation, bacteria were isolated at different periods of the fermentation. At least 30 colonies were randomly picked. This procedure was repeated for three times with different fermentation temperatures. For an additional characterization of the sourdough ecosystem the total cell count, pH and the metabolic process were followed. **Figure 4** describes the course of the cfu at 34°C, 40°C and 46°C fermentation temperature.

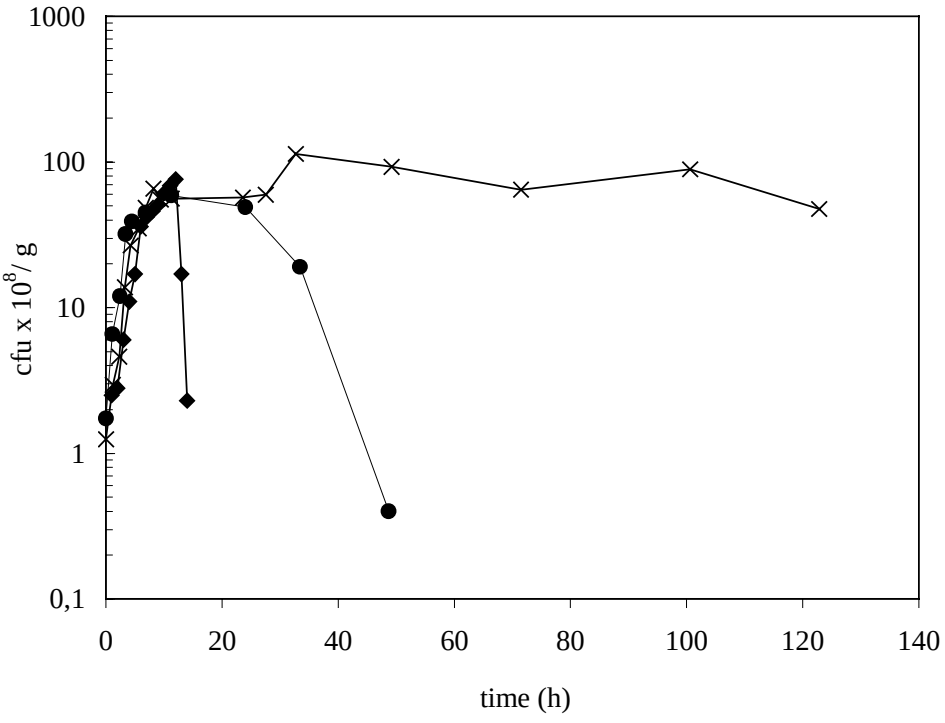

Figure 4. Development of growth calculated as cfu/ g sourdough in lab-scale fermentations at 34°C (X), 40°C (♦) and 46°C (●).

Until the beginning of the stationary phase the development can be regarded as almost temperature independent. Only the growth rate at the 46°C-fermentation was slightly higher. At all three temperatures a mean maximum of 6 x 10^9 cfu g^{-1} after 23 h was reached. While the bacteria of the 34°C and 40°C-fermentation, respectively, stayed in the stationary phase till 120 h and 55 h, respectively, at the 46°C-fermentation a very immediate decrease of the cfu after 25 h occurred. This finding correlated well with the production of metabolic products (lactate, acetate/ethanol) recorded over the fermentation time by HPLC **(Figure 5)**.

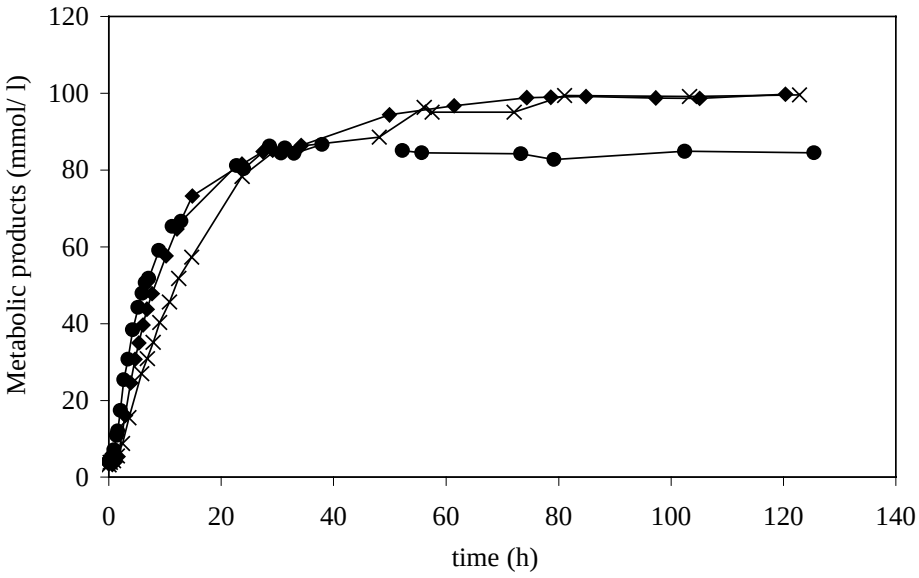

Figure 5. Comparison of the formation of the metabolic products lactate, acetate and ethanol of the homo- and heterofermentative metabolism in fermentations with 34°C (X), 40°C (♦) and 46°C (●) fermentation temperature.

The flora of the 46°C-fermentation showed the fastest acid production till 25 h, correlating with the growth curve, except that lower terminal values were obtained. In the other fermentations the maximum was reached later and higher terminal values were reached.

Since the samples were collected at different times during the rye flour fermentation, the RAPD methodology allowed us to describe the growth kinetics of the different biotypes dependent of the temperature **(Figure 6)**.

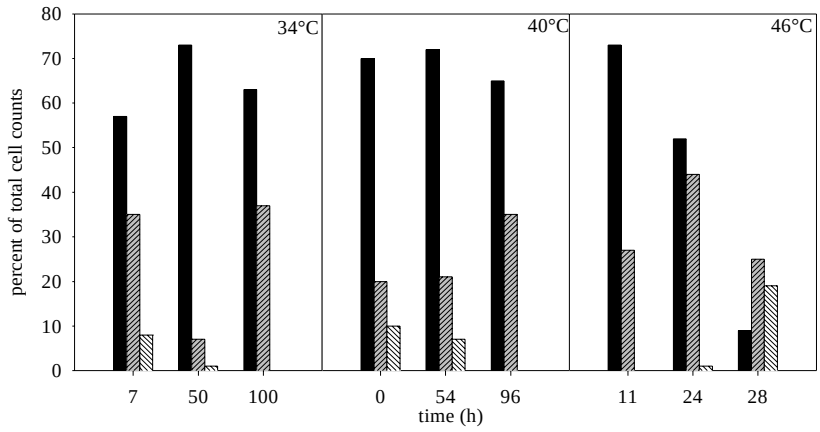

Figure 6. Percentage of relevant strains (= *L.amylovorus* I, = *L. species* III, =*L. pontis* IV) of a type II lab-scale fermentation at different temperatures, calculated by RAPD analysis.

The main component in the three fermentations was the species *L. amylovorus* I, with a percentage of the total flora between 58% and 73%. Only for the 46°C-fermentation were the heterofermentative species *L. species* III and *L. pontis* IV dominant after 24 h. During each of the fermentations a change in direction toward the heterofermentative *L. species* III with increasing fermentation time was recognized.

This shift could also be recognized by the metabolic activities **(Figure 7)**.

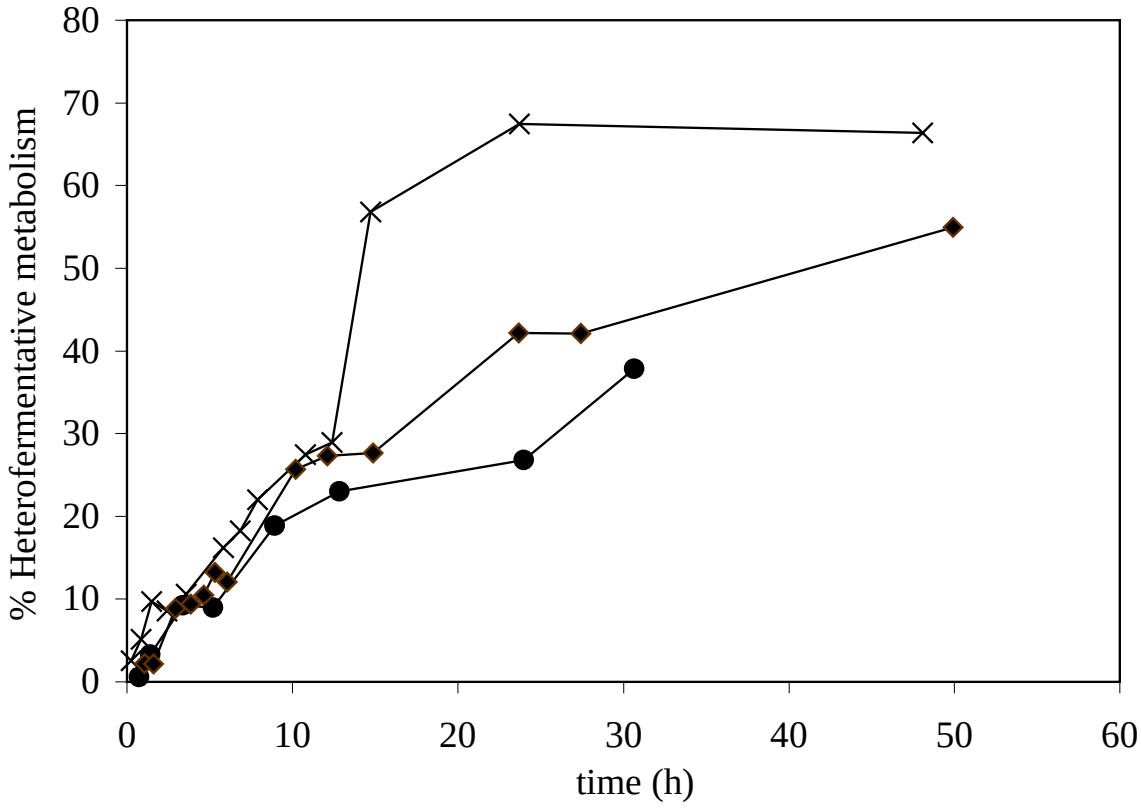

Figure 7. Percentage of products produced by heterofermentative metabolism (lactate, acetate/ethanol) in relation to the total amount of metabolic products produced by hetero- and homofermentative lactobacilli at 34°C (X), 40°C (◆) and 46°C (●). Calculations were performed on the basis of the stoechiometry of the metabolic pathway.

By the observation of the stoichiometric conversion of glucose by heterofermentative lactobacilli in relationship to the total metabolism, percental values for heterofermentative activities were calculated. Until 12 h the augmentation was temperature independent. After this point the 34°C fermentation showed the most impressive increase. After 30 h (46°C) and 50 h (34°C, 40°C) no more values could be calculated, because the differences between the metabolic end–products were to small.

Discussion

The microflora of traditional sourdoughs, which have been classified as type I, have been investigated since many years (Sugihara *et al.*, 1970; Kline & Sugihara 1971; Stolz *et al.*, 1993, Stolz *et al.*, 1995 a,b). Independent of the geographical origin this kind of sourdough is driven by strains of *L. sanfranciscensis*, selected only by the environmental conditions induced by the sourdough fermentation technology. Completely different parameters are applied at the type II sourdough fermentations, resulting in a different microbial ecosystem, whose composition and population dynamics are described here.

The bacterial flora of the industrial fermentation consisted out of four species belonging to the genus *Lactobacillus*. The lab-scale sourdough fermentation showed a consistent composition with strains belonging to the same RAPD-clusters. The only homofermentative species could be assigned to the species *L. amylovorus*. During the industrial fermentation *L. amylovorus* could not be isolated after 47 h. A similar tendency was observed at the lab-scale experiments. With increasing fermentation time the percentage of *L. amylovorus* at the total cell count decreased. After 150 h at 40°C no pattern specific for *L. amylovorus* was obtained. In contrast to diminishing numbers of *L. amylovorus*, those of the heterofermentative species *L. species* III increased. This tendency was underlined by a metabolic monitoring of the end-products lactate, acetate and ethanol.

The presence of *L. amylovorus* in type II sourdoughs has not been reported before. Strohmar (1992) investigated a rye flour sourdough fermented for at least 48h and they only found heterofermentative species. However, a similar association of *L. amylovorus* together with the heterofermentative species *L. reuteri* and *L. fermentum* in traditional sorghum doughs is described by Hamad *et al.* (1992).

L. pontis was the first described sourdough *Lactobacillus* which showed a close phylogenetic relationship to the species *L. oris*, *L. vaginalis* from human origin and

L. reuteri isolated from mammalians. Recently, Wiese *et al.* (1996) described *L. panis* , which was previously only ambiguously characterized. Like *L. pontis,* it could be assorted to the same phylogentic group to which *L. species* II, *L. species* III and *L. species* VII belong. *L. species* III was the dominating heterofermentative strain in our industrial and lab-scale fermentation, respectively. They seemed to have the highest acid-tolerance, surviving for 150 h where pH values of 3.2 and TTA values of 40 were reached. Complete 16S rDNA sequence analysis of the strains *L. species* TMW 1.666 (cluster III) and *L. species* TMW 1.655 (II) and partial sequencing (520 bp) of *L. species* TMW 1.675 revealed obvious differences of about 2% to their phylogenetic neighbors *L. vaginalis*, *L. oris* and the sourdough organisms *L. pontis* and *L. panis*. No cellular nor similarities of colony morphology to the reference strains could be observed. Therefore, it is currently not possible to decide on a final systematic position.

