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Ecological studies of the *Lactobacillus* biota in the human digestive tract and adaptation of intestinal lactobacilli to the sourdough ecosystem

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Contents

Chapter I

Scope and outline

Scope

Lactobacilli have been detected in diverse environments and are the subject of considerable research due to their commercial use in the food industry (reviewed in Hammes and Hertel, 2003). They are used in the production of foods prepared by means of a lactic acid fermentation (dairy products, fermented vegetables, fermented meats, and sourdough bread). Furthermore, lactobacilli are commonly associated with the bodies of humans and animals and are considered to benefit the health of the consumer when ingested as probiotics. Microbiological studies revealed that sixteen *Lactobacillus* species are associated with the human body, but most of these species can only be detected transiently and unpredictably.

The first part of this dissertation adresses studies on the ecology of the *Lactobacillus* biota of the human intestinal and oral tract. Studies were conducted using bacteriological culture on the selective Rogosa SL medium by incubation under traditional and alternative conditions, as well as modern molecular biological techniques, i.e. PCR-DGGE with primers specific for the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Weissella* (Walter et al., 2001).

Several intestinal *Lactobacillus* species are commonly also detected in fermented foods, but the genetic background for this ecological versatility is poorly understood. The second part of this dissertation deals with studies on the gene expression of lactobacilli in different ecosystems. The model organism is *Lactobacillus reuteri*, which has its natural habitat in the human and animal intestine (Reuter, 2001), but has also been found to dominate during type II sourdough fermentation (Meroth, 2003a). To study the genetic background that is responsible for such ecological versatility, *In Vivo* Expression Technology (IVET) was used. This technique allows the identification of genes that are highly expressed in a particular environment, e.g. sourdough or intestine.

Outline of the thesis

Chapter II gives an overview of the human gastrointestinal microbiology, with a special focus on the *Lactobacillus* biota of the oral cavity and intestinal tracts. The species composition as well as the role of lactobacilli in these habitats is taken into consideration. The last section of this chapter describes the sourdough fermentations, from a technological as well as microbiological point of view. Particular attention is given to the role played by lactic acid bacteria (LAB) during the sourdough fermentation.

Chapter III describes the application of a group-specific PCR-DGGE system to investigate various media and incubation conditions to recover LAB from human faeces. Analysis of faecal samples collected from different human subjects revealed that food-associated LAB, such as *Lactobacillus sakei* and *Leuconostoc mesenteroides*, hitherto not described as intestinal inhabitants, are more easily selected using an incubation temperature of 30°C and a modified atmosphere containing 2% O_2. Identification of randomly picked colonies grown under these conditions showed that *L. sakei* is one of the predominant food-associated LAB species, reaching counts of up to 10^6 CFU/g faeces.

This Chapter has been published in Microbial Ecology:

Dal Bello Fabio, Jens Walter, Walter P. Hammes, and Christian Hertel. 2003. Increased complexity of the species composition of lactic acid bacteria in human feces revealed by alternative incubation condition. Microb. Ecol. 45:455-63.

Chapter IV describes the investigation of the *Lactobacillus* biota of human saliva and faecal samples during a three months interval. The predominant lactobacilli were characterized by using the group-specific PCR-DGGE system as well as bacteriological culture on Rogosa SL agar. The clonal relationship of isolates belonging to any species detected in both saliva and faecal samples was evaluated by RAPD-PCR analysis. Oral isolates of the species *L. gasseri* and *L. vaginalis* showing identical RAPD types were found to persist over time, suggesting that these species are autochthonous to the oral cavity. The results of this study, together with recently published data, give strong evidence that some

lactobacilli found in human faeces are allochthonous to the intestine and originate from the oral cavity.

This Chapter has been submitted for publication in Systematic and Applied Microbiology:

Dal Bello Fabio, and Christian Hertel. 2005. Oral cavity as the natural reservoir of intestinal lactobacilli. Microb. Ecol. In Press.

Chapter V describes the application of an IVET system to identify genes of *Lactobacillus reuteri* LTH5531 specifically induced during type II sourdough fermentation. Thirty-eight sourdough induced fusions were detected, and 29 genes could be identified on the basis of the available sequence information. The study allowed insight into the transcriptional response of *Lactobacillus reuteri* to the dough environment, what founds the molecular basis to investigate bacterial properties that are likely to contribute to the ecological performance of the organism and influence the final outcome of the fermentation.

This Chapter has been submitted for publication in Applied and Environmental Microbiology:

Dal Bello Fabio, Jens Walter, Stefan Roos, Hans Jonsson, and Christian Hertel. 2005. Inducible gene expression in *Lactobacillus reuteri* LTH5531 during type II sourdough fermentation. Appl. Environ. Microbiol. In Press.

Chapter VI describes the application of the IVET system of Chapter V to identify genes of *Lactobacillus reuteri* LTH5531 specifically induced during the murine gut transit. Conclusions are drawn regarding the origin of this strain and adaptation to different habitats.

Co-authors

This dissertation comprises studies that were carried out in collaboration with several researchers. The studies with the RLF-mice described in Chapter VI (three months of research) were performed at the Department of Microbiology and Immunology, University of

Otago, New Zealand in the laboratory of Prof. Dr. Gerald W. Tannock. The work was generally supervised by PD Dr. Christian Hertel. In addition, the work presented in Chapter VI was supervised by Prof. Dr. Gerald W. Tannock and Dr. Jens Walter.

Chapter III: Dr. Jens Walter assisted the interpretation of the results and the discussion of the manuscript. Prof. Dr. Walter P. Hammes contributed by critical reading and discussing the manuscript.

Chapter V: Dr. Jens Walter provided the IVET strategy and gave advices for the construction of the genomic library. Dr. Hans Jonsson and Dr. Stefan Roos assisted by providing the genomic information of *Lactobacillus reuteri* ATCC 55730.

Chapter VI: Diane M. Loach cooperated with the RLF-mice work.

Chapter II

Introduction

The body of animals, including that of humans, is home to a vast collection of microbial species, mostly bacteria that inhabit regions that are accessible to the microbes by one or more body orifices. This collection, known as the normal microbiota, is acquired soon after birth and persists throughout life (Tannock, 1995). Some of the microbial species attain high population levels and it has been estimated that more microbial cells inhabit the human body than there are eukaryotic cells of which it is constituted (10^{14}:10^{13}; Luckey, 1972). Because of variation in physical and chemical properties of the various body sites, different microbial communities exist in the oral cavity, upper respiratory tract, gut, vagina, and on the skin. Therefore, it is possible to recognize microbial communities which are characteristic of each site (oral microbiota, gut microbiota, and so on). The largest number of bacteria resides in the distal gut (ileum and colon) of human s as well as monogastric animals. Some animal species have relatively large numbers of lactic acid-producing bacteria (lactobacilli) in the proximal gut (forestomach of rodents, crop of chickens, and pars oesophagea of pigs). This special association is due to adherence of these lactobacilli to the surface of the non-secretory epithelium lining these sites, enabling the bacteria to form biofilms that provide a bacterial inoculum of the digesta. Although a complete catalogue of the inhabitants of the gut ecosystem is not yet available, hundreds of bacterial types, predominantly obligately anaerobic species, are estimated to be capable of residing in the distal regions (Tannock, 1995). Current knowledge indicates that the human intestinal microbiota is composed of 500-1,000 different species (Xu and Gordon, 2003). Assuming 1,000 bacterial species, and using *Escherichia coli* as an arbitrarily selected representative of the community, the aggregate size of all intestinal microbial genomes may be equivalent to our own genome, and the number of genes in this "microbiome" may exceed the total number of human genes by a factor of about 100 (Xu and Gordon, 2003).

Until the 1990s, analysis of the composition of the gut microbiota relied on the use of traditional bacteriological methods of culture, microscopy and identification (O´Sullivan, 1999). Selective bacteriological culture media were essential for accurate analysis of the microbiota, because they enabled enumeration of specific bacterial population to be made (Summanen et al., 1993). Unfortunately, few culture media used in the analysis of the microbiota are absolutely selective and, in addition, not all of the species comprising a

population may be able to proliferate with equal ease on the selective medium, thus introducing biases to the results (Tannock, 1995). Even in the 1970s, researchers had observed that the total microscopic count of the bacterial cells in human faecal smears was always higher than the total viable count (CFU, colony forming units) obtained by culture on a non-selective agar medium. But this comparison was obtained by using total microscopic "clumps" counts (aggregates of bacterial cells) rather than by counting individual bacterial cells in smears (Moore and Holdeman, 1974). Total bacterial microscopic counts, utilizing the 4', 6-diamidino-2-phenylindole (DAPI) stain and computer imaging, have revealed average total bacterial cell counts in human faeces approaching 1×10^{11} per gram (wet weight) (Tannock et al., 2000). State-of-the-art bacteriological methodologies still only permit about 40% of this bacterial community to be cultivated on non-selective agar media in the laboratory (Tannock et al., 2000). Thus a large population of the bacterial cells seen in microscope smears have never been investigated. Although some of these cells may be non-viable, it is likely that many are viable but non-cultivable due to their fastidious requirements for anaerobiosis or, more likely, due to complex nutritional interactions that occur between the inhabitants of bacterial communities (Suau et al., 1999). These nutritional complexities may be difficult, if not impossible, to achieve in laboratory culture media.

16S rDNA sequence based analysis of complex microbial communities

Carl Woese's molecular phylogenetic studies of microorganisms revolutionised the understanding of biological diversity and evolution (Woese, 1987). The phylogenetic framework provided by the comparison of 16S ribosomal RNA (rRNA) gene sequences provides a conceptual approach to microbial identification and taxonomy. 16S rRNA gene (16S rDNA) sequences contain regions conserved across all bacterial species interspersed with regions (V1-V9) in which the nucleotide base sequences are variable among bacterial types (Stackebrandt and Goebel, 1994). Sometimes, the variable regions are highly species-specific. Comparison of 16S rDNA sequences can therefore be used in the identification of bacterial species and consequently, in the analysis of bacterial communities (Raskin et al., 1997). Universal or group-specific primers can be used in polymerase chain reactions (PCR) to amplify 16S rDNA from bacterial cells in natural samples. The amplified 16S rDNA sequences can be cloned, screened and sequenced. Alignment of the sequence with those stored in databanks permits the recognition of which species were represented in the habitat,

and detects those that cannot be cultivated by conventional bacteriological techniques. In a study of this type, three bacterial divisions represented 95% of the faecal microbiota of a human subject: *Bacteroides-Prevotella* group, *Clostridium coccoides* group, and *Clostridium leptum* group (Suau et al., 1999).

The study of the microbial diversity and community of the gastrointestinal tract over the time has been stimulated by the advent of fingerprinting techniques, such as denaturing or temperature gradient gel electrophoresis (DGGE or TGGE) (Muyzer and Smalla, 1998; Zoetendal et al., 1998). DGGE/TGGE of 16S rDNA PCR products are especially suited to study diversity in samples with largely unknown microbial content without cultivation. Briefly, in DGGE/TGGE fragments of the 16S rRNA genes are amplified using a primer pair, one of which has a GC-rich 5' end (GC clamp) that prevents the two DNA strands from completely dissociating even under strong denaturing conditions. During electrophoresis through a polyacrylamide gel containing denaturants, the migration of the double-stranded DNA is prevented once a domain in the PCR product reaches its melting conditions. Because of the variation in the 16S sequences of different bacterial species, chemical stability is also different; therefore different 16S "species" can be separated by this electrophoretic method. Following staining of the DNA, a banding pattern emerges that represents the diversity of the different rRNA gene sequences present in the sample. The intensity of a band is a semi-quantitative measure for the relative abundance of this sequence in the population. Bands can be excised and sequenced, and the identity determined by comparison to the databases (Muyzer and Smalla, 1998; Zoetendal et al., 1998). However, PCR poses some problems: while culture bias is removed, another bias is introduced because PCR is known to amplify DNA sequences from mixed populations with different efficiency (Reysenbach et al., 1992). Chimeric sequences can be derived during PCR where there is a mixture of template DNAs in the reaction mix (Kopczynski et al., 1994), and there can be heterogeneity with regard to 16S rDNA sequences within species, and even within a single bacterial cell (Nubel et al., 1996).