Apart from the investigation of physiological and morphological properties chemotaxonomic and genotypic methods are leading to a comprehensive classification of bacteria. The limit of these systems in the study of complex microbial ecosystems is the low number of samples that can be analyzed. Especially in fermentations of non-sterilized substrates, these complex studies have been hampered by the lack of methods for identifying and typing of *Lactobacillus* strains (Cocconcelli *et al.,* 1997). We applied M13-RAPD-PCR to elucidate the biodiversity of a special sourdough fermentation process. Environmental parameters such as increased fermentation temperature and an extended souring phase lead to a well-adapted, stable flora within this dough. An unambiguous identification on the phylogenetic level is especially important in this case, because of the widespread physiological properties which often cannot be assorted to reference organisms from other habitats as a result of adaptation to different environments. These finding is confirmed by investigations of Hamad *et al.* (1997) of sorghum sourdough and Strohmar (1992) of a rye flour sourdough. Proof that growth conditions can also markedly affect cell morphology was presented by

Schleifer *et al.* (1985), in a study in which *L. xylosus* was reclassified as *Lactococcus. lactis* subsp. *lactis* on the basis of phylogenetic data. Data indicating that it may be even impossible to distinguish lactobacilli by their physiological properties were given by Kandler & Weiss (1986): *L. reuteri* and *L. fermentum*, both associated with the lactic acid fermentation of sourdough, were indistinguishable by conventional tests, but genetically unrelated, as indicated by the difference in mol% G + C of the DNA.

Besides its potential use in characterization of the flora, RAPD-typing was a good tool for checking the long term stability of such fermentations in independent batches. The consistency and reproducibility between batches revealed RAPD-PCR as an appropriate tool for monitoring the behavior of fermentation organisms under different conditions which may be suitable for other food environments. Furthermore the transformation from an industrial process to a lab-scale fermentation was feasible without a change in the microbial composition of the flora allowing for further metabolic and ecological investigations. This information can be used to develop such processes with respect to flora composition and thus metabolic products affecting the sensory properties and staling of baked goods.

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CHAPTER II

MULTIPLEX PCR FOR THE DETECTION OF *LACTOBACILLUS PONTIS* AND TWO RELATED SPECIES IN A SOURDOUGH FERMENTATION

Summary

A specific multiplex PCR assay based on the amplification of parts of the 16S rRNA molecule was designed. Primers derived from variable regions of the 16S rRNA provided a means of easily differentiating the species *Lactobacillus pontis* and *Lactobacillus panis*. They could be clearly discriminated from the phylogenetically related species *Lactobacillus vaginalis, Lactobacillus oris* and *Lactobacillus reuteri* and from other lactobacilli commonly known to be present in sourdough. Other strains isolated together with *L. pontis* from an industrial sourdough fermentation could be clearly separated from these species by comparative sequence analysis and construction of a specific PCR primer. For a fast identification a DNA isolation protocol based on the ultrasonic lysis of cells from single colonies was developed. To demonstrate the potential of such techniques for tracking these organisms in a laboratory-scale fermentation, we combined the specific PCR assay with direct DNA extraction from the organisms in the sourdough without previous cultivation.

Introduction

The most prominent lactic acid bacteria (LAB) of the sourdough microflora belong to the genus *Lactobacillus* (Linko *et al.*, 1997). Sourdough fermentations have been categorized by Böcker and coworkers (Böcker *et al.*, 1995) by taking into account the kind of propagation and the applied fermentation parameters resulting in typical microbial communities. Sourdoughs maintained by a continuous back-slopping over an extended period have been designated type I sourdoughs. They are characterized by a microflora which is mainly dominated by *Lactobacillus sanfranciscensis* (Gänzle *et al.*, 1998). Type II sourdoughs are fermented over a longer period at elevated temperatures and higher water content. Typical lactobacilli isolated from this fermentations are *Lactobacillus pontis* and *Lactobacillus panis*, both endemic to cereal fermentations. Further species from type II fermentations have been recently itemized by Vogel *et al.* (Vogel *et al.*, 1996; Vogel *et al.*, 1999).

L. pontis is close in the phylogenetic tree to *Lactobacillus vaginalis*, *Lactobacillus oris*, and *Lactobacillus reuteri* (Vogel *et al.*, 1994). Wiese and coworkers described *L. panis* as a new species which is phylogenetically related to *L. vaginalis*, *L. oris*, and *L. reuteri* (Wiese *et al.*, 1996).

Traditionally, physiological properties of bacteria, such as their capability to degrade carbohydrates and certain enzyme activities, have been applied for identification purposes. Especially for LAB, reliable identification depending on this properties is almost impossible because of similar nutritional requirements of different species due to adaption to a certain environment (Ampe *et al.*, 1999; Hamad *et al.*, 1997). For this reason various approaches targeting the genotype of bacterial cells, which reflect the natural relationship, have been described and steadily introduced into applied and research aspects of food microbiology. During the last decade rRNA emerged as a suitable target molecule for identification purposes (Betz *et al.*, 1990; Ehrmann *et al.*, 1994; Klijn *et al.*, 1991).

In the framework of the description of *L. pontis* as a new species (Vogel *et al.*, 1994), a 16S rRNA-targeted oligonucleotide probe in the V1 region (Neefs *et al.*, 1993) of the 16S rRNA was designed. There were sufficient sequence variations to differentiate *L. pontis* from other sourdough lactobacilli. The 16S ribosomal DNA (rDNA) sequence of *L. panis* (Wiese *et al.*, 1996) revealed that this species is most closely related to *L. oris*, followed by *L. vaginalis* and *L. pontis*. The binding sites on the 16S rDNA of *L. panis* and *L. pontis* for the probe previously described for *L. pontis* are identical to each other. So far no evaluated system for the specific identification and differentiation of these two species has been available.

Therefore, we have developed 16S rDNA targeted primers for a specific PCR to distinguish these two lactobacilli. Apart from the mentioned species, primers have been designed for a third sourdough lactobacillus (*Lactobacillus* sp.) originally isolated from an industrial type II rye fermentation. Comparative sequence analysis of 16S rDNA revealed the *Lactobacillus* sp. to be an intermediate between *L. pontis and L. vaginalis*, but no final systematic position has been established up to now. To demonstrate the applicability of the PCR system to trace these organisms during fermentation, we developed a laboratory-scale fermentation similar to an industrial sourdough process.

Materials and Methods

Laboratory-scale fermentation

The fermentation was performed in a 5-liter stirred reactor (Biostat, Braun, Melsungen, Germany) at 40°C. The substrate consisted of 900 g of rye bran mixed with 2,400 ml of preheated tap water. To start the fermentation, 90 g of 48-h-old sourdough of the corresponding industrial process was added.

Sampling

Sourdough samples were collected aseptically. For the determination of the total cell count, expressed as CFU per milliliter of sourdough, samples were serially diluted 1:10 with NaCl (0.9% [wt/vol]) and plated on modified MRS (3) using a spiral plater (Spiralsystems, Inc., Cincinnati, Ohio). The plates were incubated under a modified atmosphere (90% N_2, 10% CO_2). To determine the total amount of aerobic bacteria in the flour without starter, plate count agar (Merck, Darmstadt, Germany) was used. Additionally, sourdough samples of 5 ml for the direct isolation of DNA from the organisms were taken and stored at –20°C.

Cultivation and Storage of Strains

All reference organisms (see **Table 2**) were cultivated on mMRS (Vogel *et al.*, 1994). The incubation temperatures were 30°C for *L. pontis*, *L. sanfranciscensis*, and *Lactobacillus farciminis*, 37°C for *L. panis*, *L. oris*, *L. vaginalis* and *L. reuteri*. *Lactobacillus* sp. TMW 1.655 and DSM 13145 isolated from an industrial fermentation process, and TMW 1.1104 and TMW 1.1098 isolated from previous lab-scale fermentations, were cultivated at 40°C. Stock cultures were stored at –80°C in 80% (wt/wt) glycerol.

Selection of Primers

As the primer binding site, we selected the helix 11 of the V2 region (Neefs *et al.*, 1993). The primer sequences obtained were subsequently checked with all small-subunit sequences in the Ribosomal Database Project (RDP) database (Maidak *et al.*, 1999) using the check-probe function. To improve the sensitivity of the PCR and to include a positive control for DNA accessibility for the PCR, a multiplex PCR including two universal primers (616V and 609R) and a species-specific primer as the forward primer was developed. The sequences of the amplification primers are listed in **Table 1**. Reference strains are listed in **Table 2**.

Table 1. Universal and specific primers applied in the multiplex PCR assay

Sequence[a]	Specificity	Primer
AGAGTTTGATYMTGGCTCAG	universal	616V
ACTACYNGGGTATCTAAKCC	universal	609R
AGCCATCTTTGAAAT	*L. pontis*	LaponR
AACCATCTTTTATAC	*L. panis*	LapanR
AGCCTTCTTTTATAC	*L. species*[b]	LaspecR

[a] Sequences are given in 5‘-to-3‘ direction. Mixed bases are given according to the International Union of Biochemistry code.
[b] *L. species*, *Lactobacillus* sp.

Reference strains are listed in **Table 2**.

Table 2. Strains studied and results of specific PCR as indicated by visual bands on agarose gel.

Species		PCR signal with primer[a]		
		LaponR	LapanR	LaspecR
L. pontis	ATCC 51519	+	-	-
L. pontis	LTH 2587	+	-	-
L. pontis	LTH 3572	+	-	-
L. pontis	LTH 2585	+	-	-
L. pontis	LTH 3572	+	-	-
L. panis	DSM 6035	-	+	-
L. panis	DSM 6036	-	+	-
L. species[b]	TMW 1.655	-	-	+
L. species	DSM 13145	-	-	+
L. species	TMW 1.1104	-	-	+
L. species	TMW 1.1098	-	-	+
L. sanfranciscensis	DSM 20451	-	-	-
L. farciminis	DSM 20184	-	-	-
L. oris	LMG 9848	-	-	-
L. vaginalis	LMG 12891	-	-	-
L. reuteri	DSM 20016	-	-	-

[a] See Table 1
[b] *L.* species, *Lactobacillus* sp.

DNA Isolation Protocols

DNA for the development of the PCR system was isolated in accordance with the procedure of Lewington *et al.* (Lewington *et al.*, 1987).

For the DNA isolation of single colonies, an ultrasonic lysis protocol was developed. One colony (2- to 3-mm diameter) was suspended in 100 µl lysis buffer (20 mM EDTA, 10 mM Tris [pH 7.9], 1% Triton X-100, 500 mM guanidine-HCl, 250 mM NaCl). Cells were lysed by 1 min of ultrasonication with the probe UP 50 H (Dr. Hielscher GmbH, Stahnsdorf, Germany). After the addition of 150 µl of cold (-20°C) ethanol the mixture was centrifuged over a spin column of the QIAamp tissue kit (Qiagen, Hilden, Germany) and finally eluted with 60 µl of buffer (10 mM Tris [pH 7,5]).

For isolation of bacterial DNA directly out of the sourdough a method based on enzymatic lysis was developed and evaluated. The sourdough sample of 5 ml was suspended in 10 ml of phosphate-buffered saline (PBS) (Sambrook *et al.*, 1989) and centrifuged for 5 min at 1,500 × g. Ten milliliters of the supernatant was transferred in a new tube and centrifuged for 15 min at 5,000 × g. The pellet was resuspended in 4 ml of PBS, and 1.5 ml of this suspension was transferred in an Eppendorf tube and centrifuged for 5 min at 5,000 × g to collect the cell material. The supernatant was discarded. The pellet was resuspended in 180 µl of TES (50 mM Tris-HCl, 50 mM NaCl, 10 mM EDTA [pH 8.0]) containing 20 mg of lysozyme ml^{-1}. For sufficient cell lysis it was incubated at 37°C for 40 min on a shaking platform (90 rpm). After the addition of 20 µl proteinase K and 200 µl lysis buffer AL (QIAamp tissue kit), the reaction mixture was incubated at 70°C for 30 min and for a further 30 min at 95°C. After the addition of 210 µl of cold ethanol (-20°C) it was centrifuged over a spin column (QIAamp Tissue Kit) and washed with 500 µl of the supplied buffers AW1 and AW2. The DNA was eluted with preheated (70°C) 10 mM Tris (pH 7.5) and stored at –20°C.

PCR Conditions

The amplification of the 16S rDNA was carried out in strips (Braun, Wertheim, Germany) on a Gradient master thermocycler (Eppendorff, Hamburg, Germany), in a total volume of 25 µl. Primers were obtained from Interactiva (Ulm, Germany). In order to use the three sets of reactions mixtures (a set consisted of 609R, 616V, and the specific probe for *L. pontis*, *L. panis*, or the *Lactobacillus* sp.) in parallel, the optimum annealing temperature was determined by gradient PCR between 40°C and 54°C with 1.1°C increments. The optimum annealing temperature was 46.5°C. The amplification conditions for the multiplex PCR assay with three primers were as follows: 0.5 µl of genomic DNA, 2.5 µl of 10x reaction buffer, 100 nM (each) deoxynucleoside triphosphate, 0.5 U *Taq* polymerase (Amersham Pharmacia Biotech, Piscataway, N.J.),

10 pmol of the specific primer and 616V (universal, Table 1), 2.5 pmol of primer 609R (universal, Table 1), 1% dimethyl sulfoxide, and deionized H_2O to a final volume of 25 µl. The amplification conditions were as follows: initial denaturation (94°C for 120 s) followed by 25 cycles of denaturation (94°C for 45 s), annealing (46.5°C for 60 s), and extension (72°C for 60 s). PCR products were electrophoretically separated on a 2% agarose gel and stained with ethidium bromide. As the size marker, a 100 bp ladder (Amersham Pharmacia Biotech) was used.

Results and Discussion

Figure 1 shows that it is possible to identify and discriminate between *L. pontis*, *L. panis* and the closely related *Lactobacillus* sp. with the primer combinations deduced from the variable V2 region of the 16S rDNA.