Lactobacilli in the oral cavity

The oral cavity provides a variety of epithelial surfaces and microenvironments and is the only site that contains hard non-shedding surfaces (on teeth) for microbial colonization

(Jenkinson, 1999). Potential other sites for bacterial colonization include soft, constantly replaced, epithelial surfaces. In many ways, the oral cavity represents a favourable environment for microorganisms. The temperature is maintained close to 37°C; the pH is buffered to nearly neutral; water is abundant and there is a continuous supply of nitrogen and carbon in the form of the proteins and glycoproteins which are major constituents of saliva (Hardie, 1992). However, conditions vary with respect to oxygen levels and anaerobiosis, availability of nutrients, exposure to salivary secretions or gingival crevicular fluid, masticatory forces and other variables such as oral hygiene procedures (Marsh and Martin, 1999; Hardie and Bowden, 1974). As a result of all these and other variables, the composition of the mouth microbiota is host-specific and varies considerably, from site to site and at different times (Hardie, 1992). Microbiological studies revealed that approximately 25 bacteria are present per buccal epithelium cell, whereas 10^8 bacteria are detectable in 1 ml saliva, and up to 10^{11} bacteria per gram can be isolated from dental plaque (Tannock, 1999b). More than 500 different bacterial species are known to be associated with the mouth (Moore and Holdeman, 1974), but in any particular individual there are likely to be only 50-100 species present (Hardie, 1992).

Among the different bacterial groups inhabiting the oral cavity, lactic acid bacteria (LAB), especially oral streptococci and lactobacilli, are of great importance for the host health status. Microbiological studies have revealed that lactobacilli constitute < 0.1% of either the cheek or tongue bacteria, < 0.005% of intragingival plaque, and < 1% each of the saliva and the gingival crevice bacteria (Marsh and Martin, 1999). Rogosa et al. (1953), in their classic paper on the identification of oral lactobacilli, identified 500 strains isolated from saliva specimens of 130 school children. *Lactobacillus casei* and *Lactobacillus fermentum* were the predominant species present in 59 and 45% of the samples, respectively, while *Lactobacillus acidophilus* and *Lactobacillus brevis* were present in 22 and 17% of the samples. *Lactobacillus buchneri*, *Lactobacillus cellobiosus*, *Lactobacillus plantarum* and *Lactobacillus salivarius* occurred less frequently. These findings have been confirmed by other studies on children and adults (London, 1976) that found a similar distribution of species, with *L. casei* and *L. fermentum* as the predominant species. Additionally, *L. casei* has been found to be the prevalent *Lactobacillus* species among dental plaque isolates (Basson and Van Wyk, 1982; Depaola, 1989; Hahn et al., 1989; Wijeyeweera and Kleinberg, 1989). Kneist et al. (1988) found that *Lactobacillus rhamnosus* was the dominant *Lactobacillus* in softened and hard carious dentin of 125 deciduous molars. In relative importance, this species was followed by *L. plantarum*, *L. casei*, *Lactobacillus curvatus*, *Lactobacillus xylosus* and *Lactobacillus*

coryniformis. Owing to changes in nomenclature, it should be noted that the isolated *L. casei* are probably *Lactobacillus paracasei* (Collins et al., 1989), and *L. cellobiosus* are *L. fermentum* (Vescovo et al., 1979).

Although present in carious lesions, the lactobacilli are not considered to be actively involved in caries progression. Kneist et al. (1988) and Russel and Ahmed (1978) have shown that neither *L. acidophilus*, nor *L. paracasei* (identified as *L. casei*) or *L. fermentum* are able to form plaque alone (i.e., without the participation of *Streptococcus mutans* or *Streptococcus sanguis*). Detailed studies of dental plaque show that the pioneer bacteria (first binders) to a preformed pellicle are streptococci, *Neisseria*, *Actinomyces* and *Capnocytophaga* (Jenkinson, 1999). Other bacteria co-adhere to that community and increase the complexity of the association. Dental caries is caused by the metabolism of this association, which attacks the tooth enamel. Caries is thus most probably initiated by the combined activities of several species. There is strong evidence that the progression of the disease is promoted by streptococci of the *S. mutans* group. Only at a later stage may lactobacilli multiply within the built up matrix. The frequent consumption of fermentable carbohydrates increases the production of lactic acid and changes the ecological conditions in the niche, and it follows a selection for more acidophilic bacteria among which lactobacilli, in particular *L. paracasei* and *L. rhamnosus*, are of primary importance. Nothing is known about a specific role of the multitude of *Lactobacillus* species that occur in significant but minor numbers. The effect of carbohydrates on the selective growth of LAB, and above all lactobacilli, at the expense of less acid tolerant species, is the basis of the "ecological plaque hypothesis" (Marsh, 1994). It includes, that the low pH (initiated by the streptococci) found in carious cavities favours lactobacilli. The high counts may, at least in part, be the result of caries and not the cause (Hardie and Bowden, 1974; Alaluusua et al., 1987; Wijeyeweera and Kleinberg, 1989).

Studies on the ecology of lactobacilli in the oral cavity relied mainly on bacteriological culture (Marsh, 1994). Recent bacteriological studies, supported by modern 16S rDNA based technologies, have shown that the species *L. acidophilus* (and closely related species like *Lactobacillus gasseri* and *Lactobacillus crispatus*), *L. brevis*, *L. paracasei*, *L. plantarum*, *L. rhamnosus* and *L. salivarius* are the predominant lactobacilli of the oral cavity (Ahrnè et al., 1998; Marsh and Martin, 1999). Remarkably, these species have also been frequently detected in human faeces (Ahrné et al., 1998; Tannock et al., 2000; Walter et al., 2001) and previous studies indicate that the *Lactobacillus* species composition of the oral cavity and faecal samples coincide to some extent (Ahrné et al., 1998; Mikelsaar et al., 1998). Indeed,

the saliva is the main source of bacteria entering the stomach, and consequently the intestine. Bacteria present in the saliva are a mixture of those washed from tooth, gingival crevice and soft tissue surface, together with those from food or objects introduced into mouth (Mitsuoka, 1992). Lactobacilli are present in human saliva in variable numbers, but sometimes attain a population level exceeding 10^6 CFU per ml (Ahola et al., 2002). Considering that the average output of saliva is 1000 – 1500 ml per day, up to 10^9 lactobacilli originating from the oral cavity may be daily introduced into the human gastrointestinal tract, contributing to the constitution and establishment of the intestinal *Lactobacillus* biota. These observations have recently been suggested by Bibiloni et al. (2004), but this topic still has not been investigated in-depth.

The *Lactobacillus* biota of the human intestine

Our knowledge of the intestinal biota in sections of the human intestinal tract rests on the study of samples obtained using an automatic capsule system or from post mortem cases (Reuter, 2001). It was shown that *Lactobacillus* species could be detected in all parts of the human intestine including the stomach, which is characterized by a pH of around 3.0 (2.2 – 4.2). Relatively few bacterial species can tolerate these acidic conditions and most organisms ingested with food and saliva are killed by the hydrochloric acid, reducing the population to about 10^3 CFU per ml, containing mainly lactobacilli and streptococci (enterococci; Reuter, 1965a; Drasar and Hill, 1974). The biota of the small intestine increases from $< 10^4$ bacteria per ml of digesta in the duodenum up to 10^8–10^9 bacteria per gram of faeces in the terminal ileum (Gorbach et al., 1967; Drasar and Hill, 1974; Tannock, 1995; Reuter, 2001). In the duodenum and jejunum, lactobacilli and enterococci are the dominant bacteria (Mitsuoka, 1992; Reuter, 2001). The microbiota becomes more complex in the ileum, being qualitatively similar to that of the large intestine, and the relative proportion of lactobacilli drops. Samples from the proximal and distal parts of the colon as well as from faeces show a rather similar bacterial composition, with lactobacilli being more numerous in the colon, especially in the proximal section, than in faeces (about 5×10^6 and 1×10^5 CFU/ml, respectively) (Reuter, 1965b; Marteau et al., 2001).

Insights into the intestinal microbial association have been obtained from analysis of faeces inasmuch as access to the intestines is, at least in healthy humans, extremely difficult. Human

faeces contain >10^{11} bacteria per gram (wet weight), and microbial cells make up about 55% of the solids within the colon (Tannock, 1995). More then 400 bacterial species can be isolated from one subject, with obligate anaerobe bacteria being predominant and exceeding in number facultative anaerobes by 100–1000 times (Moore and Holdemann, 1974). It is however thought that 99% of the total community consists of only 30 - 40 species (Drasar and Barrow, 1985). Among this complex community, an indigenous microbiota can be recognized, consisting of autochthonous species (e.g. species, which are able to colonize the mucosa surface of the gastrointestinal tract due to special adhesion factors including compatibility with the immunological system of the host; Reuter, 2001). These microorganisms have to be distinguished from allochthonous species, which may be present in the intestine, too, but which may only have a transient character. The presence of these strains in the intestinal tract will last for a limited time, probably only a few days (Tannock, 1999a). The bacterial numbers and composition vary considerably between different animal species and along the intestinal tract (the colon being the most highly colonized) owing to anatomical and physiological distinctions. However, the composition of the main bacterial species in human faeces remains remarkable stable over time (Zoetendal et al., 1998). The genera that are considered to be predominant include *Bacteroides*, *Eubacterium*, *Clostridium*, *Ruminococcus*, and *Bifidobacterium* (Matsuki et al., 2002; Tannock, 1995).

Lactobacillus species comprise only a minor part of the bacterial community in human faeces (Mitsuoka, 1992; Sghir et al., 2000). The role of lactobacilli in intestinal ecosystems has received much attention, especially with respect to their beneficial effect on human and animal health, e.g. when ingested as probiotics. *Lactobacillus* species can be cultured from human faeces at counts varying greatly from none to <10^9 CFU per gram faeces (Mitsuoka, 1992; Kimura et al., 1997; Tannock et al., 2000). Examination of the *Lactobacillus* populations over longer periods has revealed marked variation in the complexity and stability of these populations among human subjects (Vanhoutte et al., 2004, Walter et al., 2001). Present knowledge indicates that sixteen *Lactobacillus* species are putative inhabitant of the human gut (Vaughan et al., 2002), some of which were only recently detected by DGGE using PCR primers specific for LAB (Table 1). Studies conducted between 1960 and 1980 indicated that *L. acidophilus*, *L. fermentum*, *L. salivarius* and an anaerobic LAB, previously named *Catenabacterium catenaforme*, were the dominant autochthonous *Lactobacillus* species of humans (Lerche and Reuter, 1961; Reuter, 1965b; Mitsuoka, 1969; Moore and Holdeman, 1974; Mitsuoka et al., 1975). On the basis of current taxonomy, most of the *L. acidophilus* isolates nowadays are classified as *L. gasseri* and *L. crispatus*, and most of the