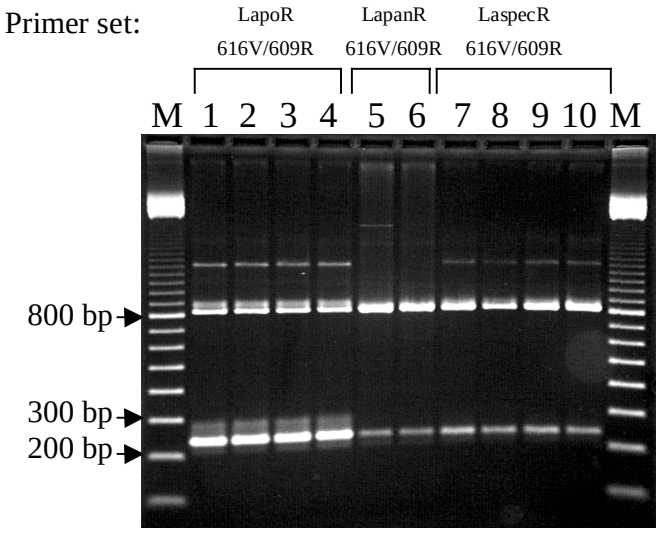

Figure 1. Multiplex PCR assay for the specific identification of *L. pontis*, *L. panis*, and the *Lactobacillus* sp. Primers indicated at the top are the specific primer for the identification of the following strains: *L. pontis* (LapoR) ATCC 51518 (lane 1), ATCC 51519 (lane 2). LTH 2587 (lane 3), and LTH 3572 (lane 4); *L. panis* (LapanR) DSM 6035 (lane 5) and DSM 6036 (lane 6); and *Lactobacillus* sp. strains (LaspecR) TMW 1.1098 (lane 7), TMW 1.1104 (lane 8), DSM 13145 (lane 9), and TMW 1.655 (lane 10). The specifically amplified fragment has a size of 236-bp (616V plus the specific reverse primer). The 800-bp fragment was amplified by the two universal primers 616V and 609R. Lane M, 100-bp ladder.

The primer combination 616V (universal) and the respective specific primers led to a 236-bp fragment for the three species. Primer 616V together with the universal reverse primer 609R allows the simultaneous amplification of a 800-bp fragment. In this way a combined specific identification in combination with a test on DNA accessibility was possible. So false-negative results could be avoided. The amplification of DNA isolated from strains of *L. pontis* and the *Lactobacillus* sp. led to an unspecific product

with 1,300 bp. We accepted this as a compromise solution in order to have the possibility to run all three identification reactions in parallel. The specificity of the PCR assay was tested on several lactobacilli (**Table 1**). No cross-reactions appeared.

To have a tool for a fast and reliable identification of single pure cultures, the already-evaluated PCR assay was combined with a fast DNA isolation procedure. Time-consuming enzymatic lysis procedures and variable susceptibility of bacteria to the lysozyme were overcome by ultrasonic treatment of the cells, with subsequent purification and concentration by binding DNA to a silica matrix. The cell material of a single colony was found to be sufficient for the PCR. **Figure 2** illustrates a typical result for the amplification of DNA of representative strains of the investigated sourdough lactobacilli.

Figure 2. 16S rDNA amplification of DNA isolated from a single colony of representative strains of the species *L. pontis* ATCC 51519 (lane 1), *L. panis* DSM 6035 (lane 2), and *Lactobacillus* sp. strain DSM 13145 (lane 3). The fragments were amplified with the universal primer 616V and the respecitive species-specific reverse primers. The 800-bp fragment was amplified by the two universal primers 616V and 609R. Lane M, 100-bp ladder.

The described PCR assay in combination with the fast-lysis procedure of even small amounts of cell material has major advantages over classical identification techniques, as physiological and biochemical identification is time-consuming and often not reliable. This is especially true for *L. pontis*, which exhibits a wide strain-dependent range of fermented carbohydrates, making a clear identification difficult. The fast ultrasonic lysis made it possible to identify this lactobacillus at the species level in 4 h. The application of this species-specific PCR technique for a rapid identification provides an attracting alternative to conventional methods.

For the evaluation of the applicability of multiplex PCR for monitoring the lactobacilli from a mixed culture without prior cultivation, laboratory-scale sourdough fermentation was set up. The fermentation was based on rye bran and was started with sourdough from the corresponding industrial process which had been fermented for 48 h at similar conditions. Previous microbiological investigations of this as well as of laboratory-scale processes by randomly amplified polymorphic DNA typing and 16S rDNA sequence analysis showed that strains of the *Lactobacillus* sp., *L. pontis*, and *L.actobacillus amylovorus* predominated in the flora (unpublished results). As shown in **Figure 3**, it was possible to specifically amplify DNA from *L. pontis* and the *Lactobacillus* sp.

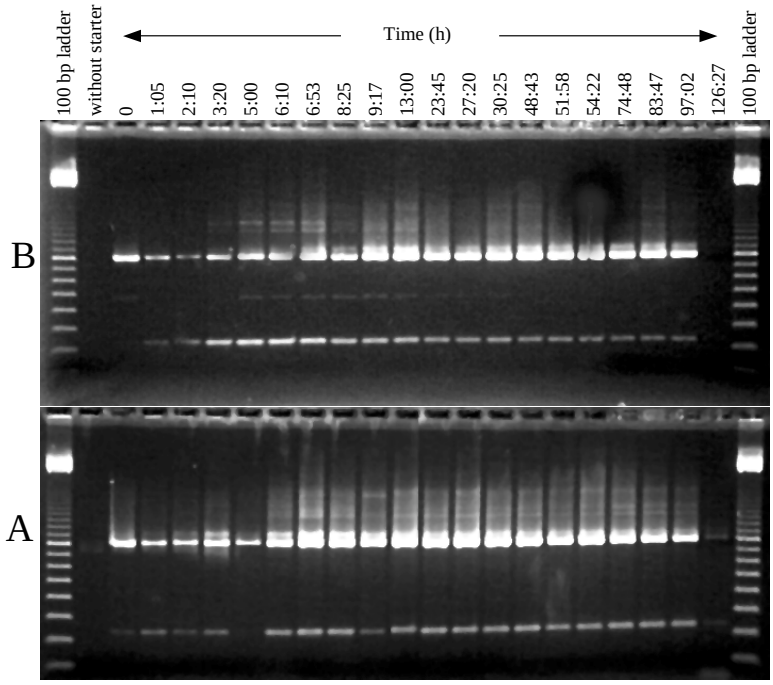

Figure 1. Specific amplification with primers for *L. pontis* (A) and the *Lactobacillus* sp. (B) of DNA extracted directly from the sourdough broth. The fermentation was monitored for over 126 h, starting from 0 h. At this time the starter was added. Without starter, sample before starter addition.

L. panis could not be identified in this fermentation, which resulted in the 800-bp fragment of the universal primers 616V and 609R only (a picture of the gel is not shown). Samples before starter addition showed no specific signal in both cases, even the universal primers targeting any bacterial DNA gave almost no signal (A) or no signal (B). This was confirmed by the classical microbiological investigation of plating on plate count agar, where a total cell count of 10 to 100 CFU was determined. On mMRS no LAB growth could be determined before starter was added. Directly after inoculation the total cell count was 9.3×10^6 CFU ml sourdough^{-1} , reaching its maximum of 3.2×10^9 CFU ml sourough^{-1} after 24 h and dropping to a final cell count of 5.5×10^7 CFU ml sourdough^{-1} after 126 h. At this time for both primer combinations a signal with the two universal primers could be obtained, but only for *L. pontis* could a weak signal be recognized. During the fermentation the intensities of signals for both the universally amplified product and the specific fragment increased, becoming lower after 54 h and disappearing completely for *Lactobacillus* sp. after 126 h. The total cell count at the end of the fermentation seems to be a detection limit for directly extracted DNA from sourdough with this method. This limit seems to be high in comparison to those from other investigations in this field. For example, Zapparoli & Torriani (1997) could amplify 10^2 CFU ml of diluted sourdough^{-1}. Nevertheless the aim of this study was not to detect such small amounts but to have a fast and easy tool for detecting dominant lactobacilli from such fermentations. The direct isolation of total bacterial community DNA with a subsequent specific PCR can be considered a valuable tool for monitoring these lactobacilli in mixed microbial populations.

Moreover, no genotypic method to identify *L. panis* was available. Only a combined identification with *L. pontis*, not discrimination between these two species, was possible. With the presented specific PCR it could be demonstrated that it is possible to differentiate between these closely related species.

The *Lactobacillus* sp., a dominant element in the investigated fermentation, could be clearly discriminated from phylogenetically related species by genotypic

identification techniques. To clarify the definitive phylogenetic position, further investigations are being prepared.

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CHAPTER III

LACTOBACILLUS FRUMENTI SP. NOV., A NEW LACTIC ACID BACTERIUM ISOLATED FROM RYE-BRAN FERMENTATIONS WITH A LONG FERMENTATION PERIOD

Summary

Within the framework of the characterization of the microflora of an industrial sourdough fermentation, strains of *Lactobacillus amylovorus*, *L. pontis* and two other strains were isolated which could not be associated with a valid species. These latter strains were Gram-positive, catalase-negative, non-spore-forming, non-motile rods that could be clearly differentiated from known species by 16S rDNA sequence analysis.

For further characterization, the morphological, physiological (sugar fermentation, formation of D/L-lactate, hydrolysis of arginine, growth temperature, CO_2 production) and chemotaxonomic (G+C content, cell wall composition, SDS-PAGE of whole-cell proteins) properties have been determined.

Fitting of the complete 16S rDNA sequence into alignments of 16.000 of such sequences, together with the subsequent phylogenetic calculations, allowed the reconstruction of a phylogenetic tree. These data showed that the two strains were phylogenetically related but formed an independent cluster distinct from their closest neighbours, *L. pontis*, *Lactobacillus panis*, *Lactobacillus oris*, *Lactobacillus vaginalis* and *Lactobacillus reuteri*.

The results of DNA-DNA hybridization experiments indicated that the new two isolates represent a new *Lactobacillus* species, for which the name *Lactobacillus frumenti* is *proposed;* the type strain of this species is DSM 13145^T (= LMG 19473).

Introduction

Lactic acid bacteria (LAB), especially the genera of *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Lactobacillus,* play an important role in the fermentative food and feed production (Herrero *et al.*, 1996). In non-lactic fermentations, lactobacilli, in particular, contribute to a huge variety of spontaneously fermented indigenous foods and beverages as well as to biotechnological processes carried out under controlled conditions.

The fermentation of starchy substrate can be found in many countries. Numerous reports have elucidated the microbial populations in such fermentations, e.g. fermentation of sour cassava (Figueroa *et al.*, 1995, Giraud *et al.*, 1998, Morlon-Gyot *et al.* 1998), ogi (Johansson *et al.*, 1995), pozol (Ampe, 1999) and kishra (Hamad *et al.*, 1997). In Europe, cereal fermentations are mainly applied to the brewing industry, providing sourmashes, and to baking, in which sourdough plays an important role in the preparation of bread dough to improve dough machinability, breadcrumb structure, keeping properties and flavour (Salovaara, 1998). The microbial ecology of such cereal fermentations is determined by several exogenous and endogenous parameters, as reported by Vogel *et al.* (1996). Whilst a wide variety of LAB, e.g. *Lactobacillius brevis*, *Lactobacillus alimentarius*, *Lactobacillus farciminis*, *Weissella confusa or Enterococcus faecium* can be found in spontaneous homemade sourdough fermentations, *Lactobacillus sanfranciscensis*, *Lactobacillus pontis* and *Lactobacillus panis* are endemic in cereal fermentations, because their competitive metabolism has adapted to this environment. Böcker *et al.* (1995) introduced a classification of industrial sourdough fermentations which takes the kind of propagation and the manner of preparation into consideration, resulting in typical bacterial communities. Whilst *L. sanfranciscensis* constitutes a stable element (Böcker *et al.*, 1990) in the so-called type I doughs, micro-organisms found in liquid doughs with an extended fermentation period and higher temperatures (type II) belong to the species *L. pontis*,

L. panis, *Lactobacillus reuteri*, *Lactobacillus fermentum* and *Lactobacillus amylovorus* (Vogel *et al.*, 1999).

In this paper we report the description of a new *Lactobacillus* species from an industrial type II rye-bran fermentation and for which we propose the name *Lactobacillus frumenti*.

Methods

Strains, Medium and Culture Conditions

Strains were isolated from an industrial rye-bran sourdough fermentation. For cultivation, the modified MRS (mMRS) medium described by Vogel *et al.*, (1994) was prepared with an additional modification whereby 90% of the water was replaced with a rye-bran extract. For the preparation of 1 l of this extract, 40 g rye bran and 2 g malted wheat meal together with 0.8 g trypsin were incubated for 24 h at 50 °C. The liquid was separated by filtration. Strains of *L. frumenti* were at 40 °C in line with the fermentation temperature of the sourdough from which they were isolated. The other strains were incubated at the temperature recommended by the respective strain collection. Solid media were incubated under a modified atmosphere (N_2 : CO_2 90% : 10%, v/v). Cultures were maintained at –80°C in glycerol (a pellet of 10 ml overnight culture in 500 µl fresh medium to 500 µl glycerol). The purity of the cultures was checked microscopically and by preparing streak cultures. The two isolates that are described here as the new species *L. frumenti* sp. nov. bear the numbers of the strain collection of the Technische Mikrobiologie Weihenstephan, i.e. TMW 1.655 and TMW 1.666. Strain TMW 1.666 was deposited as type strain of the species as LMG 19473^T and DSM 13145^T, and will be referred to as the latter in this paper. The following type strains were used as reference organisms: *Lactobacillus vaginalis* DSM 5837^T, *L. pontis* DSM 8475^T, *L. panis* DSM 6035^T, *L. reuteri* DSM 20016^T, *L. oris* DSM 4864^T, *Lactobacillus. buchneri* DSM 20057^T.

Physiological characterization

Sugar-fermentation patterns were determined by using a microtitre plate assay. The reproducibility was verified by repeated analysis using cultures grown on mMRS. All sugar solutions (2% w/w) were sterilised by filtration through a 0.2 µm filter. Cells were suspended in a medium containing (per 250 ml) 5 g peptone from casein, 1 g of

meat extract, 3.5 g yeast extract, 125 mg cysteine, 250 µl Tween 80, 100 mg bromcresol purple, 2.5 g sodium acetate, 250 mg citric acid (ammonium salt), 62.5 mg KH_2PO_4, 125 mg Na_2HPO_4, 100 mg $MgSO_4$, 5 mg $MnSO_4$ and 5 mg $FeSO_4$. The solution was autoclaved for 15 min at 121°C. A 2 ml of an overnight culture of each strain was centrifuged and washed three times with 2 ml PBS buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, made to 1 l with dH_2O, pH 7.4). The cell concentration was adjusted with water to an OD_{578} of 1; 45 µl of this cell suspension was added to 1155 µl test medium. From this cell suspension, aliquots of 40 µl were added to each of the wells. In the first well, sugar was replaced with sterile water. To avoid evaporation and to guarantee anaerobic conditions, the wells were covered with paraffin. The change of the indicator from purple to yellow was documented after 24, 48 and 168 h. Only definitive changes were rated as positive results. To evaluate the system, additional experiments were carried out using the API 50 CH kit (bioMérieux).

The formation of the lactate isomeres in the fermented broth was determined enzymically using the DL-lactate test kit (Boehringer Mannheim).

Arginine hydrolysis was determined according the methods described by Sharpe (1979).

Morphological Characteristics

Cell morphology was studied using phase-contrast microscopy. Gram-determinations were performed using the KOH method of Gregersen (1978).

Whole-Cell Protein Analysis

For the preparation of cell extracts, 70-80 mg (wet weight) of bacterial cells was treated as described by Vogel *et al.* (1994). Electrophoresis was performed as described by Laemmli (1970), using a 12% (v/v) SDS-polyacrylamide separating gel. Gels were run in a Mini-Protean II electrophoresis cell (BioRad), coomassie-stained and dried on a vacuum gel dryer. The protein patterns were compared visually. The

LMW electrophoresis calibration kit (Amersham Pharmacia Biotech) was used as a size maker.

DNA Base Composition

The G + C contents (mol%) of the DNA of strains TMW 1.655 and DSM 13145^T were determined using an HPLC analytical method. The experiments were performed by the DSMZ and were carried out using the protocol previously described by Tamaoka & Komagata (1984). Wild-type lambda phage DNA was used as the standard (Meshbah *et al.*, 1989). The G + C content (mol%) was determined according to Meshbah *et al.* (1989).

Cell Wall

The peptidoglycan structure of the cell wall was determined by the DSMZ.

DNA Isolation

DNA was isolated according a protocol described by Marmur (1961), with some modifications. One hour before cells were harvested, penicillin G (Sigma) was added to inhibit the synthesis of cross-linkages of the cell wall and therefore to facilitate the lysis. A wet weight of 70 mg cells was used for the DNA isolation. After the protocol, lysis was completed after 45-90 min after the addition of lysozyme and mutanolysine. For some strains, more effective lysis was obtained by using an overnight lysis at 4°C and a subsequent proteinase K treatment at 60°C for 1 h, before continuation with the normal protocol. The purified and vacuum-dried DNA was dissolved in 2 x SSC (0.3 M NaCl, 0.03 M Na_3 citrate 2 H_2O, pH 7.0). This DNA preparation served for the DNA-DNA hybridization experiments as well as for 16S rDNA amplification. The purity of the culture subjected to DNA isolation was proved as described above before the addition of penicillin. Furthermore, DNA preparations were checked for their

authenticity in a randomly amplified polymorphic DNA (RAPD)-PCR assay (Paramithiotis *et al.*, 2000), comparing the patterns with those of primary preparations.

DNA-DNA Hybridization

The determination of DNA homology values was carried out by using chromosomal DNA (chrDNA) from strain DSM 13145^T and from *L. pontis* DSM 8475^{T}as probes. The chrDNA probes were labelled by nick translation using a kit system (Promega) incorporating biotin-21-dUTP (Clontech) for 2h at 16°C. The DNA (5 µg) of selected type strains was transferred by using a dot-blot block (Stratagene) on a positively charged nylon membrane (Boehringer Mannheim) and then fixed by incubation at 80°C for 1 h. The DNA of *L. buchneri* DSM 20054^T was included to check the sensitivity of the system for a more distantly related *Lactobacillus* species. The hybridization procedure was carried out as described by Ehrmann *et al.* (1994), except for the hybridization and washing steps, which were performed with alternative buffers described in the protocol of Engler-Blum *et al.* (1993). To determine the accessibility of the DNA, a parallel hybridization was carried out with a 5‘ biotin-labelled universal probe (612R, 5´-GTAAGGTTYTNCGCGT-3´) targeting the 16S rDNA. For the hybridization, 100 ng chrDNA probe 20 pmol oligo probe (each per ml hybridization solution) were used. Hybridization was performed at 65°C for the chrDNA probe and 42°C for the oligo probe. The detection system consisted of a combination of alkaline phosphatase and the substrate CDP-Star (Boehriner Mannheim). Membranes were exposed to a Kodak X-Omat film (Sigma-Aldrich). The spot intensity was calculated with the IMAGE MASTER 2D Elite software (Amersham Pharmacia Biotech). Calculations of homology values was performed in principal as described by Liebl *et al.* (1991).

Additional DNA-DNA hybridization experiments for *L. frumenti* DSM 13145^T, *L. frumenti* TMW 1.655 and *L. vaginalis* DSM 5837^T were performed by the DSMZ according to the protocol of De Ley *et al.* (1970), but with the modifications described

by Huß *et al.* (1983) and Escara & Hutton (1980). The renaturation rates were calculated according to Jahnke (1992) with the program TRANSFER : BAS.

16S rDNA Amplification and Sequencing

PCR-mediated amplification of the complete 16S rDNA was carried out in a Gradient Master thermocycler (Eppendorf). All reagents were from Amersham Pharmacia Biotech, unless otherwise indicated. The amplification conditions were as follows: 1 µl genomic DNA, 10 µl 10x reaction buffer, 200 nM each of the four deoxynucleotides, 1.5 U *Taq* polymerase, 20 pmol each primer (Interactiva) (616V, 5‘-AGAGTTTGATYMTGGCTCAG-3‘; 630R, 5‘-CAKAAAGGAGGTGATCC-3‘) and dH_2O to a final volume of 100 µl. The amplification conditions were as follows: 94 °C for 2 min; 30 cycles of 94 °C for 45 s, 52°C for 1 min, 72 °C for 30 s; 94 °C for 1 min, 72 °C for 4 min. PCR products were purified by the QIAquick PCR purification kit (Quiagen) and were eluted with 60 µl elution buffer. DNA sequences were determined by the chain-termination method (Sanger *et al.*, 1977) using the Thermo Sequenase fluorescence-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Life Science) and separated on an ALF express sequencer (Amersham Pharmacia Biotech) or, alternatively, using the ABI Prism Dye Terminator Cycle Sequencing Kit (Perkin Elmer) on an ABI 373 stretch sequencing system by a commercial service (SequiServe, Germany). For sequencing, the amplification primer 616V together with the internal primers 609R [5’-ACT AC(CT) (AGC)GG GTA TCT AA(GT) CC-3’], 612R [5’-GTA AGG TT(CT) T(AGCT)C GCG T-3’], 607R [5’-ACG TGT GTA GCC C-3’], 606R [5’-T(AG)A CGG (GC)C(AG) GTG TGT ACA-3’] and 607V [5’-GGG CTA CAC ACG TGC-3’] were used.

Phylogenetic Analysis

The complete 16S rDNA sequences of *L. frumenti* DSM 13145^T and TMW 1.655 were fitted into alignments of approximately 16000 homologous full and partial primary structures available in public databases (Ludwig, 1995). Distance-matrix, maximum-parsimony and maximum-likelihood methods were applied for tree reconstruction as implemented in the ARB software package (W. Ludwig & O. Strunk; http://www.mikro.biologie.tu-muenchen.de/pub/ARB/documentation). Different data-sets varying with respect to included outgroup reference sequences as well as alignment positions were analysed.

Results

Colony and Cell Morphology

On mMRS, the colonies of *L. frumenti* DSM 13145^T were white with regular sharp edges and after 3 d growth, they were 1 mm in diameter. The colonies of TMW 1.655 were of a similar shape but were a little smaller and appeared whitish and transparent.

The cells of strains TMW 1.655 and *L. frumenti* DSM 13145^T were non-spore-forming, non-motile rods, that occurred singly or in pairs (seldom in chains). In liquid cultures of mMRS after 24 h at 40 °C, cells of TMW 1.655 had a tendency to form nest-shaped aggregations. Depending on the period of growth, the ends of the cells were bent like hooks. The cells of *L. frumenti* DSM 13145^T were more regular in shape than those of TMW 1.655. The size of a single cell was difficult to determine, because of the heterogeneity. The cell length was between 3 and 5 µm and the diameter was 0.3 µm. The KOH test indicated a Gram-positive behaviour.

Physiological and Biochemical Properties

The sugar patterns and further physiological and biochemical characteristics of *L. frumenti* strains and the reference organisms are listed in **Table 1**.

Melezitose, rhamnose, methyl α-D-mannoside and L-arabinose were fermented only by strain *L. frumenti* DSM 13145^T. 5-ketogluconate was fermented only by strain TMW 1.655.

Table 1. Physiological and biochemical characteristics of *L. ceralis* and selected reference organisms

−, Negative Reaction; +, positive reaction; ND, not determined. All strains contain DL-lactate, produce CO_2 from glucose, and produce acid from glucose, maltose and sucrose.

Characteristic	*L. frumenti* DSM 13145^T	*L. frumenti* TMW 1.655	*L. panis* DSM 6035^{T*}	*L. pontis* DSM 8475^T†	*L. vaginalis* DSM 5837^T‡	*L. oris* DSM 4864^T§	*L. reuteri* DSM 20016^T‖
NH_3 from arginine	+	+	–	+	+	–	+
Growth at 15°C/45°C	+/–	+/–	+/–	+/+	+/–	+/–	+/–
Peptidoglycan type	L-Lys-D-Asp	L-Lys-D-Asp	L-Lys-D-Asp	Orn-D-Asp	Orn-D-Asp	L-Lys-D-Asp	L-Lys-D-Asp
DNA G + C content (mol%)	48.3 ± 0.2¶	44.4 ± 0.3¶	48.3 ± 0.3¶	53-56¶	38-41**	49-51**	40-42**
Acid production from:							
DL-Arabinose	+/–	–/–	+/–	–/–	–	+/–	ND/+
Ribose	+	+	+	+	–	+	+
DL-Xylose	–	–	+	–	–	+/-	–/ND
Glucose	+	+	+	–	+	+	+
Fructose	+	+	+	+	ND	+	+
Mannose	+	+	+	–	ND	+	–
Mannitol	+	+	–	–	–	–	–
Sorbitol	+	+	ND	–	–	–	–
N-Acetylglucosamine	+	+	–	–	–	–	–
Amygdalin	+	+	–	–	ND	+	–
Arbutin	+	+	–	–	–	+	–
Aesculin	+	+	+	–	–	–	–
Salicin	+	+	+	–	–	–	–
Cellobiose	+	+	–	–	–	+	–
Lactose	+	+	+	–	+	+	+
Melibiose	+	+	+	–	+	+	+
Trehalose	+	+	–	–	–	+	–
Melezitose	+	–	–	–	–	–	–
Raffinose	+	+	+	–	+	+	+
Gluconate	+	+	–	–	–	+	+

* Data are from Wiese *et al*. (1996).
† Data are from Vogel *et al*. (1994); acid production from sugars was tested in this study.
‡ Data from Embley *et al*. (1989).
§ Data from Farrow & Collins (1988)
‖ Data from Kandler *et al*. (1980) and Axelsson & Lindgren (1987).
¶ Determined by the HPLC method.
** Determined by the thermal denaturation method.

SDS-PAGE Pattern

Both strains of *L. frumenti* were included in a comparison of SDS-PAGE pattern of the whole-cell proteins together with those of the type strains of the closest phylogenetic neighbours **(Figure 1).** The protein patterns of *L. frumenti* DSM 13145^T and TMW 1.655 were very similar to each other and they could be clearly discriminated from the others.

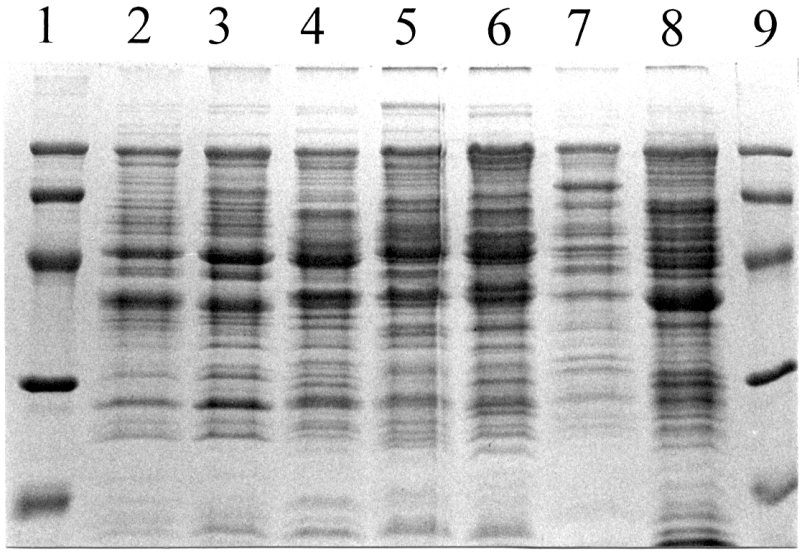

Figure 1. SDS-PAGE protein patterns. Lanes: 2, *L. frumenti* DSM 13145^T; 3, strain TMW 1.655; 4, *L. pontis* DSM 8475^T; 5, *L. panis* DSM 6035^T; 6, *L. reuteri* DSM 20016^T; 7, *L. oris* DSM 4864^T ; and 8, *L. vaginalis* DSM 5837^T. Lanes 1 and 9: molecular mass standards (94, 67, 43, 30, 20 kDa).