L. fermentum strains belong to *Lactobacillus reuteri* (Mitsuoka, 1992; Reuter, 2001). Isolates identified as *C. catenaforme* were later identified as non-motile variants of *Lactobacillus ruminis* (Reuter, 2001). In a recent study, Tannock et al. (2000) investigated the succession of lactobacilli in faeces of 10 human subjects over a period of fifteen months. The dominant and persistent species were found to belong to the species *L. ruminis* and *L. salivarius*. *L. acidophilus*, *L. crispatus* and *L. gasseri* were regularly detected in the faeces, but strains of these species did not persist over time. Investigation of human faecal samples using PCR-DGGE in combination with primers specific for LAB revealed that fluctuations were observed at the species level (Walter et al., 2001; Heilig et al., 2002). This was in contrast to PCR-DGGE profiles generated with universal bacterial primers that indicated that the composition of the microbiota with regard to the numerically dominant bacterial species was very stable (Zoetendal et al., 1998; Tannock et al., 2000). *L. ruminis* was also detected by PCR-DGGE as the predominant species over several months, and *L. salivarius*, *L. acidophilus*, *L. crispatus* and *L. gasseri* could be detected regularly (Walter et al., 2001; Heilig et al., 2002). *L. reuteri*, once considered to be part of the autochthonous *Lactobacillus* biota of humans (Mitsuoka, 1992; Reuter, 2001), has been rarely detected in human faecal samples in recent studies either by culture or by nucleic acid-based methods of analysis (Ahrné et al., 1998; Tannock et al., 2000; Walter et al., 2001; Heilig et al., 2002). Furthermore, these studies indicated that lactobacilli such as *L. paracasei*, *L. rhamnosus*, *Lactobacillus delbrueckii*, *L. brevis*, *Lactobacillus johnsonii*, *L. plantarum* and *L. fermentum* are rather transient, persist for limited times, or in undetectable low numbers that may increase in response to dietary factors or changes in the host's conditions. This conclusion is in accordance with findings of Bunte et al. (2000), Jacobsen et al. (1999), and Reuter (1965a) showing that some food-associated lactobacilli survive the passage through the intestinal tract. Other food-associated lactobacilli, especially *Lactobacillus sakei* and *L. curvatus*, could be detected by direct analyses of 16S rRNA genes using specific primers but not by bacteriological culture on Rogosa SL agar (Walter et al., 2001; Heilig et al., 2002).

The presence of the lactic microbiota, and especially lactobacilli, in the digestive tract has historically been considered as beneficial to the host. At the beginning of the last century, Elie Metchnikoff (1845–1916) stated that toxic substances produced by members of the intestinal microbiota are absorbed from the intestinal tract and contribute to the aging process (Tannock, 1995). Microbes capable of degrading proteins (putrefaction), releasing ammonia, amines and indole were considered harmful, and bacteria like lactobacilli (which ferment carbohydrates to obtain energy and have little proteolytic activity) were thought to be

beneficial (Metchnikoff 1907, 1908). The extent to which lactobacilli colonizing the intestine contribute to the health of a healthy human is still hypothetical. Recent ecological studies revealed that only a minority of human subjects contains "autochthonous" (persistent) *Lactobacillus* strains and some did not contain any culturable lactobacilli (Tannock et al., 2000; Walter et al., 2001). It has never been reported that these subjects are less healthy or more susceptible to infections. Nevertheless, lactobacilli are considered to benefit the health of the consumer when ingested as probiotics (Mitsuoka, 1992; Vaughan et al., 1999). *In vitro*, and, to some extend, *in vivo* studies have shown that lactobacilli are effective against intestinal disorders such as lactose intolerance (Fernandes et al., 1992; Saavedra, 2001) and diarrhoea (Fernandes et al., 1992; Saavedra, 2001; Van Niel et al., 2002). Moreover, some *Lactobacillus* strains have been shown to be effective in stimulation of the immune system of the host (Isolauri et al., 2001; Perdigon et al., 2001), i.e. modulation of cytokine gene expression, stimulation of phagocytosis by peripheral blood leucocytes, and an increase of serum IgA and IgM titres (Schiffrin et al., 1995; McCracken and Gaskins, 1999; Haller et al., 2000). Finally, lactobacilli have been reported to reduce pro-carcinogenic enzymes in faeces and to have the potential to bind and degrade carcinogens (Fernandes et al., 1992; Hirayama and Rafter, 1999). Many of the effects attributed to the ingestion of lactobacilli and other LAB including probiotics, however, remain convoluted, medically and scientifically unsubstantiated, and it is rare that specific health claims can be made (Sanders, 1993; Tannock, 1999b). Probiotics products have not been subjected to large scale trials of efficacy that are used in the pharmaceutical industry. Therefore, without these trials and subsequent approval by fastidious regulatory agencies such as the FDA (USA), probiotics will continue to languish in the self-care health market (Tannock, 2003). More research is necessary to confirm the influence of ingested lactobacilli on the health of the consumer and to understand the mechanisms lying behind these effects.

Table 1. *Lactobacillus* species commonly detected in the intestine (faecal samples), oral cavity and associated with food and probiotics products.

Species	Oral Cavity	Faeces	Food	Probiotic
L. crispatus	+	+		+
L. gasseri	+	+		+
L. reuteri		+	+/-	+
L. ruminis		+		
L. salivarius	+	+		
L. acidophilus	+	+		+
L. brevis	+	+	+	
L. casei	+	+	+	+
L. delbrueckii		+	+	
L. fermentum	+	+	+	
L. johnsonii		+		+
L. paracasei	+	+	+	+
L. plantarum	+	+	+	+
L. rhamnosus	+	+	+	+
L. sakei	+	-*	+	
L. curvatus	+	-*	+	

*Only detected by PCR-DGGE (Walter et al., 2001).

Sourdough fermentation

Addition of water to flour will inevitably lead to acid and gas production in the resulting dough. This observation has been made early in history and was exploited to produce leavened bread as staple food of diet. Over the centuries, traditional sourdough fermentation processes have evolved empirically, bringing about a large variety of breads and baked goods (Rothe et al., 1973). The products are characterised by their unique flavour, enhanced shelf life and nutritional values, and favourable technological properties (Hammes and Gänzle, 1997; Salovaara, 1998). Based on common principles used in artisanal and industrial processes, Böcker et al. (1995) defined three types of sourdoughs. Type I sourdoughs are produced with traditional techniques and are characterized by continuous (daily) propagation to keep microorganisms in an active state, as indicated by high metabolic activity, above all with regard to leavening, i.e. gas production. The process is performed at temperatures of <30°C, and examples of baked goods so obtained are San Francisco sourdough French bread, panettone, and three-stage sourdough rye bread. The industrialization of the baking process of rye bread led to the development of type II sourdoughs, which serves mainly as dough acidifiers. These sourdoughs are fermented for long periods (up to 5 days) at temperatures of >30°C, and high dough yields permit pumping of the dough. The microorganisms are commonly in the late stationary phase and therefore exhibit restricted metabolic activity only. Type III sourdoughs are dried doughs which are used as acidifier supplements and aroma carriers. Doughs of type II and III require the addition of baker's yeast for leavening. Yeast preparations usually contain LAB, which contribute to acidification and aroma development in pre-doughs used for the production of soda crackers (Fields et al., 1982).

The microbial ecology of the sourdough fermentation is determined by ecological factors described by Hammes and Gänzle (1997). Endogenous factors are determined by the chemical and microbiological components of the dough, and exogenous factors are determined by the temperature and atmosphere employed. In practice, strong effects are exerted by process parameters such as dough yield, amount and composition of the starter, number of propagation steps, and fermentation time. The impact of these parameters during continuous propagation of sourdough causes the selection of a characteristic microbiota. At the start of fermentation, Gram-negative enteric bacteria are dominant, but during the sourdough fermentation, the microbiota gradually alters to the Gram-positive LAB and yeasts (Loenner et al., 1986). In mature rye sourdough LAB range from 1×10^9 to 3×10^9 CFU per gram sourdough, and yeasts from 1×10^6 to 5×10^7 CFU per gram sourdough (Loenner and Ahrné,

1995; Hammes et al., 2004). Microbiological studies have revealed that 43 species of LAB, mostly species of the genus *Lactobacillus*, and more than 23 species of yeasts, especially of the genera *Saccharomyces* and *Candida* (Brandt, 2001; Ottogalli et al., 1996), occur in sourdough. Recently, investigations of sourdough fermentations allowed the identification and description of several new *Lactobacillus* species, thus raising the interest on this microbial ecosystem (Corsetti et al., 2005; Vancanneyt et al., 2005; Valcheva et al., 2005). For investigation of the microbial population of sourdoughs, traditionally cultivation methods in combination with phenotypic (physiological and biochemical) and genotypic (randomly amplified polymorphic DNA [RAPD] and specific PCR) identification techniques have been used (Spicher, 1984; Creemers-Molenaar et al., 1985; Okada et al., 1992; Böcker et al., 1995; Vogel et al., 1996; Corsetti et al., 2001). However, these studies, based on culturing techniques, are laborious and time-consuming, and have often focused on the analysis of end products. Recently, two LAB-specific PCR-DGGE systems (Meroth et al. 2003a) and a fungi-specific PCR-DGGE system (Meroth et al., 2003b) were developed to monitor the microbiota population dynamics in sourdough fermentation processes. The results of these studies indicate that PCR-DGGE constitute a suitable tool for the design of sourdough fermentation processes to ensure the development of a desired microbiota. Furthermore, application of these PCR-DGGE systems will allow a rapid detection of undesirable changes in the bacterial population caused by fluctuating qualities of ingredients or false fermentation conditions, thus permitting adjustments to be made by technological measures.

Sourdough contains metabolically active microorganisms. During fermentation, the main role of the yeasts is to provide the CO_2 necessary for leavening. Apart from this, yeasts produce fermentation by-products, such as glycerol and acetic acid, which contribute to the improvement of the quality of the final product (Rossi, 1996). Glycerol, which is a by-product of the ethanolic fermentation, accumulates under osmotic stress conditions and is important for leavening of sweet doughs (Myers et al., 1997; Attfield and Klestas, 2000). The role of LAB in sourdough is multiple. Production of organic acids, with the consequent reduction of the pH, is necessary when rye flour is used in bread making. Production of acetic acid is important, since it modifies the flavour and protects the final product from bacterial and mould spoilage. Finally, sourdough LAB are also of importance for the production of amylases, proteinases and bacteriocins. Proteolytic events during sourdough fermentation have been shown to improve bread flavour (Thiele et al., 2002). Flavour is influenced by microbial modification of amino acids during fermentation and thermal degradation during baking (Schieberle, 1996). Ornithine, proline, isoleucine, phenylalanine and methionine, in

particular, are precursors for flavour active compounds. The hydrolysis of proteins in sourdough is attributable to cereal proteases (Bleukx et al., 1997; Thiele et al., 2002), and sourdough LAB selected for their high proteolytic acticity also contribute to the hydrolysis of wheat proteins in a strain specific manner (Di Cagno et al., 2002). However, Thiele et al. (2003) recently showed that proteolytic degradation of gluten proteins and depolymerization of the gluten macropolymer can be mainly attributed to dough pH and enzyme activity.