Phylogenetic Position

For both strain TMW 1.655 and *L. frumenti* DSM 13145^T, 1561 bp of the 16S rDNA were sequenced. **Fig. 2** shows a phylogenetic tree based on these sequence data and reflects the phylogenetic position of *L. frumenti*.

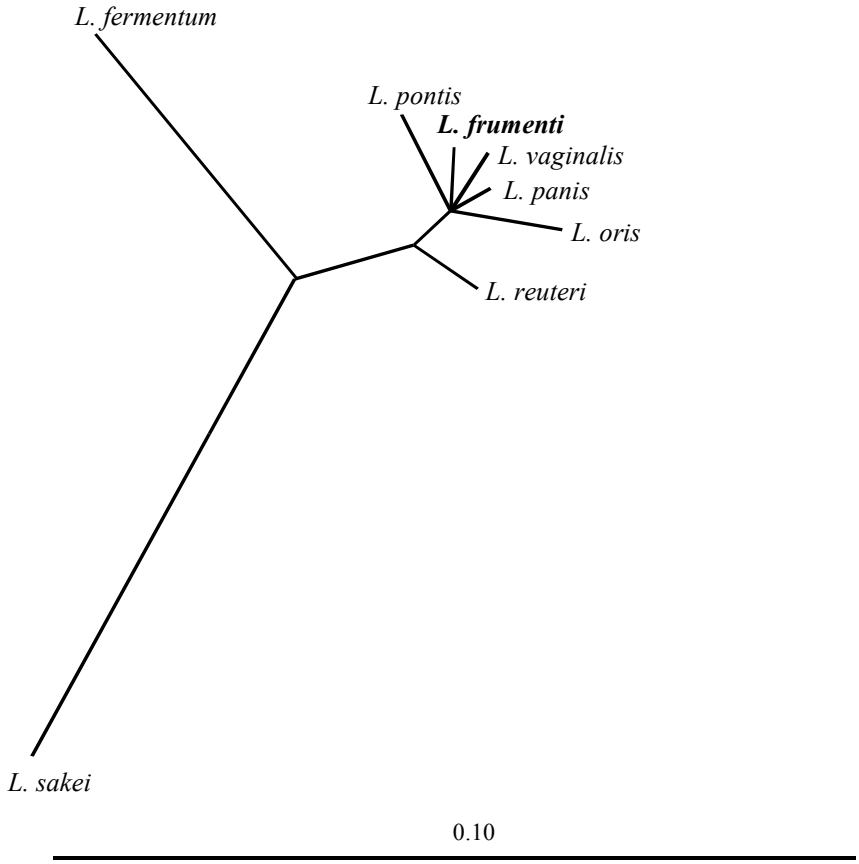

Figure 2. Phylogenetic tree demonstrating the relationship between *L. frumenti* DSM 13145^T and the most closely related lactobacilli. The tree was constructed by using a maximum-likelihood approach and was based on a data-set that included only positions present in at least 50% of all available *Lactobacillus* 16S rRNA sequences. Bar, 10% homology divergence.

The percentage similarities between *L. frumenti* and the type strains of the other species were as follows: *L. vaginalis,* 97.7%; *L. panis,* 97.6%; *L. pontis*, 97.3%; *L. oris,* 96.8%; *L. reuteri*, 94.3%. The 16S rRNA sequence of *Lactobacillus sakei* was used as an outgroup rRNA reference.

DNA Homology

The DNA-DNA hybridization studies were performed with chrDNA from *L. frumenti* DSM 13145^T against the DNA of the relevant type strains exhibiting a strong phylogenetic relationship to *L. frumenti*. To evaluate the hybridization system for the calculation of DNA-DNA homology values, DNA from *L. pontis* DSM 8475^T served as a probe against the same strains. Because of the high homology values between the *L. frumenti* strains and *L. vaginalis*, both strains were included in an additional spectrophotometrical DNA-DNA hybridization experiment against the latter to verify their independent position as a new species **(Table 2)**.

Table 2. Percentage DNA-DNA hybridization results

Strain	***L. frumenti* DSM 13145^T**	***L. pontis* DSM 8475^T**	***L. vaginalis* DSM 5837^T**
L. pontis DSM 8475^T	48	100	ND
L. panis DSM 6035^T	49	43	ND.
L. oris DSM 4864^T	66	29	ND.
L. vaginalis DSM 5837^T	74	21	ND.
L. reuteri DSM 20016^T	49	20	ND.
L. buchneri DSM 20057^T	27	12	ND.
L. frumenti DSM 13145^T	100	46	41*
L. frumenti TMW 1.655	90	33	45*

* Data were obtained by the spectrophotometric method.

Discussion

Reliable identification techniques are fundamental to the unequivocal description of natural fermentation communities. Although methods that target the phentotypes of bacteria lead to an understanding of the physiological properties, for an increasing number of species, identification remains ambiguous. The 16S rDNA molecule emerged as the main target for identification purposes, reflecting the natural relationship of prokaryotes, and has improved our knowledge on the generic and suprageneric relationship among LAB. Within the framework of microbial investigations of long-term rye-bran fermentations, we isolated *Lactobacillus* strains which were identified by comparative 16S rDNA sequence analysis. They were phylogenetically most closely related to *L. vaginalis,* but formed an independent cluster. On the basis of comparative sequence analysis and phylogenetic calculations, Schleifer & Ludwig (1995) proposed a species-specific grouping of LAB. Thus, *L. frumenti* strains clustered in the *L. reuteri* subgroup of the *Lactobacillus casei-Pediococcus* group, comprising only obligate heterofermentative lactobacilli, namely *L. oris*, *L. panis*, *L. pontis*, *L. vaginalis*, *L. reuteri* and *L. fermentum*. Starting from these 16S rDNA data and comparative sequence analyis, sufficient sequence variation in the V2 region (Neefs *et al.*, 1990) of the 16S rDNA was feasible, which offered the possibility of distinguishing *L. frumenti* from other even closely related species **(Figure 3)**.

	193																208	
L. frumenti	5‘	U	A	G	U	A	U	A	A	A	A	G	A	A	G	G	C	U 3‘
L. vaginalis	5‘	.	C	.	.	U	.	C	.	.	.	.	.	U	.	.	U	. 3‘
L. oris	5‘	.	C	C	A	.	.	.	.	.	.	A	G	.	.	.	U	. 3‘
L. panis	5‘	.	C	.	.	.	.	.	.	.	.	.	.	U	.	.	U	. 3‘
L. pontis	5‘	.	G	A	.	U	.	C	.	.	.	.	.	U	.	.	.	. 3‘
L. reuteri	5‘	.	U	C	.	n	G	G	.	.	.	.	.	U	.	.	.	. 3‘

Figure 3. Diagnostic region 193-208 (Brosius *et al.*, 1981) of the 16S rRNA, which shows sufficient sequence variability to differentiate *L. frumenti* from other lactic acid bacteria. Dots indicate sequence identity to the *L. frumenti* sequence.

A PCR-based system that allows *L. frumenti* to be differentiated from these and other sourdough lactobacilli has already been described by Müller *et. al.* (2000). This supports the hypothesis that strains DSM 13145^T and TMW 1.655 should be separated from other members of the *L. reuteri* group.

Despite the close phylogenetic relationships of species within the *L. reuteri* group, G + C contents range between 38 and 54 mol%. The closest phylogenetic neighbor of *L. frumenti* (with a 16S rDNA similarity of 97.7%) is *L. vaginalis*. This relationship is confirmed by their similar G + C content (43.3 versus 41 mol%). The biggest difference in the G + C contents was shown with *L. pontis.* In this case, no correlation with 16S rRNA homology data can be deduced.

The relatedness of *L. frumenti* and *L. vaginalis* with respect to G + C content and 16S rRNA sequence data contrasts with the chemical composition of the cell walls of these micro-organisms. *L. frumenti* has a cell wall of the peptidoglycan type [A4α (L-Lys-D-Asp)], which constitutes the main feature of species of the Cb group (Hammes & Vogel, 1995). The cell wall of *L. vaginalis* is in the same grouop but lysine is replaced by ornithine, which can be seen as additional proof of the status of *L. frumenti* as an independent species.

SDS-PAGE pattern analysis of whole-cell proteins has proven to be a reliable tool for the discrimination of even closely related species (Pot *et. al.,* 1993). SDS-PAGE comparisons (in which the two strains of *L. frumenti* exhibited almost identical patterns) allowed *L. frumenti* DSM 13145^T and TMW 1.655 to be clearly differentiated from the species *L. pontis*, *L. panis*, *L. oris*, *L. vaginalis* and *L. reuteri*. This, again can be seen as a further proof of the independent status of *L. frumenti* as new species.

The determination of DNA reassociation values constitutes a meaningful method for unequivocal species description (Stackebrandt & Goebel, 1994). In particular, when closely related species (≥ 97% rRNA homology) are inspected, the resolution power of 16S rRNA sequences is limited (Fox *et al.*, 1992), but DNA similarity can range between 10 and 100% (Stackebrandt & Goebel, 1994). The threshold value for a phylogenetic definition of a species, as proposed by Wayne *et al.* (1987), should not

exceed 70%. In our case, the DNA-DNA hybridization studies clearly adhered to this principle. For *L. vaginalis* and *L. pontis,* rRNA similarity values with respect to *L. frumenti* were 97.7 and 97.3%, respectively. However, DNA-DNA homology values between *L. frumenti* and *L. vaginalis* and between *L. frumenti* and *L. pontis* amounted to 74 and 48%, respectively. The spectrophotometric technique used as reference method for the determination of homology values for the closest relative, *L. vaginalis*, produced lower values between *L. vaginalis* and TMW 1.655 or DSM 13145^T (41 and 45%, respectively). The homology values obtained by dot-blot hybridization of *L. frumenti* against the other species were confirmed by alternative hybridization with the DNA of *L. pontis*. The value for similarity to *L. frumenti* (46%) was almost identical to that determined by hybridization of *L. frumenti* against *L. pontis* (48%). Moreover, the similarity calculated between *L. pontis* and *L. panis* was identical (43%) to the value determined by the spectrophotometric technique (Wiese *et al.*, 1996). Summarizing the DNA hybridization results (**Table 2**), all species could be clearly separated from *L. frumenti*, indicating its independent status as a species.

In view of the phylogenetic evidence (16S rDNA, DNA-DNA homology) presented, the protein-pattern differences measured by SDS-PAGE and the phenotypic distinctiveness of DSM 13145^T and TMW 1.655, it is proposed that these strains belong to a new species, *L. frumenti* sp. nov..

Description of *Lactobacillus frumenti* sp. nov. (Müller, Ehrmann and Vogel)

Lactobacillus frumenti (fru.men'ti. L. gen. N. *frumenti* from cereal).

The cells are Gram-positive, non-motile, non-spore-forming rods that occur singly or in pairs (seldom in chains). Growth was observed up to 45 °C but not at 15 °C. They are facultatively anaerobic, catalase-negative and obligately heterofermentative. The L-isomer constitutes more than 85% of the total lactic acid content. Acid is produced from L-arabinose, ribose, galactose, glucose, fructose, mannose, mannitol, sorbitol, N-acetylglucosamine, amygdalin, arbutin, aesculin (hydrolysed), salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, raffinose and gluconate. Melezitose, rhamnose, methyl α-D-mannoside and L-arabinose were fermented only by strain DSM 13145^T. 5-Ketogluconate was fermented only by strain TMW 1.655. The type strain is *Lactobacillus frumenti* DSM 13145^T (= LMG 19473^T).

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CHAPTER IV

POLYPHASIC IDENTIFICATION OF WILD YEAST STRAINS ISOLATED FROM GREEK SOURDOUGHS

Summary

A total of forty-five wild yeast strains were isolated from five traditional Greek sourdoughs. Strains were identified using the classical identification technique along with the sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole cell proteins (SDS-PAGE), Fourier transform-infrared spectroscopy (FT-IR) and the randomly amplified polymorphic DNA-polymerase chain reaction analysis (RAPD-PCR). The latter methods confirmed the classical identification. According to the results obtained, 14 strains were identified as *Saccharomyces cerevisiae* strains, twenty five as *Pichia membranaefaciens* strains and six as *Yarrowia lipolytica.*

Introduction

Yeasts are one of the most important groups of microorganisms related with human nutrition. They have been used for millennia and they are still used in bread making and in the production of alcoholic beverages. Furthermore, several yeast species such as *Candida albicans* and *Cryptococcus neoformans* are of medical importance, while many species like *Zygosaccharomyces bailii*, *Pichia membranaefaciens* and *Saccharomyces cerevisiae* may cause spoilage in a wide range of foods (Deak & Beuchat, 1996; Kockova-Kratochvilova, 1990).