The sourdough lactobacilli studied most intensely include the species *Lactobacillus sanfranciscensis*, *L. reuteri* and *L. pontis* isolated from traditional and modern rye and wheat fermentations (Vogel et al., 1999). The metabolism of strains of these species has been characterised with respect to their utilization of maltose (Stolz et al., 1993), electron acceptors (Stolz et al, 1995a, 1995b), arginine metabolism (Hammes et al, 1996) and proteolytic activity (Gobbetti et al., 1996). Following these investigations the competitiveness of heterofermentative lactobacilli in sourdough can be explained mainly by their combined use of maltose and electron acceptors (Vogel et al., 1999). Maltose is continuously delivered by flour amylases and/or by *Lactobacillus amylovorus* present in type II doughs. While the growth requirements of lactobacilli and yeasts with respect to pH, temperature and organic acids (Gänzle et al., 1998) as well as metabolic interactions between these organisms (Gobbetti, 1998) may contribute to their stable coexistence in sourdough, a symbiotic role of yeasts could further be the supply of fructose (Saunders et al., 1972), which is used as electron acceptor by the lactobacilli to increase their energy yield. The practical relevance of this interaction is the change in the lactate/acetate ratio affecting the baking and sensorial properties of sourdough bread (Röcken et al., 1992; Martinez-Anaya et al., 1994). Several bacteriocins produced by *L. reuteri* have been characterised and strains of *L. sanfranciscensis* have been reported to produce inhibitory activity that was attributed to proteinaceous compounds (Corsetti et al., 1996). Growth of *L. sanfranciscensis* in wheat flour hydrolysate results in formation of acetic and caproic acids, which exert an inhibitory effect against moulds (Corsetti et al., 1998). Some strains of *L. reuteri* isolated from sourdough produce antagonistic compounds not fitting the general bacteriocin definition. Reuterin (2-hydroxy-propanal) is a product of glycerol metabolism by *L. reuteri*, resulting in inhibition of a wide range of Gram-positive as well as Gram-negative organisms. Furthermore, *L. reuteri* LTH2584 produces reutericyclin, a novel antibiotic with a molecular weight of 349 (Gänzle, 1998). This compound is active against a broad range of Gram-positive bacteria including spoilage organisms and pathogens as *Staphylococcus aureus*, *Enterococcus faecalis*, *Listeria monocytogenes* and *Bacillus cereus*, as well as vegetative cells and spores of rope forming

bacilli. Recently, it has been suggested that reutericyclin production gives a strong competitive advantage to strains of *L. reuteri*, and therefore is responsible for their persistence over many years in industrial sourdough fermentations (Gänzle, 2004; Gänzle and Vogel, 2003).

The use of sourdough results in the improvement of the quality of the resulting bread. Through the metabolism of the yeasts and LAB the properties of the flour are improved, as well as aroma, taste, nutritive value and shelf life of the bread. The flavour of sourdough bread depends on many factors such as ingredients (Kenny et al., 2000), additives (Ravi et al.,2000), sourdough fermentation, leavening of the bread dough (Vollmar and Meuser, 1992), as well as the baking process (Morad and D´Appolonia, 1980). During the sourdough fermentation, metabolic products are produced by LAB and the yeasts, e.g. acetic acid, lactic acid and ethanol, which contribute to the bread flavour. Of particular interest as aroma precursors are the free amino acids and the peptides that take part to the Maillard reactions (Ames, 1990). Physicochemical changes (e.g. staling, firming) and microbiological spoilage (e.g. mould growth) reduce the shelf life of bread. The retrogradation of starch toward a more crystalline form is considered the primary cause of bread staling (Gray and Bemiller, 2003). Acidification by sourdough LAB, microbial hydrolysis of starch and protelolysis affect physicochemical changes throughout bread storage including a positive effect in delaying both bread firmness and staling. The effect of biological acidification depends on the level of acidity produced and/or the sourdough LAB strains (Gil et al., 1997; Armero and Collar, 1998; Corsetti et al., 2000).

Strains of species detected in type II sourdough fermentations are frequently found in the intestinal tracts of mammals and birds (Kurzak et al., 1998; Leser et al., 2002; Tannock et al., 2000; Walter et al., 2001). In particular, virtually all species of lactobacilli detected in pig intestines are also recognized as organisms predominating in type II sourdough fermentations (Böcker et al., 1995; Leser et al., 2002; Simpson et al., 2000; Vogel et al., 1999), although evidence for the occurrence of *L. pontis* and *Lactobacillus panis* in pig intestines is based on culture-independent techniques only. The presence of identical *Lactobacillus* species in both sourdough and intestine raises the question as to the genetic background responsible for such ecological fitness. Recently, it was shown that formation of exopolysaccharides (EPS) was most frequently found in intestinal isolates as well as type II sourdough strains of the species *L. reuteri*, *L. pontis*, and *L. frumenti* (Tieking et al., 2003). In this study, the authors showed that strains of these *Lactobacillus* species are able to produce the EPS during the sourdough

fermentation (*in situ*). EPS from lactobacilli may positively influence the intestinal biota, because oligofructose and fructans of the levan- and inulin-types are known to selectively stimulate the growth of bifidobacteria (Bouhnik et al., 1999; Marx et al., 2000; Dal Bello et al., 2001). Possible health benefits achieved through stimulation of the growth and metabolism of bifidobacteria by dietary oligofructose or fructans have been proposed (Fiordaliso et al., 1995; Le Blay et al., 1999; Molis et al., 1996; Roberfroid, 1996; Taper and Roberfroid, 1999; Yamamoto et al., 1999). Remarkably, the levan produced by the sourdough strain *L. sanfranciscensis* LTH2590 is metabolized by bifidobacteria (Korakli et al., 2002) and have been shown to selectively stimulate the growth of bifidobacteria *in vitro* (Dal Bello et al., 2001). The links between type II sourdough and intestinal microbiota on the levels of species composition and EPS production may prove to be helpful for the further development of pre- and probiotic concepts, as recently suggested by Tieking et al. (2002).

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Chapter III

Increased complexity of the species composition of lactic acid bacteria in human feces revealed by alternative incubation condition

Abstract

Denaturing gradient gel electrophoresis (DGGE) of DNA fragments generated by PCR with primers specific for lactic acid bacteria (LAB) was applied to investigate various media and incubation conditions to recover LAB from human faeces. Samples were plated on selective and non-selective media and incubated under standard condition (37°C, anaerobiosis) for faecal LAB as well as alternative condition (30°C, 2% O_2). PCR-DGGE analyses of resuspended bacterial biomass (RBB) obtained from agar plates revealed that the species composition of the recovered LAB was affected stronger by the incubation condition than by the used medium. It was observed that food-associated LAB, such as *Lactobacillus sakei* and *Leuconostoc mesenteroides*, hitherto not described as intestinal inhabitants, are more easily selected when the alternative incubation condition is used. Identification of randomly picked colonies grown under the alternative condition showed that *L. sakei* is one of the predominant food-associated LAB species, reaching counts of up to 10^6 CFU/g faeces. Comparison of the results of bacteriological culture with those obtained by PCR-DGGE analysis of the RBB showed that investigation of RBB is a fast and reliable method to gain insight into the species composition of culturable LAB in faeces.

Dal Bello, F., J. Walter, W. P. Hammes, and C. Hertel. 2003. Increased complexity of the species composition of lactic acid bacteria in human feces revealed by alternative incubation condition. Microbial Ecology 45:455-63.

Introduction

The human intestine is colonized by a complex microbiota (23). Characterization of its composition using modern molecular methods revealed that a large portion of the bacterial community has not yet been cultivated (24). Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) of 16S rDNA amplicons have proven to be suitable tools for the analysis of the intestinal microbiota including non-cultured bacteria. They permit the detection of species and changes in community structure both quickly and economically (27, 29). Briefly, total bacterial DNA from the habitat of interest is extracted and a region with variable nucleotide base sequence of the 16S rRNA gene is amplified by PCR. The resulting mixture of 16S rDNA fragments is subjected to electrophoresis on a denaturing gradient, established in a polyacrylamide gel with urea and formamide or increasing temperature, in order to separate the fragments and generate a 'genetic fingerprint' of the community (17).

The presence of lactic acid bacteria (LAB) in samples taken from faeces or sections of the intestinal tract has traditionally been shown by bacteriological culture. However, only a relatively limited number of species, in particular those belonging to the genus *Lactobacillus*, can be routinely detected (15, 21, 23). Some *Lactobacillus* species, especially those used for probiotic products have received considerable attention because of their putative health-promoting properties (20, 26). Analysis of parts of the 16S rRNA gene amplified directly from DNA of human faecal samples using group specific PCR primers in combination with DGGE revealed that food-associated LAB species, e.g. *Lactobacillus sakei*, *Lactobacillus curvatus*, *Leuconostoc mesenteroides*, *Leuconostoc argentinum*, *Pediococcus pentosaceus*, and *Pediococcus acidilactici* are present regularly (12, 27). By comparison of PCR-DGGE results with those of traditional bacteriological culture it became evident that most of these species cannot be cultured from faecal samples by plating on Rogosa agar and incubating at 37°C (27). As PCR-DGGE also detects dead cells, it is not possible to make conclusions about the viability of the bacteria (17). So far, nearly all bacteriological studies relied on the use of selective media such as Rogosa, acidified MRS or LAMVAB to detect faecal LAB (19). These selective media may exert a stress on certain strains, and furthermore the commonly used incubation temperature of 37°C for culturing might not be optimal for food-associated bacteria, thus generating a bias in the interpretation of the bacterial composition of the faecal microbiota.

In this study we describe the application of standard and alternative incubation conditions in combination with various media to recover LAB from human faecal samples. The species composition of faecal samples as well as that of resuspended biomass from faecal bacteria grown on agar plates used in culturing experiments were determined by PCR-DGGE. Moreover, to identify the predominant culturable LAB, the 16S rDNA from randomly picked colonies grown on agar plates after incubation at alternative condition were partially sequenced to identify the isolate to the species level.

Methods

Bacterial strains, media and growth conditions

Growth studies were performed using MRS (Difco), Rogosa SL (Difco) and LAMVAB (10) medium as well as the following bacteria: *L. curvatus* DSM 20019T, *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 20081T, *Lactobacillus fermentum* DSM 20052T, *Lactobacillus paracasei* DSM 5622T, *Lactobacillus plantarum* DSM 20174T, *L. sakei* DSM 20017T, *Lactobacillus reuteri* DSM 20016T, *Lc. mesenteroides* ssp. *cremoris* DSM 20346T, and *P. pentosaceus* DSM 20336T. For construction of the DGGE identification ladder the following strains were used: *Lactobacillus* acidophilus DSM 20079T, *L. curvatus* DSM 20019T, *Lactobacillus crispatus* DSM 20584T, *Lactobacillus gasseri* DSM 20243T, *L. plantarum* DSM 20174T, *L. reuteri* DSM 20016T, *Lactobacillus ruminis* DSM 20403T, *L. sakei* DSM 20017T, *Lactobacillus salivarius* ssp. *salicinius* DSM 20554T, and *Lc. mesenteroides* ssp. *cremoris* DSM 20346T. Human faecal samples were cultivated on the following media: Rogosa SL agar, LAMVAB agar, MRS agar (non-acidified, pH 6.2), Columbia Blood Agar (5) supplemented with 5.0 g glucose per litre, 2.0 g K_2HPO_4 per litre, 0.2 g $MgSO_4$ x $7H_2O$ per litre, and 0.05 g $MnSO_4$ x $4H_2O$ per litre (pH 5.8), and modified Rogosa agar without sodium acetate but supplemented with 2.5 g of each fructose and maltose per litre (pH 5.8). Media were incubated at 37°C under anaerobic conditions (2% H_2, 10% CO_2 and 88% N_2) as well as at 30°C under a modified atmosphere (2% O_2, 10% CO_2 and 88% N_2).

Investigation of faecal samples

Faecal samples were obtained from five healthy subjects who received no antibiotic treatment for at least 6 months prior to this study and had no restriction on their diet, except for omitting probiotic products. Samples from two studies were investigated. In the first study faecal samples were obtained from four subjects (I, II, III, IV; two female, two male) aged 25 – 38 years. Freshly collected samples were placed in an anaerobic glove box. An aliquot (1 g) was diluted 10 fold in cryoprotective broth (3) and stored at -80°C for DNA extraction and PCR-DGGE at a later opportunity. Another aliquot (1 g) was serially diluted as described previously (27), plated on the media described above and incubated under both the conditions investigated in this study. After 48 h of incubation, the bacterial counts from the different media were determined. The bacterial biomass from the plates of all the different media, on which the 10^{-2} dilution was plated, was harvested with a sterile spreader using 4 ml of cryoprotective broth. This resuspended bacterial biomass (RBB) was stored at -80°C (4) for PCR-DGGE analysis at a later stage.

In the second study (6 months later) faecal samples were collected from five subjects (subjects I to IV and an additional subject V, aged 24, male). The samples were treated and investigated as described for the first study with the exception that only Rogosa SL agar was used. In addition, 20 to 30 colonies were picked randomly from agar plates that contained a total of 30 to 300 colonies and which were incubated at 30°C under modified atmosphere conditions. The isolates were subcultured and subjected to PCR-DGGE analysis and 16S rDNA sequencing.

PCR-DGGE analysis and 16S rDNA sequencing

DNA was extracted from faecal samples, RBB and the subcultured isolates as described previously for faecal samples (27) with the following modification: for the isolates the initial washing steps were omitted. PCR with specific primers Lac1-Lac2GC and the subsequent DGGE were performed as described previously (27). The bands in the profile were identified by comparing the migration distances of the amplicons in DGGE gels with those of the identification ladder (28). In addition, the DNA fragments were excised, purified, and sequenced as described previously (27). At least 3 isolates showing identical DGGE patterns were further analyzed by sequencing of the first 900 bp of the 16S rDNA (14). To determine the closest relatives of the partial 16S rDNA sequences, a GeneBank DNA database search

was conducted. A similarity of > 98% to 16S rDNA sequences of type strains was used as the criterion for identification.

Results

Cultivation of LAB from human faecal samples

To investigate the ability to cultivate food-associated LAB under the standard incubation condition (37°C, anaerobiosis) used for recovering LAB from faecal samples, growth studies were performed with various LAB type strains using MRS, Rogosa, and LAMVAB media. Neither *L. sakei* nor *Lc. mesenteroides* ssp. *cremoris* grew on these agar plates. In addition, *L. delbrueckii* ssp. *bulgaricus* did not grow on LAMVAB medium. Since most food-fermenting LAB are routinely cultivated at lower temperatures and under semi-anaerobic condition, we investigated the growth of such bacteria under alternative conditions (30°C in an atmosphere of 2% O_2, 10% CO_2 and 88% N_2). Using this alternative incubation condition, *L. sakei* and *Lc. mesenteroides* ssp. *cremoris* grew well on MRS medium but only poorly on Rogosa and LAMVAB media. LAMVAB medium failed again to support the growth of *L. delbrueckii* ssp. *bulgaricus*. The type strains of the species *L. curvatus*, *L. fermentum*, *L. paracasei*, *L. plantarum*, *L. reuteri*, and *P. pentosaceus* grew well on all media and at both incubation conditions.

In the first study we investigated the ability to cultivate LAB from faecal samples of 4 healthy subjects (I to IV) on the selective media Rogosa and LAMVAB as well as on the poorly selective medium MRS under both incubation conditions. Furthermore, we used the non-selective Columbia Blood agar and modified Rogosa (without sodium acetate) media, as these should not exert stress on the bacteria. As shown in Table 1, comparisons of bacterial cell counts revealed that highest numbers were recovered on the non-selective media incubated anaerobically at 37°C. On the other hand, similar counts were obtained for the selective media (Rogosa and LAMVAB), independent from the incubation temperature and atmosphere, and the non-selective media under the alternative incubation conditions. The results suggest that the alternative incubation condition (30°C, 2% O_2) contributes to the inhibition of most of the intestinal microbiota apart from the LAB and other facultative anaerobes.

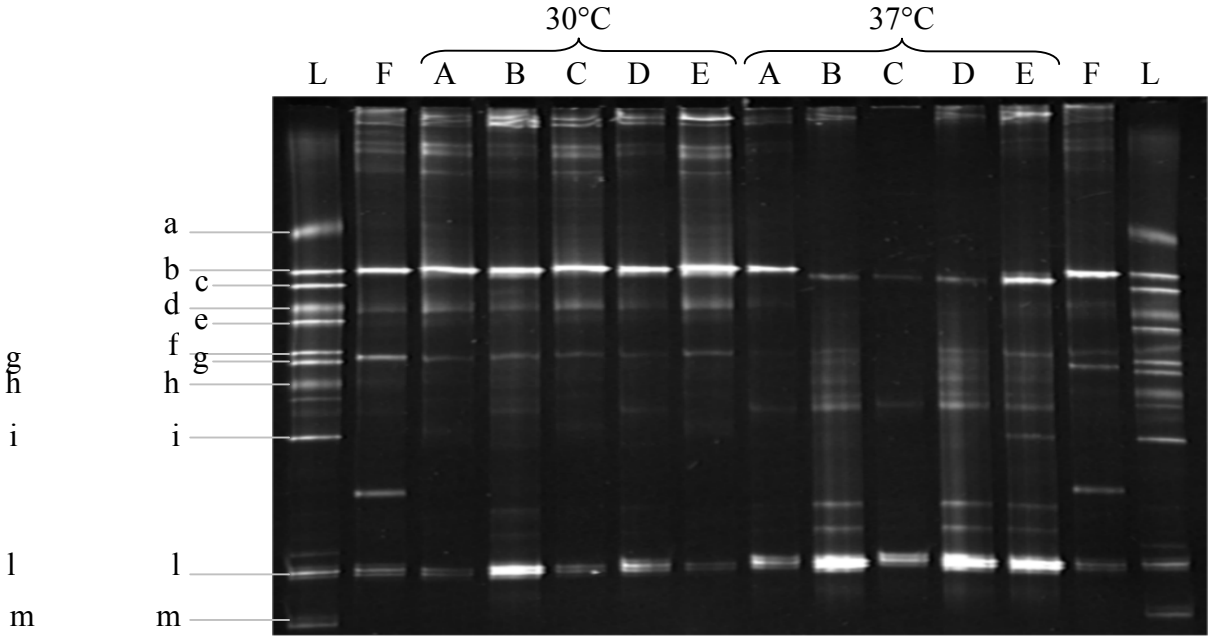

Figure 1. PCR-DGGE analysis of 16S rDNA fragments generated by PCR with primer pair Lac1-Lac2GC and DNA isolated from faecal samples (F) of subject I and the corresponding RBB from MRS (A), Rogosa (B), Rogosa modified (C), LAMVAB (D), and Columbia Blood Agar (E). L, identification ladder comprising the following type strains: a, *L. plantarum*; b, *L. sakei*; c, *L. curvatus*; d, *L. gasseri*; e, *L. acidophilus*; f, *L. crispatus*; g, *L. salivarius*; h, *Lc. mesenteroides* ssp. *cremoris*; i, *L. ruminis*; l, *L. paracasei*; m, *L. reuteri*. The bands indicated by arrows were excised and identified by sequencing (for results see Table 2).

Table 1. Bacterial counts (log CFU/g) of faecal samples determined under different incubation conditions.

	30°C with 2% O_2					37°C, anaerobiosis				
Subject	Rogosa SL	LAMVAB	MRS	Columbia Blood Agar	Rogosa modified	Rogosa SL	LAMVAB	MRS	Columbia Blood Agar	Rogosa modified
I	6.2	6.1	6.4	6.5	6.7	6.0	6.0	9.7	9.7	9.4
II	6.1	6.1	7.2	7.1	7.3	7.0	6.3	8.1	10.3	9.1
III	2.6	2.5	7.9	7.8	8.1	4.0	n.d.*	7.5	10.0	9.0
IV	4.7	4.8	5.6	5.6	5.6	4.7	4.7	10.1	10.4	9.7

* n.d., not detected.

PCR-DGGE analysis of faecal samples and RBB

DNA was isolated from the faecal samples (subject I to IV) and RBB obtained from the different media. To rapidly identify the LAB grown under the selected conditions and present in the faecal samples, the extracted DNA was subjected to PCR-DGGE in combination with primers specific for the genera *Lactobacillus, Pediococcus*, *Leuconostoc*, and *Weissella*. The results obtained from subject I are shown in Fig. 1 as an example, and in Fig. 2 the results are shown for all of the subjects and using Rogosa medium. The results are also summarized in Table 2, which shows the subject-specific composition of the LAB microbiota.

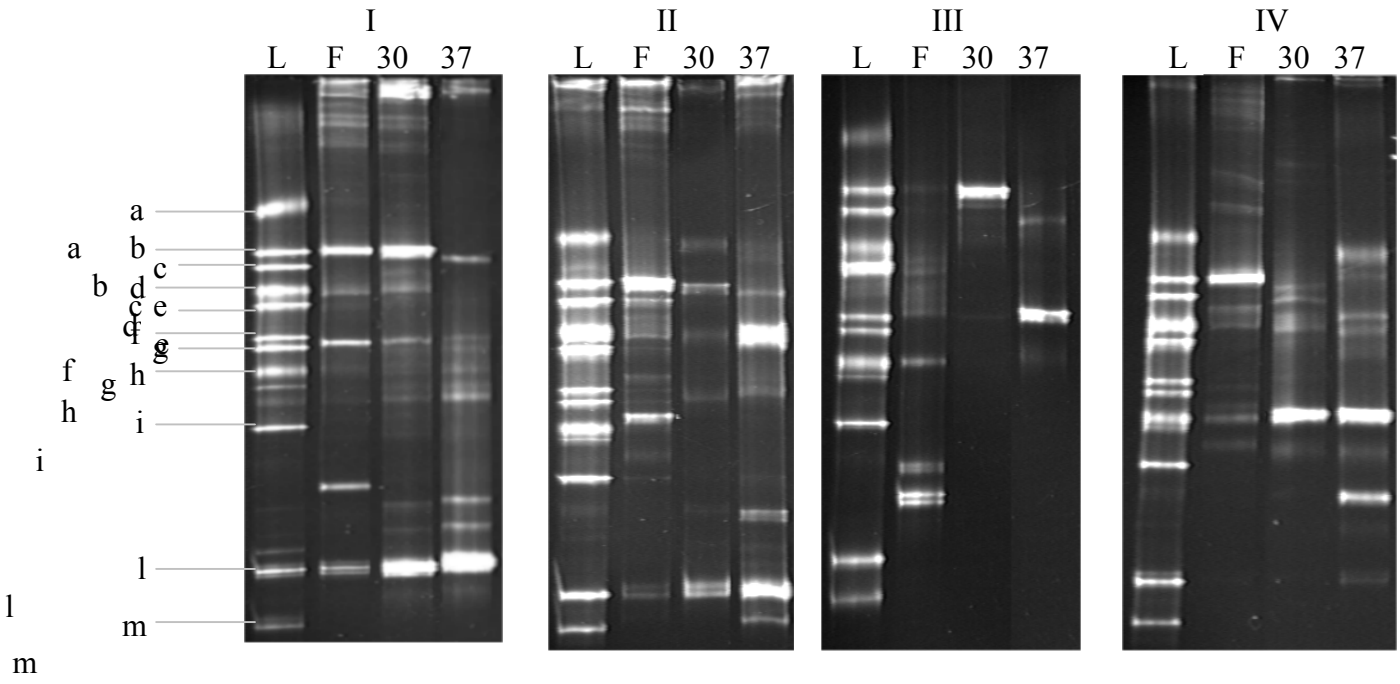

Figure 2. DGGE analysis of PCR-amplified 16S rDNA fragments obtained with primer pair Lac1-Lac2GC and DNA isolated from faecal samples (F) and RBB from Rogosa at 30°C with modified atmosphere (30) and 37°C in anaerobiosis (37) from subjects I to IV. L, identification ladder (see Fig. 1). The bands indicated by arrows were excised and identified by sequencing (for results see Table 2).

Table 2. Species of LAB detected by PCR-DGGE in faecal samples and the RBB obtained from all of the five different media.