In bread making many different functional properties have been defined for yeasts. Leavening is their main role (Sugihara *et al.*, 1971) and the production of either primary or secondary metabolites, such as alcohols, esters and carbonyl compounds, is of major importance for the formation of the characteristic bread flavor (Martinez-Anaya *et al.*, 1990; Collar *et al.*, 1994; Hansen & Hansen, 1994; Damiani *et al.*, 1996). Through their enzymatic activities, mainly promoted by amylolytic, proteolytic and lipolytic enzymes, yeasts can affect not only the organoleptic characteristics, but the overall appearance of the final product as well (Martinez-Anaya, 1996; Collar *et al.*, 1994; Antuna & Martinez-Anaya, 1993).

Recent studies on cereal fermentations mainly focused on the characterization of sourdough fermentations based on wheat or rye flour as raw materials. Basically, three types of sourdough fermentations can be distinguished (Böcker *et al.*, 1995) (i) type I doughs sustained by repeated inoculation at ambient temperature (20-30°C) according to traditional procedures, (ii) type II doughs using elevated temperatures, longer fermentation times and higher water content, mostly used in industrialized processes to produce bakery pre-products, and (iii) type III processes initiated by defined starter cultures. Predominance of certain microorganisms, as far as spontaneous cereal fermentations are concerned, is decided by the degree od adaptation of the microorganisms to the respective environment. As a general rule, lactic acid bacteria

are the predominant microorganisms and in many cases yeasts are present in significant numbers (Vogel *et al.*, 1999).

It is generally accepted that the use of sourdough in bread making yields in products of high quality in terms of sensory characteristics and shelf-life. Furthermore the sourdough bread preparation process is technologically essential for baking whole rye meal bread to improve dough machinability, bread crumb properties and flavor (Salovaara, 1998). This is due to the synergistic effect of yeasts and lactic acid bacteria (LAB) present in this product (Roecken & Voysey, 1995; Linko *et al.*, 1997; Rosenquist & Hansen, 1998). According to this, isolation, identification and study of the biochemical and technological features of these microorganisms is of great technological importance. The detailed study of sourdough microflora can contribute to the design and production of starter cultures for industrial scale applications.

Nowadays, a great number of identification techniques are available. Beside the classical phenotypic methods relying on morphological, physiological and biochemical criteria, investigations with chemotaxonomic methods (SDS-PAGE of whole cell proteins, fatty acid analysis, FT-IR spectroscopy) and genotypic methods (electrophoretic karyotyping, (PCR)-RFLP, AFLP, rRNA analysis, RAPD) are becoming more and more applied in the field of food microbiology for characterization and identification of yeasts (Baleiras Couto *et al.*, 1994; Baleiras Couto *et al.*, 1995; Kosse *et al.*, 1997; Noronha-da-Costa *et al.*, 1996; Romano *et al.*, 1996; Kümmerle *et al.*, 1998; Vancanneyt *et al.*, 1991; Mäntynen *et al.*, 1999).

We have chosen a polyphasic approach for the characterization of sourdough yeast, combining FT-IR spectroscopy together with the PCR-based molecular technique RAPD fingerprinting and the conventional biochemical/morphological characterization.

Traditional identification rely upon several physiological and biochemical tests. The mode of vegetative and sexual reproduction along with the ability to ferment sugars and aerobically growth on various carbon and nitrogen compounds together with growth under stressing conditions are important in identification. Although this

procedure is time-consuming and laborious and only leads to species level identification, is still regarded as the reference one.

SDS-PAGE of whole cell proteins has been successfully used in bacterial and yeast identification (Vancanneyt *et al.*, 1991). It is a valuable criterion for classifying microorganisms at the species level, when reliable reference patterns are available, because of its high levels of sensitivity and reproducibility (Vancanneyt *et al.*, 1991).

FT-IR spectroscopy originally used in chemical analysis for the identification of substances has been applied for the first time to identify microorganisms (Naumann *et al.*, 1994). In principle a FT-IR spectrum of the whole cell is compared with a reference spectrum library. Very recently Kümmerle (1998) demonstrated the relevance of this technique for food microbiology. A reference database for yeasts with about 900 spectra has been built up by which food-borne yeasts have been successfully identified.

RAPD, first introduced by Williams *et al.* (1990), relies on the amplification of fragments with only a single short primer present. In order to allow annealing of the primer to the target DNA, the annealing step of the reaction is run under low stringency conditions. Together with a random, non-specific primer sequence species up to strain specific fingerprints are generated. Since these days the RAPD technique was applied to many problems both in fungal and bacterial microbiology mainly in the characterization of complex habitats or the differentiation of isolates (Tompkins *et al.*, 1996; Laidlaw *et al.*, 1996). The reason of the integration of different kinds of data and information leading to a consensus type of taxonomy (Vandamme *et al.*, 1996) was to overcome the limits of each single method and to improve the reliability of the whole expression.

Greek traditional wheat sourdoughs belong to type I. Up to now there is no data concerning chemical and microbiological characteristics of traditional Greek sourdoughs. One of the most common traditional Greek wheat sourdoughs is based on mixing flour and tap water (40-50°C) in the presence of basil (*Ocimum basilicum*), an aromatic plant closely related to the country's tradition and religion. The dough is

subjected to fermentation for one day at ambient temperature (25-35°C). After one or two subsequent refreshments and overnight fermentation, it is ready to serve as an inoculum for bread making. The sourdoughs that have been examined were prepared according to this procedure.

The aim of this work was not only to provide data concerning chemical and microbiological characteristics for both household and semi-industrial traditional Greek wheat sourdoughs, but to present a polyphasic approach to yeast identification as well.

Materials and Methods

Yeast Strains

All wild yeast strains have been isolated from Greek sourdoughs, three from household scale fermentations (sourdough I-III) and two from bakeries (sourdough IV, V). Strain numbers are given according to the strain collection (ACA-YC) of the Laboratory of Dairy Research of Agricultural University of Athens. Reference strains for RAPD have been obtained from the CBS (*Candida ovalis* CBS 7298^T, *Yarrowia lipolytica* CBS 6164), from the DSMZ (*Zygosacharomyces rouxii* DSM 2531, *Debaromyces hansenii* DSM 70244, *Pichia membranaefaciens* DSM 70366, *Saccharomyces cerevisiae* DSM 70449^T) and from the NCYC (*S. cerevisiae* NCYC 1200) collections. Reference strains included in the FT-IR analysis, others than for RAPD-PCR, were *Y. lipolytica* CBS 6124^T, *Y. lipolytica* M331, *P. membranaefaciens* CBS 107^T and *S. cerevisiae* CBS 5900.

Sampling

Sourdough samples were collected aseptically, stored at 4°C and analyzed the same day.

pH and Total Titratable Acidity (TTA) Measurement

10 g of the sample was homogenized with 90 ml of sterile distilled water. The pH value was recorded and the acidity was titrated using 0.1N NaOH to final pH 8.5. The TTA was expressed in ml 0.1N NaOH.

Isolation of Yeast and Lactic Acid Bacteria

Samples (10 g) were diluted 1:10 with 90 ml NaCl (0.9% w/v). Numbers of colony forming units (CFU) were determined by serial dilutions on yeast glucose chloramphenicol (YGC) agar for yeasts and on modified Rogosa (mMRS) agar,

containing 2% w/v maltose for LAB. Incubation temperature was 25°C for yeasts and 30°C for LAB. Purification of strains was performed by successive subculturing. Yeast and LAB strains were stored at –80°C in 80% glycerol.

Selection of Yeast Isolates

Yeast colonies were grouped according to their morphological characteristics and this grouping was confirmed by microscopical observation. From each colony type, one colony was selected in order to perform polyphasic taxonomy.

Identification of Yeast Strains

Classical identification was performed according to Barnett *et al.* (1990) and Kreger-van Rij (1987), with the aid of a computer-based yeast identification program (Barnett *et al.*, 1985).

SDS-PAGE of Whole Cell Proteins

Cultivation of yeast, preparation of cell-free extracts and protein electrophoresis were performed according to Vancanneyt *et al.* (1991). Gel evaluation and cluster analysis were carried out as in the case of RAPD-PCR patterns.

FT-IR Spectroscopy

All spectra were determined according to Kümmerle *et al.* (1998): Yeast strains were cultivated on YGC agar (Merck, Darmstadt, Germany) at 27°C for 24 hours as a confluent lawn. Cells were suspended in 100 µl of distilled water. Of this suspensions 35 µl each were transferred onto the 15 windows of a SnSe wheel and were dried in an incubator at 42°C. The transparent cell films were recorded with a IFS-28B FT-IR spectrometer (Bruker) in the range of 4000 to 600 cm^{-1} wave numbers. Data were processed with the software OPUS, version 2-2, for microbiological identification (Bruker). For creating a distance matrix the spectral windows 3,030 to 2,830, 1,350 to

1,200 and 900 to 700 cm^{-1} with no weighting factors were used. The dendrogram was calculated with an average–linkage algorithm using a reprolevel of 30 for each spectral window. Good identification was received when the spectral distance (SD) to a reference spectrum was below 1.

DNA Preparation

5 ml of an overnight culture was pelleted by centrifugation (5000g for15 min) and resuspended in 180 µl ATL lysis buffer (QIAamp Tissue Kit; Quiagen, Hilden, Germany) according to the tissue protocol. Cells were lysed by 2 min of ultrasonic treatment with a sonication sonde (UP 50 H, Dr. Hielscher GmbH, Germany). The subsequent procedures were carried out by means of the tissue protocol. DNA was eluted with 200 µl of preheated (70°C) distilled water.

DNA Fingerprinting

Fingerprinting using RAPD-PCR was performed in TopYield ™ Strips (Nunc, Denmark) in a total volume of 50 µl. The amplification primer was the M13V universal primer (5‘ – GTT TTC CCA GTC ACG AC – 3‘). The PCR mixture contained 1 µl genomic DNA, 5 µl 10x reaction buffer, 5 mM $MgCl_2$, 200 nM each of the 4 deoxynucleotides, 1.5 U Taq polymerase (all components from Amersham Pharmacia Biotech, Freiburg, Germany) and 20 pmol of primer M13V (Interactiva, Ulm, Germany). The PCR reactions were carried out on a Hybaid Omni Gene thermocycler (MWG-Biotech, Ebersberg, Germany). The program was: (96°C/3 min-35°C/5 min-75°C/5 min)3x; (96°C/1 min-55°C/2 min-75°C/3 min) 32x.

Electrophoresis

All PCR products (10 µl DNA + 10 µl water + 5 µl AGS loading dye) were electrophoretically separated on a 1.5% agarose gel (0.5xTBE) in a MWG-Biotech chamber (20 cm x 25 cm) for three hours at constant 170 V. As size marker encompassing the whole range of fragments 1 µg per lane of the BioSizer™ (AGS,

Heidelberg, Germany) was loaded. After 15 min staining with ethidium bromide patterns were digitally saved by the E.A.S.Y. system (Herolab, Griesheim, Germany).

Cluster Analysis

Pictures were evaluated by the Gel Compar 4.1 package (Applied Math, Kortrijk, Belgium). As clustering algorithm the Unweighted Pair Group Method using arithmetic averages (UPGMA) was applied. Similarities between RAPD patterns were calculated using the Pearson product-moment correlation coefficient (r%).

Results and Discussion

Chemical Characterization of the Sourdoughs

Results concerning acidity of the five sourdoughs tested are given in Table 1. Values obtained varied according to the origin of the sample. The pH ranged from 4.09 to 3.58, the TTA between 10.0 and 29.0. For samples I, II, III and V, pH and TTA values recorded were similar to those reported in the literature (Roecken and Voysey, 1995) and they could be easily correlated with the respective LAB population **(Table 2)**. However, in the case of sample IV (a bakery sourdough), the high TTA value of 29.0 could hardly be explained only by the respective pH of 3.70. It could be only attributed to a strong buffer capacity, due to external additives or to endogenous metabolites.

Table 1. Acidity of the sourdoughs tested

Sourdough	pH	TTA (ml 0.1 N NaOH)
I	3.75	15.6
II	3.58	20.0
III	3.78	14.0
IV	3.70	29.0
V	4.09	10.0

TTA = total titrable acidity

Microbiological Characterization of the Sourdoughs

In the homemade sourdoughs, yeast and LAB populations (Table 2) ranged from 8×10^6 to 8.4×10^7 and from 7×10^7 to 6.1×10^9, respectively, while the ratio between yeast and lactic acid bacteria varied from 1:8 to 1:73. These values are in agreement with the literature data (ROECKEN and VOYSEY, 1995; GOBBETTI et al., 1994)

and that could be considered as an indication that no addition of commercial yeast took place and that these doughs were spontaneously fermented. On the contrary, in sourdoughs originating from bakeries, yeast populations were much higher than those of lactic acid bacteria. This was probably due to the fact that commercial yeast was added for leavening acceleration.

Table. 2. Microbial counts of the sourdough tested

Sourdough	yeasts (CFU x 10^7)	LAB (CFU x 10^7)
I	0.9	7
II	0.8	20
III	8.4	610
IV	0.2	0.05
V	0.3	0.06

CFU = colony forming units, LAB = lactic acid bacteria

The yeast colonies observed were grouped according to their morphological characteristics. Five colony types were distinguished in sourdough I, while four, two, one and one in sourdoughs II, III, IV and V, respectively. From each colony type, initially one colony was selected. However, in some cases during the purification procedure some colonies exhibited a variety on morphology; these colonies were also purified and thus finally more colonies were selected for identification. This way, twenty-two colony types were selected from sourdough I, while seven, eleven, one and four from sourdoughs II, III, IV and V, respectively. In this meaning, homemade sourdoughs compared to the bakeries ones showed higher variety of colony morphology.