Subject	Bacterial species [c]	PCR-DGGE analysis of		
		faecal samples	RBB from agar plates incubated at 30°C with 2% O_2	RBB from agar plates incubated anaerobically at 37°C
I	*L. sakei*	+	+	- (+[d,e])
	L. brevis	-	-	+
	L. delbrueckii ssp. *bulgaricus*	+	+	+
	L. parabuchneri	-	-	+[f]
	Lc. carnosum[a]	+	-	-
	L. casei group[d]	+	+	+
II	*L. plantarum* group[d]	-	+	-
	L. sakei	+	+	+(-[g])
	L. curvatus [d]	+	-	-
	L. gasseri	+	+	+
	L. delbrueckii ssp. *lactis*	+	-	+[f]
	P. acidilactici[b]	-	-	+
	L. casei group[d]	+	+	+
	L. reuteri[d]	-	-	+
III	*L. sakei*	-	+	-
	L. crispatus	-	-	- (+[h])
	Lc. mesenteroides[a]	+	+	-
	Lc. gelidum[a]	+	-	-
	Lc. carnosum[a]	+	-	-
IV	*L. sakei*	+	-	-
	P. pentosaceus[b]	+	+	+
	P. acidilactici[b]	-	-	+[i]

[a] *Lc.*, *Leuconostoc*;

[b] *P.*, *Pediococcus*

[c] Species were identified by sequencing of the DNA fragments upon excision from the gel

[d] Identified by comparison of the DGGE profiles with those obtained from reference strains

[e] Exclusively on MRS

[f] Exclusively on Columbia Blood Agar

[g] Not on Rogosa

[h] Exclusively on Rogosa

[i] Not on LAMVAB

Not all of the species present in faeces were also detected in the RBB, e.g. *L. curvatus* (subject II), *Leuconostoc gelidum* (subject III), *Leuconostoc carnosum* (subjects I and III) and *L. sakei* (subject IV). Furthermore, differences were noted for the two growth conditions (30°C, 2% O_2 and 37°C, anaerobiosis). For example, *Lc. mesenteroides* (subject III), *L. plantarum* (subject II) and *L. sakei* (subject III) were detectable exclusively in the RBB from 30°C. For subject I, *L. sakei* was detectable in the RBB from all media at 30°C but at 37°C exclusively in the RBB from MRS medium. For subject II, growth of *L. sakei* occurred at 30°C but not at 37°C in the RBB from Rogosa medium. For subject III, *L. crispatus* was detectable in the RBB from Rogosa at 37°C only. This species was probably overgrown on the non-selective media by total anaerobic members of the intestinal microbiota and did not grow on LAMVAB (Table 1). For subject I and II, *Lactobacillus parabuchneri* and *L. delbrueckii* ssp. *lactis*, respectively, were detectable only in the RBB of Columbia Blood Agar (37°C). These results demonstrated that the influence of the modified atmosphere has a more pronounced effect on the growth of faecal LAB than the different media. Furthermore, food-associated species such as *L. sakei* and *Lc. mesenteroides* can be more easily selected by the alternative incubation condition. Both species were detected in the RBB obtained from all of the five media, although the type strains grew poorly on Rogosa and LAMVAB media (see above).

Quantification of LAB species grown under the alternative incubation condition

To quantify the culturable LAB species, which predominate after incubation under the alternative incubation condition, a second study was performed with faecal samples from five subjects (including subject I to IV which participate in the first study). For subjects I and II similar cell counts were determined on Rogosa agar under both incubation conditions, whereas for the other subjects the cell counts were higher under the standard rather than the alternative incubation condition. Colonies were randomly picked from agar plates incubated under the alternative incubation condition and subjected to species identification. The results are summarized in Table 3. *L. sakei* was recovered from 3 subjects as one of the predominant species of the LAB community selected on Rogosa agar. The estimated cell counts were in the order of 10^6 and 10^4 CFU/g faeces for subjects II and I, respectively. *Weissella cibaria/kimchii* was isolated from subject I, and remarkably, both species have previously not been detected in faecal samples. All the remaining isolates belong to species considered to be associated with the intestinal content. For three subjects (I, II, and V) PCR-DGGE analysis was performed using the DNA isolated from the faecal samples as well as the RBB obtained from the Rogosa agar plates (Fig. 3).

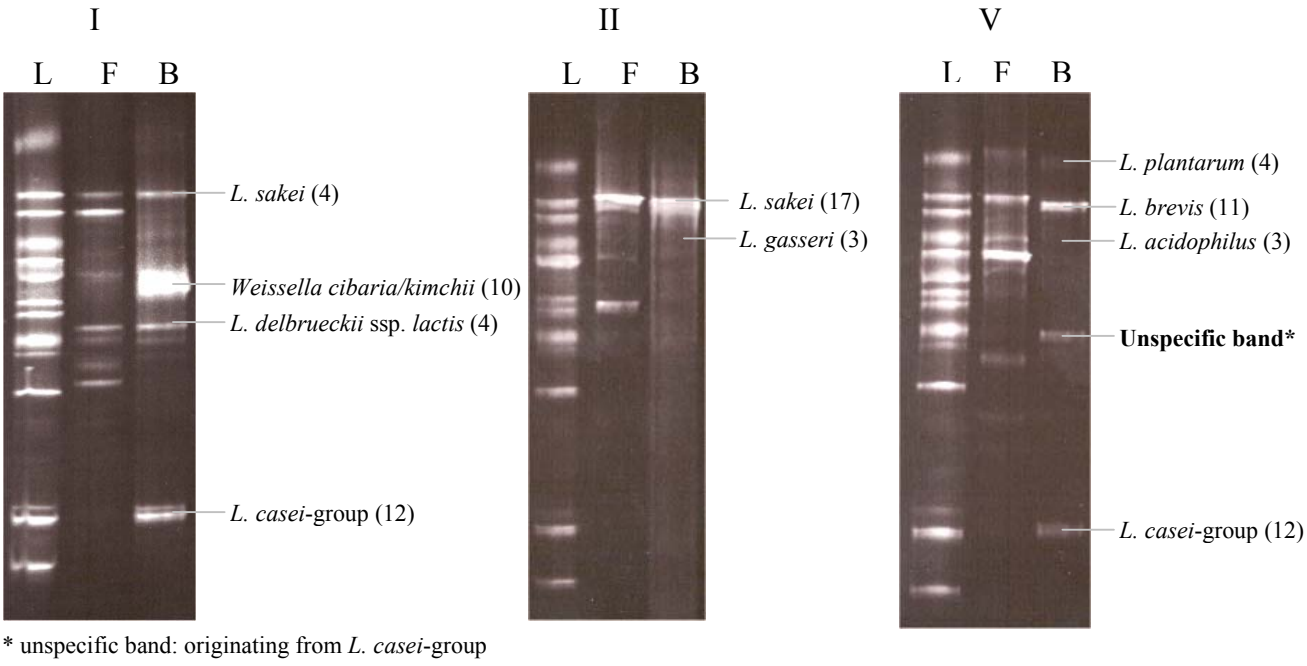

Figure 3. PCR-DGGE analysis of 16S rDNA fragments generated by PCR with primer pair Lac1-Lac2GC and DNA isolated from faecal samples (F) of subjects I, II, and V and the RBB obtained from Rogosa agar (B) incubated at 30°C. Species were identified by comparison of the PCR-DGGE patterns of the RBB with those obtained from the single isolates which were identified by 16S rDNA sequence analysis. The number of randomly picked colonies allotted to the corresponding species are given in parenthesis (see also Table 3).

Comparison of the results with those of bacteriological culture (Table 3) revealed that all cultured LAB species were detected, and no additional major band became visible. Thus, the specific PCR-DGGE analysis of RBB obtained from agar plates provides a representative view of the predominant LAB species growing on the plates. As observed in the preceding study, the DGGE patterns obtained from faecal samples differed from those of the RBB. Comparison of the DGGE patterns obtained from the faecal and RBB samples of subject I-IV with those obtained in the first study revealed a subject specific variation in composition and stability of the LAB community (data not shown), which is in agreement with previous observations (12, 27).

Table 3. Bacterial cell counts of faecal samples and species identification of randomly picked colonies from Rogosa agar plates incubated under the alternative incubation condition (30°C, 2% O_2).

Subject	Total counts (log CFU/g) [a]	Number of investigated colonies	Species [c]	Share of colonies	Estimated counts [e] (log CFU/g)
I	5.2 (5.6)	30	*L. sakei*	4	4.3
			Weissella cibaria/kimchii[d]	10	4.7
			L. delbrueckii ssp. *lactis*	4	4.3
			L. casei-group	12	4.8
II	6.2 (7.4)	20	*L. sakei*	17	6.1
			L. gasseri	3	5.4
III	2.3 (5.5)	2 [b]	*L. sakei*	2	2.3
IV	2.5 (7.8)	7 [b]	*L. casei*-group	6	2.4
V	4.9 (7.6)	30	*L. plantarum*	4	4.0
			L. brevis	11	4.5
			L. acidophilus	3	3.9
			L. casei-group	12	4.5

[a] Counts determined under standard condition (37°C, anaerobiosis) are given in parenthesis

[b] Number of colonies was limited due to the low cell counts

[c] Species identification based on the sequencing of the first 900 bp of the 16S rDNA

[d] No differentiation was possible on the basis of the partial 16S rDNA sequence

[e] Counts were estimated on the basis of the total counts and the share of the identified colonies within the total investigated colonies

Discussion

Studies of LAB in the human intestinal tract have mainly focused on the genus *Lactobacillus*, and their results suggest that only a few species (*L. ruminis*, *L. salivarius*, *L. reuteri, L. gasseri*) are truly autochthonous (21, 24). Other species such as *L. plantarum*, *L. brevis*, *L. fermentum* and members of the *L. casei*-group are considered to be transient and to probably originate from food. Our studies showed that the species composition of viable LAB is more complex than previously recognized. We demonstrated that additional food-associated LAB, in particular *L. sakei* and *Lc. mesenteroides*, can be cultivated from human fecal samples when an alternative incubation condition is used (30°C, 2% O_2). These species have so far neither been described as intestinal inhabitants, nor cultured from faecal samples, but have previously been detected by PCR-DGGE in the faeces of humans from different geographical origin (12, 27). In most subjects of this study we identified *L. sakei* and occasionally *Lc. mesenteroides* as the predominant LAB species growing under the alternative incubation condition (Table 3). *Lc. mesenteroides* was detected in the faeces of subject III by culturing and PCR-DGGE exclusively in the first study, indicating fluctuations in the occurrence of this species. In some subjects, *L. sakei* as well as the food-associated *L. curvatus*, *Lc. carnosum* and *Lc. gelidum* were detected by PCR-DGGE as predominant species in the faecal samples, but these could not be detected in the RBB (Table 2). The target DNA may originate from living cells in a non-culturable state or from dead cells, or is released from cells that have lysed in the intestinal tract. Our results are consistent with the assumption that a large amount of LAB passing the gastrointestinal tract in the digesta remains viable. These organisms may even have an impact on the consuming individuals, which is comparable to that of probiotics, e.g. by affecting the immune system and the health of the host (6, 7, 16, 18).

By using bacteriological culture we showed that *L. sakei* is one of the predominant food-associated *Lactobacillus* species that occurs in human faeces and at counts as high as 10^6 CFU/g (subject II). Our finding is supported by the results of PCR-DGGE analysis of faecal samples (12, 27) because this method detects the 90 to 99% most numerous species in the LAB community. This may be explained by the frequent occurrence of this species in the food environment. *L. sakei* has been isolated from meat, sausages, fermenting sake, and sauerkraut, and is one of the major spoilage organisms for vacuum-packaged meat products (9). In addition, it is an important component of starter cultures used for production of fermented meat products, in which high counts of approximately 10^9 CFU/g are observed (8). Thus, all these foods may contribute to an uptake of *L. sakei* into the intestinal tract and,

depending on the diet, fluctuations may occur as was previously observed by PCR-DGGE analysis (27).

For analysis of the species composition we used the LAB-specific PCR-DGGE analysis of RBB. Comparison of the results obtained with those from identification of randomly picked isolates showed that the investigation of RBB represents a fast and reliable method to gain insight into the species composition of the culturable LAB of faecal samples. We also used this technique to compare different media for the cultivation of LAB from faecal samples under different incubation conditions. In general, the same LAB composition was detected on all media under identical incubation conditions, indicating that the selective principles of Rogosa and LAMVAB media exert only little effect on the growth of LAB. However, exceptions noted were *L. sakei*, which did not grow on Rogosa at 37°C (subject II), and *L. crispatus*, which was not detected on LAMVAB at 37°C (subject III). Our observation that LAMVAB did not support the growth of all the lactobacilli is in agreement with that of Hartemink and Rombouts (11) and Jackson *et al.* (13). In addition, these authors found that Rogosa is not suitable for the enumeration of lactobacilli after incubation for 72 h, as most of the isolates were identified as bifidobacteria. In fact, other research groups have used Rogosa medium for enumeration of bifidobacteria after 96 h of incubation and described that the medium is highly selective for lactobacilli after 48 h of incubation only (24, 27). Finally, in our study *L. delbrueckii* ssp. *lactis* and *L. parabuchneri* were detected only in the RBB from Columbia Blood Agar at 37°C. This medium is described to have the potential to recover fastidious or sub-lethally damaged bacteria (2).

Recent molecular studies have revealed that a substantial part of the intestinal microbiota can not be cultured under laboratory conditions and has therefore never been investigated (25). Nevertheless, these non-cultured bacteria may have an impact on the health of the host (1). To increase our understanding of the role of these currently unknown bacteria in health and disease, they need to be cultured to enable such novel investigations. PCR-DGGE analysis of RBB constitutes a suitable tool for the design of alternative media and culture conditions that will finally support the growth of the hitherto non-cultured bacteria.

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Chapter IV

Oral cavity as natural reservoir for intestinal lactobacilli

Abstract

Ecological studies indicate that most *Lactobacillus* species found in the human gastrointestinal tract are likely to be transient (allochthonous), originating from either the oral cavity or food. In order to investigate if oral lactobacilli constitute a part of the fecal *Lactobacillus* biota, the *Lactobacillus* biota of saliva and feces of three human subjects were investigated and compared at two time-points in a three months interval. Bacteriological culture, performed by incubation under standard (37°C, anaerobic) and alternative (30°C, microaerobic) conditions, as well as PCR-DGGE with group-specific primers were used to characterize the predominant lactobacilli. Cell counts varied among the subjects and over time, reaching up to 10^7 CFU/ml in saliva and 5 x 10^6 CFU/g in fecal samples. The species composition of the *Lactobacillus* biota of human saliva and feces was found to be subject-specific and fluctuated to some degree, but the species *Lactobacillus gasseri*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus* and *Lactobacillus vaginalis* were detected at both time-points in saliva and fecal samples of individual subjects. RAPD-PCR analysis indicated that several strains of these species were present both in the oral cavity and in the fecal samples of the same subject. Oral isolates of the species *L. gasseri* and *L. vaginalis* showing identical RAPD types were found to persist over time, suggesting that these species are autochthonous to the oral cavity. Our results together with recent published data give strong evidence that some lactobacilli found in human feces are allochthonous to the intestine and originate from the oral cavity.

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Introduction

The microbiota of the human gastrointestinal tract (GIT) constitutes a complex community (28, 29). More than 400 different bacterial species have been identified, with population levels of up to 10^{11} cells/g feces (wet weight) (21, 30). Species of the genus *Lactobacillus* can be cultivated from human feces with cell counts of up to 10^9 CFU/g (15, 20, 31). Investigation of the *Lactobacillus* population over extended periods of time has revealed marked variations in cell counts as well as species composition among human subjects (3, 27, 31, 32). Sixteen *Lactobacillus* species are commonly isolated from fecal samples (4), but it has been suggested that only the species *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus reuteri*, *Lactobacillus ruminis*, and *Lactobacillus salivarius* are truly autochthonous to the human GIT (25, 31). The remaining *Lactobacillus* species are detected transiently and unpredictably, and therefore are considered allochthonous organisms.

Allochthonous lactobacilli are introduced regularly into the GIT because they are ubiquitous in nature, especially in association with fermented and non-fermented foods (12). Thus, depending on individual consumption habits, these lactobacilli are likely to be transferred day by day through the stomach and small intestine into the large bowel and can be detected in human feces (7, 13, 33). Moreover, lactobacilli are also present in all parts of the human digestive tract, including the mouth, stomach and small intestine (20, 25). They can be detected in human saliva in variable numbers but sometimes attain a population level exceeding 10^6 CFU/ml (1). Considering that the average output of saliva is 1,000 to 1,500 ml per day, it has been suggested that some *Lactobacillus* species detected in the gastrointestinal tract originate from the oral cavity (4). Microbiological studies have shown that the species *Lactobacillus acidophilus* (and closely related species like *L. gasseri* and *L. crispatus*), *Lactobacillus brevis*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus* and *L. salivarius* are the predominant lactobacilli of the oral cavity (2, 17). Remarkably, these species have also been frequently detected in human feces (2, 31, 33, 7), and previous studies indicate that the *Lactobacillus* species composition of the oral cavity and fecal samples coincide to some extent (2, 19).

In order to study if lactobacilli inhabiting the oral cavity are detectable in fecal samples we investigated the *Lactobacillus* biota of saliva and fecal samples obtained from three healthy human subjects at two different time-points. For rapid identification of the predominating *Lactobacillus* species, PCR-DGGE with primers specific for the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Weissella* (33) was applied. Isolates of the dominating

lactobacilli were recovered from saliva and fecal samples and characterized by 16S rDNA sequence and RAPD-PCR analysis. The results confirmed that oral *Lactobacillus* species are also detectable in human feces and, according to the RAPD-PCR, indicated that *Lactobacillus* strains of the human saliva constitute a part of the fecal isolates.

Methods

Media and growth conditions

Lactic acid bacteria (LAB) from fecal and saliva samples were cultivated on Rogosa SL agar (Difco) supplemented with 0.5 g/l bromcresol green. Plates were incubated anaerobically at 37°C (standard conditions) as well as microaerobically (2% O_2, 10% CO_2 and 88% N_2) at 30°C (alternative conditions). Purification and isolation of selected colonies was performed under the appropriate growth conditions on MRS broth and/or agar (Difco) supplemented with 0.5 g/l bromcresol green.

Collection and treatment of fecal and saliva samples

Saliva and fecal samples from three healthy subjects (I, II, III; male) aged 27-31 years were analyzed at two time-points (1 and 2) spaced by three months. Saliva samples (ca. 4 ml) were taken early in the morning before brushing the teeth. The day after, fecal samples were collected. All samples were immediately introduced in an anaerobic glove box, serially diluted as described previously (7), plated in triplicate on Rogosa SL agar containing bromcresol green and incubated under both conditions. In addition, 1 ml of the saliva sample and 1 ml of the 10-fold diluted fecal sample were stored at –80°C for later DNA extraction and PCR-DGGE. After 48 h of incubation of the agar plates, the bacterial counts were determined. From the agar plates on which the lowest dilutions were plated (10^{-1} for saliva; 10^{-2} for fecal samples), the bacterial biomass was harvested with a sterile spreader using 4 ml of cryoprotective broth (6). This resuspended bacterial biomass (RBB) was stored at -80°C for PCR-DGGE analysis at a later stage (10). The agar plates containing 30 to 300 colonies were incubated for further 24 h. Thereafter, for each different colony form up to three isolates were picked, purified and stored for further analysis.

DNA extraction and PCR-DGGE analysis

DNA was extracted from fecal and saliva samples as well as RBBs as described previously (7). PCR with the specific primers Lac1-Lac2GC as well as DGGE were performed as described previously (33). The bands in the profile were identified by comparing the migration distances of the amplicons in the DGGE gels with those of reference strains. Additionally, bands were excised, purified, sequenced and identified as described previously (33).

Characterization and comparison of the isolates

The recovered isolates were subjected to DNA extraction using the High Pure DNA extraction kit (Roche) and identified by sequence analysis of the first 1000 bp of the 16S rDNA (7). For each subject, isolates belonging to the same species and detected in both saliva and fecal samples were analyzed by RAPD-PCR with the primer M13V (MWG-Biotech) as described by Meroth et al. (18).

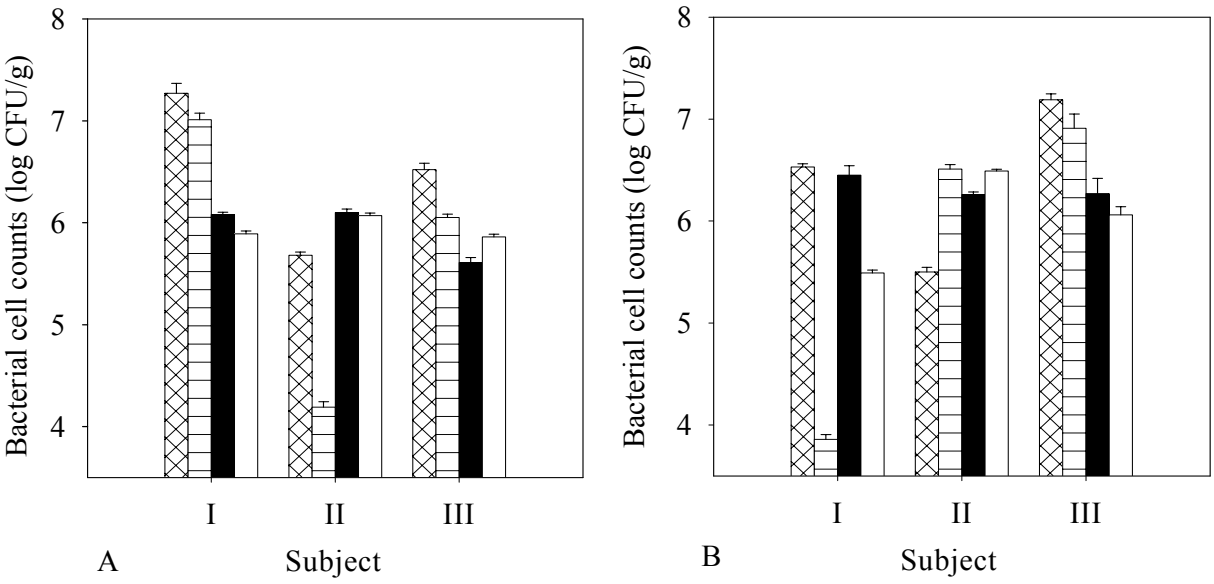

Figure 1. LAB cell counts of human saliva (crossed and horizontal-lined bars) and fecal (black and white bars) samples obtained from the three subjects at two time-points spaced by three months. Rogosa SL agar plates were incubated anaerobically at 37°C (horizontal-lined and white bars) or microaerobically at 30°C (crossed and black bars).

Results

Cultivation of LAB from human saliva and fecal samples

The results of bacteriological culture are depicted in Fig. 1. Cell counts of LAB in fecal and saliva samples differed among the subjects and fluctuated over time. Differences were more pronounced for the saliva samples. In some cases the application of the alternative incubation condition permitted to recover higher cell counts than the use of the standard incubation condition.

PCR-DGGE analysis of saliva and fecal samples

For characterization of the LAB species composition, PCR-DGGE was performed using primers specific for the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella*. DNA was extracted from saliva and fecal samples as well as from RBB consisting of the bacterial biomass grown on the lowest dilution Rogosa SL agar plate. The results, summarized in Table 1, indicated that the LAB species composition is subject-specific and identical species occurred in saliva and feces. In particular, the species *L. gasseri* (all subjects), *L. sakei* (subject I) and *L. vaginalis* (subject I and III), as well as species belonging to the *L. casei* group (subject I and II), were found in both saliva and fecal samples of the same subject.