Identification of Yeast Strains by their Biochemical Characteristics

With the traditional identification technique using various physiological and morphological characteristics, identification to species level was achieved. Out of forty-five colony types, fourteen were identified as *Saccharomyces cerevisiae*, twenty-five as *Pichia membranaefaciens* and six as *Yarrowia lipolytica* In sourdough I, one colony was identified as *Saccharomyces cerevisiae* and twenty-one colonies as *Pichia membranaefaciens*. In sourdough II, one colony as *Saccharomyces cerevisiae* and six colonies as *Yarrowia lipolytica*. In sourdough III, seven colonies as *Saccharomyces cerevisiae* and four colonies as *Pichia membranaefaciens*. Finally, in sourdoughs IV and V, one and four colonies as *Saccharomyces cerevisiae*, respectively. According to these results and taking into account the CFU that each colony type represented, a conclusion could be drawn concerning the distribution of yeast species in the examined sourdoughs **(Table 3)**.

Table 3. Distribution of yeast species in the examined sourdoughs

	CFU x 10^7				
Sourdough:	I	II	III	IV	V
Species					
S. cerevisiae	0.6	0.7	6.4	0.2	0.3
P. membranaefaciens	0.3	-	2.0	-	-
Y. lipolytica	-	0.1	-	-	-

In order to confirm these results SDS-PAGE of whole cell proteins, FT-IR spectroscopy and RAPD-PCR analysis were used.

In order to confirm these results SDS-PAGE of whole cell proteins, FT-IR spectroscopy and RAPD-PCR analysis were used.

Characterization of Yeast Strains with SDS-PAGE Analysis

The comparison of whole cell protein patterns has proven to be very reliable for comparing and grouping of closely related strains. SDS-PAGE provides with discriminative information at or below the species level (Vandamme *et al.*, 1996).

In **Figure 1** the whole cell protein patterns of the yeast isolates along with their correlation are shown.

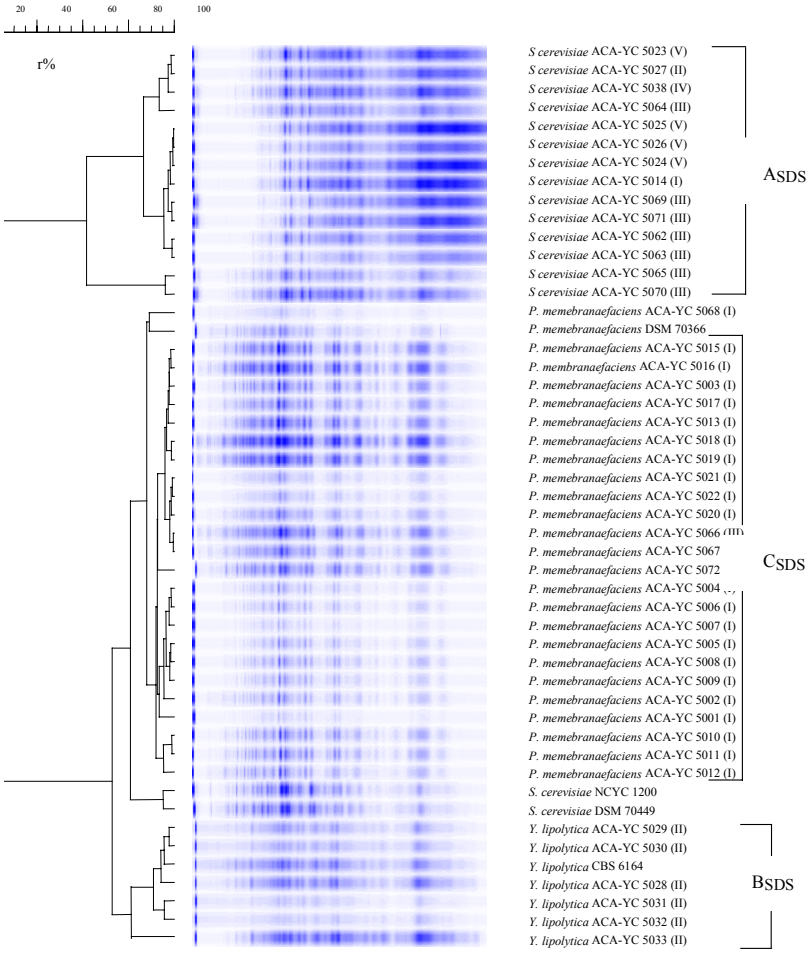

Figure 1. Cluster-analysis of SDS-PAGE patterns calculated by the unweighted average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%). The roman numbers in parenthesis are pointing out the mentioned origin of the respective yeast strain. For the type strains the number of the strain collection is used.

In order to confirm the result of the classical identification, reference strains were included. All strains were grouped into three major clusters representing the yeast species as identified by the classical identification. Cluster A contains all *S. cerevisiae* strains isolated from traditional Greek sourdough. The reference strains of *S. cerevisiae* showed a correlation of 62% with the sourdough isolates. This low correlation could be attributed to the distortion of the patterns due to the smear observed at the area of the low molecular weight proteins of the electrophoretic profile since the similarity was high at the area of high molecular weight proteins. This smear was probably caused by insufficient washing of the cells and it is most likely remaining of the growth medium consisting of complexes of proteins with polysaccharides. Although the cultivation of the cells with the preparation of cell-free extracts was repeated, the problem persisted. In cluster C all *P. membranaefaciens* isolates were included. The respective reference strain showed a correlation of 85% with the sourdough isolates. Finally, all *Y. lipolytica* strains were included in cluster B. The respective reference strains showed a correlation between 80% and 96% with the sourdough isolates.

FT-IR Spectra of Yeast Strains

A dendrogram of the wild yeast strains together with selected reference strains is shown in **Figure 2**.

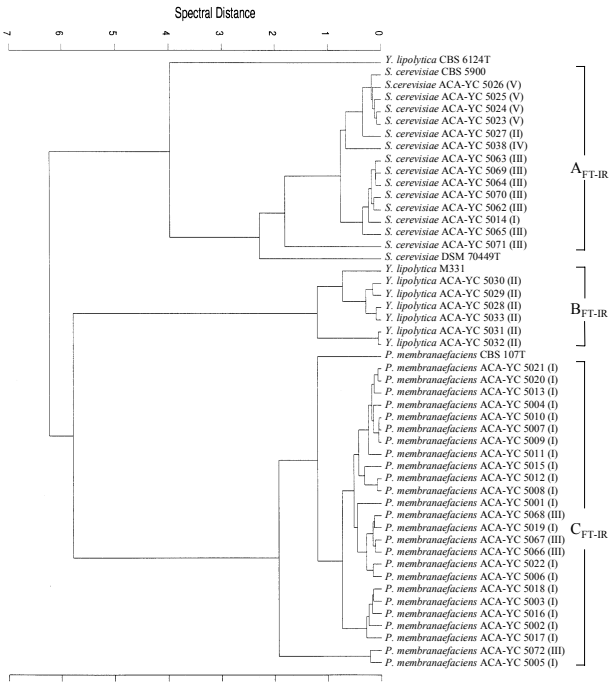

Figure 2. Dendrogramm of the spectra of 45 isolates and 5 reference strains. The dendrogram was calculated by an average linkage algorithm. The spectral window 3030 to 2830, 1350 to 1200 and 900 to 700 wave length without weightening were used. Roman numbers parenthesis are indicating the sourdough the strains were isolated from.

Figure 3 is showing the original spectras of selected strains the calculation of the dendrogram is based on.

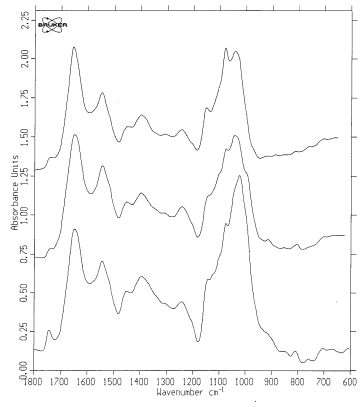

Figure. 3. Original spectra in the meaningful range of 1800 to 600 cm^{-1} wave length of an isolate of *Pichia membranaefaciens* (bottom), *Saccharomyces cerevisiae* (middle) and *Yarrowia lipolytica* (top), each.

The dendrogram is divided in three distinct clusters. Cluster A contains 14 isolates with the strain *S. cerevisiae* CBS 5900; the type strain has a major distance. Cluster B includes 6 isolates with the reference strain *Y. lipolytica* M331, an isolate from dairy environment. The type strain of *Y. lipolytica* does not cluster with the phenon B. Cluster C contains 25 isolates together with the type strain of *P. membranaefaciens*. The clustering of all isolates together with the reference strains was in agreement with the findings of classical identification.

Characterization of Yeast Flora in Sourdoughs with RAPD Analysis

A crucial point for the construction of meaningful patterns is the isolation of DNA out of the examined strain. Conventional methods are often based on the enzymatic lysis of the cell wall followed by time consuming purification steps with organic solvents. While the yield of DNA is dependent on the degree of lysis it can differ between strains due to their cell wall structure. For a rapid and reliable

extraction a modified procedure of the Quiagen Tissue Kit was used. The disruption of cells by ultrasonic treatment in a buffer containing chaotropic salts and the separation and purification of the released DNA by binding at the silica matrix of the spin columns emerged as a appropriate method. In contrast to the findings of Davin-Regli et al. (1995) our own results indicated that pattern quality is almost not affected by DNA concentration. **Figure 4** depicts a digitized picture of the RAPD-PCR generated banding patterns after cluster analysis.

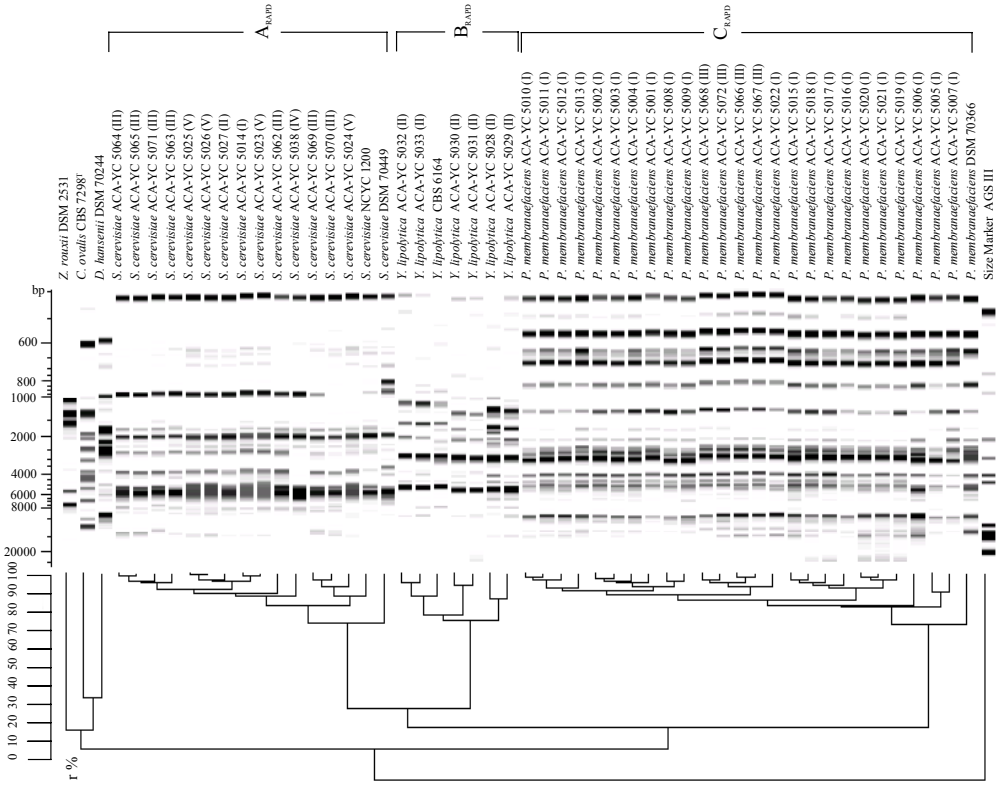

Figure 4. Cluster analysis of RAPD patterns calculated by the unweighted average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%). As shown by the metric scale and the DNA size marker fragments between a range of 20 kb and 500 bp are included in the analysis. The roman numbers in parenthesis are pointing out the mentioned origin of the respective yeast strain. For the type strains the number of the strain collection is used.

All strains isolated out of the 5 sourdoughs from different homemade and accordingly bakeries sourdough could be grouped into the 3 major clusters A, B, C standing for *S. cerevisiae, Y. lipolytica* and *P. membranaefaciens*, respectively. For a confirmation of the allocation, type strains have been enclosed in the investigation. In cluster A the *S. cerevisiae* reference strains showed a correlation between 72% and 88% to the sourdough isolates. The minimum correlation between the representatives of cluster A was 84%, whereby no significant difference between the different habitats could be observed. For cluster B similar correlation values can be realized. Sourdough II was the only habitat where *Y. lipolytica* has been isolated from. The strains TMW 3.103 and TMW 3.104 exhibited a little lower correlation of 75% to the rest where it was between 80% and 93%. The largest cluster is that for *P. membranaefaciens*. Almost all isolates from sourdough I are belonging to cluster C, whereas only four isolates from the sourdough III matched in this cluster. The minimum correlation of the isolates is 84%. The reference strain DSM 70366 showed a obvious absence of bands resulting in correlation factor of 72% in comparison to the rest.

RAPD fingerprinting of yeast strains isolated from 5 Greek sourdoughs of different origin proved to be a good and economical tool for the characterization of yeasts from these complex habitats. Besides a description of their diversity it could be demonstrated that unknown isolates easily could be identified by the inclusion of reference organisms. In combination with the fast DNA isolation procedure results can be obtained within 2 days after cultivation, which offers obvious advantages beside classical identification procedures. In comparison, SDS-PAGE, FT-IR spectroscopy and RAPD showed the same efficiency at species level discrimination. The major advantage of RAPD in opposite to SDS-PAGE and FT-IR is that there is no necessity to cultivate organisms under standardized conditions, because it relies on genotypic properties. The main advantage of SDS-PAGE against FT-IR and RAPD is that it is less expensive. The pronounced advantage of identification with FT-IR is that one strain can be identified within merely 10 minutes work and that this method is a very

simple and low-cost procedure. Contrary to SDS-PAGE and RAPD a huge reference library for food-borne yeasts is available.

In this respect, SDS-PAGE, FT-IR and RAPD-PCR analysis are powerful tools for identification of yeasts. All methods confirmed the identification results obtained when the classical technique was used.

S. cerevisiae strains were isolated from all five sourdoughs examined. Depending on the origin of the sample, *P. membranaefaciens* or *Y. lipolytica* could also be found. When *S. cerevisiae* was associated with *P. membranaefaciens* the ratio ranged from 1:0.5-1:0.31 with *S. cerevisiae* dominating. This could be explained by the fact that *S. cerevisiae* isolates could ferment all sugars present in the dough, i.e. glucose, fructose, sucrose and maltose. Regardless the glucose repression, *S. cerevisiae* has an advantage towards other yeast species that can not ferment all these carbon sources. *P. membranaefaciens* isolates could only ferment glucose, but about 8 times slower than *S. cerevisiae* isolates do. When *S. cerevisiae* was associated with *Y. lipolytica* the ratio was 1:0.14 with *S. cerevisiae* dominating. This could be explained by the fact that *Y. lipolytica* isolates possesses no fermentative metabolism, while they oxidize only glucose. These facts along with the occurrence of *S. cerevisiae* strains in every sourdough tested led to the conclusion that this species represents the indigenous yeast microflora while *P. membranaefaciens* and *Y. lipolytica* appeared to be a random microflora of traditional greek sourdoughs.

The presence of *S. cerevisiae* in the sourdough microflora is very often mentioned (Ottogalli et al., 1996; Rossi, 1996; Gobetti, 1994) and it seems that, in many cases, is part of the indigenous flora. Nevertheless, its overall presence could be the result of the extensive use of *S. cerevisiae* in bakeries. The presence of *P. membranaefaciens* strains in sourdough microflora is also mentioned by Rossi (1996) and it has been found in traditional Portuguese bread doughs (Almeida & Pais, 1996). For *Y. lipolytica* it is the first time that the presence of this species in sourdough microflora is reported. Both species are able to grow at low pH foodstuffs and thus the

presence of both can be justified, even though *Y. lipolytica* is mostly associated with meat and dairy products.

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ADDITIONAL INSIGHTS AND CONCLUSIONS

The consequent application of molecular techniques to investigate microbial ecosystems in food fermentations is an important prerequisite for the better understanding of the respective processes. This was demonstrated for the recalcitrant microorganisms of sourdough fermentations. The results obtained not only provide a profound insight in the discriminatory power of the respective state of the art tools. New useful methods were developed to describe occurrence and behavior of lactobacilli in the sourdough ecosystem, and a so far unmatched overview is provided on the dynamics of the LAB sourdough flora. Besides the scientific insights in the ecology of these fermentation the organisms involved, and their behavior in response to external and internal process parameters conclusions may be drawn for industrial applications, to ensure product quality, to improve the process, steering it in a suitable way or to construct novel processes on a rationale basis.

Historically, methods to achieve all these insights changed dramatically during the last 20 years. At the beginning of modern microbiology microorganisms have been classified by their phenotypic properties, like certain metabolic activities or their morphology. This aspects are sufficient for the construction of classification schemes and still important for the screening and evaluation of strains with desired physiological properties. However, for fast and reliable identification and moreover detection purposes these methods are improper due to the variability of the phenotype of bacteria as a fact for example of the adaptation to special environments, mutations or the loss of plasmids. Especially for LAB, differences in fermentation patterns are small giving raise to uncertain results. In many cases and as demonstrated in this work species from cereal fermentations exhibit a strong strain dependent phenotype making a clear identification almost impossible. Therefore, to cope with the demands of modern food microbiology for a fast and unequivocal identification more accurate methods are desired. In the last decade methods targeting the genotype achieved the greatest importance. Aside from the latter, chemotaxonomic methods meaning the

setup of a classification scheme based on the chemical characterization of cellular components like whole cell protein patterns, fatty acid analyses, determination of the peptidoglycan type, or the G+C content of the DNA are applied. However, with the breakthrough and establishment of genotypic methods they are losing importance, but are still necessary in polyphasic approaches as for example for the description of new taxons, and to determine a strains suitability to conduct a process.

Today the phylogeny of microorganisms is reflected in a genealogical tree divided into the three domains *Bacteria*, *Archeae* and *Eucarya* is based on rRNA sequences. The 16S rRNA-based trees are reflecting the phylogenetic relationship among prokaryotes and therefore had a major impact on their taxonomy. Nucleotide sequencing and other analytical techniques – most notably, PCR-based DNA amplification methods – have dramatically expanded the rRNA sequence database leading to a more and more detailed classification of species. Culture independent rRNA approaches led to additional insights of bacterial ecosystems. These theoretical awareness of the significance of the rRNA represents a framework for applied approaches to answer practical problems in environmental and food microbiology. A fundamental technical progress to realize this was the introduction of the polymerase chain reaction. In the following approaches we used to investigate the ecology of cereal fermentations are presented and basic conclusions are drawn.

A fundamental question at the beginning of our investigations was if the LAB grown in the laboratory are really representing the in situ diversity of the fermentation flora or if these microorganisms that grow on isolation plates are the ones best adapted to the artificial growth conditions and are not necessarily those that are metabolically active or abundant in the environment. This would drastically influence the results of methods including a pre-selection by a cultivation step and therefore introducing bias. The main steps of this investigation were the extraction of total community DNA from the sourdough, amplification of the 16S rDNA, cloning the 16S rDNA-plasmid-construct into *E. coli*, reamplification of the 16S rDNA inserts, and finally characterization of the inserts. For the DNA extraction we used a strategy described in

Chapter II. For the amplification primers with a Not I cleavage site were used, making an easy sticky end ligation into the vector possible. After cloning, insert-positive clones were picked followed by a reamplification of the insert. To characterize the insert we sequenced only a single base (T), which allowed a higher throughput and resulted in meaningful patterns. The inclusion of reference organisms usually found in such fermentations allowed a direct allocation. For a definitive identification inserts were sequenced. Finally it could be demonstrated that the sourdough flora of the investigated system consisted mainly of *L. amylovorus* and *L. pontis*. This correlated well with previous culture based investigations. It can be concluded, that on the one hand side methods based on the direct DNA isolation from the sourdough are appropriate for the description of such fermentations and on the other hand methods including a cultivation step are reflecting the actually present flora. For a routine analysis of food fermentations this method would be too time consuming.

The intention of a further culture independent study was the fast and simultaneous monitoring of the fermentation flora throughout the fermentation time. LAB and bacteria in general may exhibit species dependent length polymorphisms within the same variable regions on the 16S rDNA. When amplifying this region from mixed culture DNA using the same universal primers products with different length are generated. These differences may amount only a few base pairs making a highly resolving electrophoresis necessary. We detected the PCR-products by using a Cy-5 labeled primer on an sequencer. The development was followed of a lab-scale sourdough fermentation described in **Chapter I**. The flora of this fermentation was mainly composed of *L. amylovorus*, *L. pontis* and the closely related *L. frumenti* as described in **Chapter III**. With the ss-amplified rDNA-FLP (single stranded amplified ribosomal DNA fragment length polymorphism) two peaks could be detected one with 269 bp representing *L. amylovorus* and one with 273 bp representing both species, *L.*

pontis and *L. frumenti* (Vogel *et al.*, 1999[1]). The calculation of the peak area allowed a semi-quantitative estimation about the presence of these organisms. These results were in good correlation with the numbers calculated after RAPD analysis (**Chapter I**). The developed method has also been successfully applied to monitor crop associated lactobacilli in ducks during a feeding experiment. It can be concluded that this method constitutes an ideal possibility for a simultaneous monitoring of several species. Besides the detection, quantification and recording of population dynamics are possible. A prerequisite for this method is to know how the flora is composed and that the component species have a suitable rRNA fragment length polymorphism.

In **Chapter II** a culture independent method was described demonstrating the possibility of a fast and specific identification and detection of sourdough LAB. A PCR system with primers specific for the closely related species *L. pontis*, *L. panis* and *L. frumenti* (see **Chapter III**) allowed the differentiation of these species and on the other side the tracking during a sourdough fermentation. Such PCR systems may have practical applications like the survey of industrial fermentations or to verify species identity. This may especially be true for sourdough LAB like *L. pontis* which is known to exhibit a heterogeneous fermentation profile and morphological appearance, and therefore is hardly identified by the latter.

RAPD-PCR is another genotypic method we applied for the characterization of the microflora of cereal fermentations. In contrast to rRNA based methods, RAPD is reflecting polymorphisms on the entire genome leading to the differentiation of genomovars. At first RAPD is a typing method leading to a characteristic fingerprint of single organisms. These fingerprints may than be used for discrimination from other isolates or to proof the authenticity of strains. Potential industrial applications could be the specific detection of starter organisms with certain properties. We did an investigation where the persistence of strains *of L. sanfranciscensis* - a major element in this industrial starter preparation - was controlled, when a different flour type was

[1] **Vogel, R. F., Knorr, R., Müller, M. R. A., Steudel, U., Gänzle, M. G. & Ehrmann, M. A. (1999).** Non dairy lactic fermentations: the cereal world. *Antonie van Leeuwenhoek* **76**, 403-411.

fermented spontaneously. The comparison of RAPD patterns of the original strains with the patterns of the new isolates, showed that the strains were still present besides some other species. This makes clear that RAPD typing is also applicable for routine analyses.

RAPD typing is moreover an appropriate tool for ecological investigations of microbial communities to obtain an qualitative and quantitative overview of the microbial composition. Representatives of each cluster can be identified (see rDNA sequencing) making a fast and reliable description of such fermentations feasible. We applied this strategy for an microbial investigation of indigenous Greek sourdough fermentations. In comparison to a physiological characterization this approach revealed to be faster and the result were more definite. The major part of isolates belong to *L. sanfranciscensis* almost independent of the origin, followed by *Weissella* species, *Pediococcus* species, *Enterococcus* species, and additional *Lactobacillus* species. The same sourdoughs have been investigated on their yeast flora (see **Chapter IV**). It was demonstrated that this RAPD typing technique is not only limited to LAB. The cluster analyses was consistent with other typing methods, like SDS-PAGE and FT-IR analyses. Furthermore, the wild yeast strains could be identified by including reference and type strains in the analyses.

A basic question in taxonomy is the differentiation below the species and subspecies level. RAPD typing is often leading to similar patterns with polymorphisms within one species or clearly set aside patterns leading to a differentiation of genomovars which could even be used for identification at the strain level. This may especially be useful for the evaluation of starter organisms with particular properties. For this reason and to seek out if there exist regional strain varieties of isolates of *L. sanfranciscensis* from various sourdoughs and starters from different locations in Europe have been investigated by RAPD typing. The major part of the strains grouped together in 7 clusters. A correlation to the provenance could not be made. An explanation for this may be due to commercial starters, which are not only limited to industrial but also to artesanal products or because they originate from artesanal ones.

To evaluate the discriminatory power of RAPD these typing method was compared with AFLP derived clusters (Leissner *et al.*, 2000[2]). In this study it could be demonstrated that the AFLP technique originally developed for analyses in the field of eucaryotes is suitable as well as for applied problems in the field of LAB. Within the genera *Lactobacillus*, *Pediococcus*, *Enterococcus* and *Weissella* the organisms could be unequivocally distinguished at the species level. A comparison with the RAPD generated patterns showed good correlation, whereas the RAPD patterns exhibited more polymorphisms within a species.

In **Chapter I** another strategy including RAPD typing was presented. The main targets of this study have been to check the stability of the microbial composition in a lab-scale fermentation when started with industrial sourdough. After several refreshment steps randomly picked clones were subjected for RAPD typing by what a percental composition could be calculated. The established fermentation was monitored in the same way and the influence of external parameters like temperature was determined. The consistency of the RAPD patterns over a longer time and their independence of DNA quality and PCR batches allowed a database setup by which subsequent patterns were allocated and strains were identified. Finally, it could be demonstrated that it is possible to entirely describe the microbiological status of an industrial process, to transfer it into a lab-scale fermentation with a similar flora and that RAPD typing may be a suitable to tool to verify this model.

Apart from the taxonomic investigations but as a result of the genotypic characterization and the phylogenetic classification of the sourdough isolates adhesion studies have been performed (Müller *et al.*, 1998[3]). Many isolates from cereal fermentations showed a close phylogenetic relationship or even identicalness to species from the human environment like *L. pontis* or *L. frumenti to L. vaginalis*, *L. oris* and *L. reuteri* or *L. amylovorus* to *L. acidophilus* or *L. johnsonii*. This fact was the

[2] **Leissner, C. E. W., Müller, M. R. A., Niessen, L., Ehrmann, M. A. & Vogel, R. F. (2000).** Use of the AFLP fingerprinting method for the differentiation and identification of lactic acid bacteria. *Biospektrum Sonderausgabe*, 155.

[3] **Müller, M. R. A., Rouvet, M., Brassart, D., Böcker, G., Ehrmann, M. A. & Vogel, R. F. (1998).** Adhesion of *Lactobacillus* strains from cereal fermentations to human intestinal cells. *Int Dairy Journal* **8**, 584

motivation to investigate the adhesion properties of LAB from the cereal environment to human Caco-2 cells as an important feature for probiotic cultures. Strains of *L. reuteri*, *L. amylovorus* and *L. fermentum* from different cereal fermentation showed adhesion to the Caco-2 cells as revealed by liquid scintillation counting and laser scanning microscopy. This capability renders them possible probiotic organisms but further investigations have to follow.