Table 1. LAB species detected by PCR-DGGE analysis of saliva, fecal samples as well as RBBs.

Subject	Bacterial species	LAB species detected at time-point 1 / time-point 2 by PCR-DGGE analysis of					
		saliva			feces		
		saliva sample	RBB, 30°C microanaerobic	RBB, 37°C anaerobic	fecal sample	RBB, 30°C microanaerobic	RBB, 37°C anaerobic
I	*L. gasseri*	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +
	Lactobacillus casei group	- / -	+ / -	- / +	+ / -	+ / -	+ / +
	Lactobacillus pentosus	- / -	- / -	- / -	- / -	- / -	- / +
	L. salivarius	+ / -	- / -	- / -	- / -	- / -	- / -
	Lactobacillus sakei	- / +	- / +	- / -	- / +	- / +	- / +
	Lactobacillus vaginalis	- / -	+ / -	+ / +	- / -	+ / -	+ / +
II	*Lactobacillus fermentum*	+ / +	+ / +	+ / +	- / -	- / -	- / -
	L. gasseri	+ / +	+ / -	+ / -	- / -	+ /-	- / +
	L. casei group	- / -	+ / +	+ / +	+ / -	- / +	+ / +
	L. sakei	- / -	- / -	- / -	- / +	- / -	- / -
	L. vaginalis	- / -	- / -	- / +	- / -	- / -	- / -
	Pediococcus pentosaceus	- / -	- / -	- / -	- / -	+ / -	+ / -
III	*Lactobacillus delbrueckii*	- / -	- / -	- / -	- / -	- / -	+ / +
	L. brevis	- / -	- / +	- / -	- / -	- / -	- / -
	L. crispatus	- / -	- / -	- / -	+ / -	- / -	- / -
	L. gasseri	+ / +	+ / +	+ / -	+ / -	+ / -	+ / -
	L. casei group	- / -	- / -	- / -	- / -	+ / -	- / -
	L. sakei	- / -	- / -	- / -	- / +	+ / +	- / +
	L. vaginalis	+ / -	+ / +	+ / +	+ / -	+ / -	+ / -

Table 2. Species of LAB isolated from saliva and fecal samples at time-points 1 and 2 spaced by three months.

Subject	Bacterial species	LAB species detected at time-point 1 / time-point 2 in			
		feces by cultivation at		saliva by cultivation at	
		30°C, microaerobic	37°C, anaerobic	30°C, microaerobic	37°C, anaerobic
I	*L. gasseri*	+ / +	+ / +	- / -	- / +
	L. paracasei	+ / +	+ / -	- / -	- / +
	Lactobacillus pentosus	- / -	- / +	- / -	- / -
	L. rhamnosus	- / -	- / +	- / -	- / -
	L. sakei	- / +	- / +	- / -	- / -
	L. vaginalis	+ / -	+ / +	- / -	- / +
	V. atypica	- / -	- / -	- / -	+ / +
II	*L. fermentum*	- / -	- / -	+ / +	+ / +
	L. gasseri	**+ / +**	**- / -**	**+ / -**	**+ / +**
	Lactobacillus parabuchneri	+ / -	- / -	- / -	- / -
	L. paracasei	- / -	- / -	+ / +	+ / +
	L. rhamnosus	+ / +	+ / +	+ / -	- / -
	L. sakei	- / -	- / +	- / +	- / -
	L. vaginalis	- / -	- / +	- / -	- / +
	Pediococcus pentosaceus	+ / -	+ / -	- / -	- / -
	V. atypica	- / -	- / -	- / -	- / +
III	*L. delbrueckii*	- / -	+ / +	- / -	- / -
	L. fermentum	- / -	- / -	- / -	- / +
	L. gasseri	+ / -	+ / -	+ / -	- / -
	L. paracasei	- / -	- / +	- / -	- / -
	L. rhamnosus	+ / -	- / -	- / -	+ / -
	L. sakei	- / +	- / -	- / -	- / -
	L. vaginalis	+ / -	- / -	+ / +	+ / +
	Leuconostoc mesenteroides	- / +	- / -	- / -	- / -
	V. atypica	- / +	- / -	- / -	+ / -

Characterization and comparison of LAB isolates

To investigate the clonal relationships among the LAB from feces and saliva, up to three isolates of each colony form were subjected to species identification by 16S rDNA sequence analysis and typing by RAPD-PCR. As shown in Table 2, species identification of the isolates confirmed the presence of identical *Lactobacillus* species in both saliva and fecal samples, i.e. *L. gasseri* and *L. vaginalis* (all subjects), *L. paracasei* (subject I), *L. rhamnosus* (subject II and III) and *L. sakei* (subject II). Furthermore, for each subject some *Lactobacillus* species were found to predominate at both time-points, e.g. subject II, *L. gasseri* (feces and saliva), *L. rhamnosus* (feces), *L. fermentum* and *L. paracasei* (saliva). Comparison of the results of species identification (Table 2) with those obtained by PCR-DGGE analysis of the RBB (Table 1) confirmed that PCR-DGGE analysis of RBB obtained from agar plates has the potential to give an insight into the viable predominant LAB species. However, for the saliva samples of subject I, PCR-DGGE analysis of RBBs obtained from agar plates incubated microaerobically at 30°C revealed the presence of several lactobacilli (Table 1) although none of the isolates could be allotted to the genus *Lactobacillus* (Table 2). Under the alternative incubation condition, the recovered isolates grown on Rogosa SL agar were found to belong to the genus *Streptococcus* (data not shown). Several *Lactobacillus* species detected by PCR-DGGE analysis of the RBB could not be found among the corresponding isolates, indicating that selection based on the colony form may lead to underestimate the real bacterial diversity of the samples.

The results of RAPD-PCR with the DNA of the *Lactobacillus* isolates belonging to species detected in both saliva and feces and/or persisting over time are summarized in Table 3. For all subjects some isolates of several *Lactobacillus* species were found to exhibit identical RAPD types and to occur in both saliva and fecal samples. The RAPD types of isolates belonging to *L. gasseri* (depicted in Fig. 2 as example), *L. paracasei* or *L. vaginalis* recovered from the saliva sample of subject I (time-point 2) were all detected in the fecal samples. For subject II, the RAPD types of all fecal *L. gasseri* and *L. rhamnosus* type Lr1 isolates were found among the saliva isolates. For subject III, *L. gasseri* type Lg8, *L. rhamnosus* type Lr4 and all the fecal *L. vaginalis* RAPD types were identified during analysis of the saliva isolates. For some *Lactobacillus* species the RAPD types of the isolates obtained from the same compartment changed over the time, e.g. *L. gasseri* and *L. paracasei* isolates (feces, subject I) and *L. vaginalis* (saliva, subject III). On the other hand, in some cases the RAPD types of isolates remained stable during the time, e.g. *L. vaginalis* (feces, subject I) and *L. gasseri* (saliva, subject II).

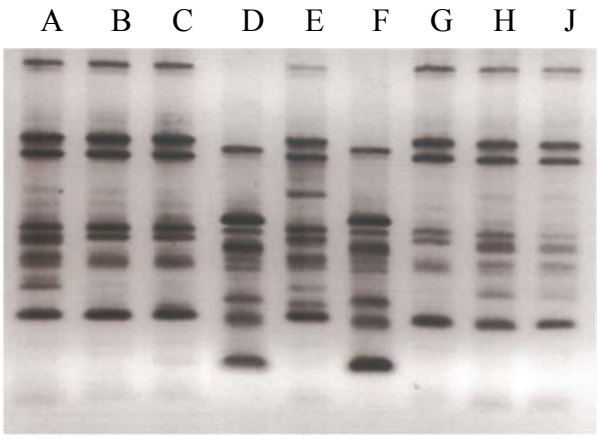

Figure 2. RAPD-PCR profiles of *L. gasseri* isolates recovered from saliva and fecal samples of subject I. A and B, isolated from fecal sample at time-point 1; C, isolated from saliva sample at time-point 1; D to J, isolated from fecal sample at time-point 2.

Table 3. Identification of different strains among the isolates from saliva and fecal samples during study I and study II. Each letter correspond to a different strain/RAPD pattern. In parenthesis: number of isolates showing the same RAPD pattern.

Subject	Bacterial species	RAPD types detected in			
		saliva at		feces at	
		time-point 0	time-point 1	time-point 0	time-point 1
I	*L. gasseri*	-	Lg1	Lg1, Lg2	Lg1, Lg3, Lg4
	L. paracasei	-	Lp1	Lp2, Lp3, Lp4	Lp1
	L. vaginalis	-	Lv1	Lv1	Lv1
II	*L. fermentum*	Lf1, Lf2, Lf3	Lf1, Lf3	-	-
	L. gasseri	Lg5, Lg6, Lg7	-	Lg5, Lg6	Lg5
	L. paracasei	-	Lp5	Lp6, Lp7	-
	L. rhamnosus	Lr1, Lr2	-	Lr3	Lr1
	L. sakei	-	Ls1	-	Ls2, Ls3
	L. vaginalis	-	Lv4	-	Lv2, Lv3
III	*L. delbrueckii*	-	-	Ld1	Ld1
	L. gasseri	Lg8	-	Lg8, Lg9, Lg10	-
	L. rhamnosus	Lr4, Lr5	-	Lr4	-
	L. vaginalis	Lv5, Lv6	Lv6, Lv7, Lv8	Lv5, Lv6	-

Discussion

Using bacteriological culture and PCR-DGGE we showed that the *Lactobacillus* species composition of human saliva coincides to some extent with that of feces (Table 1 and 2). In particular, *L. gasseri*, *L. paracasei*, *L. rhamnosus*, and *L. vaginalis* were most commonly detected among the predominant lactobacilli in the saliva and fecal samples of the three subjects. These results are consistent with the findings of Ahrnè et al. (2), who also identified *L. paracasei* and *L. rhamnosus* among the isolates mostly recovered from oral and rectal mucosa of 42 volunteers. Remarkably, identical RAPD types occurred among oral and fecal *Lactobacillus* isolates (Table 3). The RAPD-PCR has proven to be a suitable tool for molecular typing of lactobacilli (8, 24), especially of the *L. acidophilus* group (16). Provided that identical strains exhibit identical RAPD patterns, our results indicate that some of the intestinal lactobacilli indeed originate from the oral cavity. Together with previous findings that numerous *Lactobacillus* species detected in fecal samples probably derive from food (7, 13, 33), it is tempting to speculate that the majority of *Lactobacillus* species in the human intestine is allochthonous. Thus, particular attention should be paid when describing an isolate as "intestinal", as the mere isolation of lactobacilli from human feces does not constitute a certainty of their intestinal origin.

The species *L. gasseri* and *L. vaginalis* have only been described in association with humans and animals (12). We observed that strains of these species persisted in human saliva over a three months interval (Table 3). These findings suggest that both *Lactobacillus* species belong to the autochthonous biota of the oral cavity. Indeed, *L. gasseri* has often been described as a common member of the oral *Lactobacillus* biota (e.g. 17, 22, 26). However, *L. vaginalis* has only been rarely detected in oral samples (22). The failure in previous detection of *L. vaginalis* may be due to phenotypic misclassifications (12) or the relatively late description of this species (9). In our study *L. gasseri* and *L. vaginalis* were found to be predominant in the oral *Lactobacillus* biota (Table 1 and 2), reaching a population density of up to 10^7 CFU/ml saliva (Fig. 1). Assuming an average output of saliva of about 1,000 ml per day, up to 10^{10} CFU of the autochthonous oral lactobacilli enter the human intestine each day. Provided that these lactobacilli survive the intestinal passage, the constant load of oral lactobacilli leads to a wrong description of the autochthonous intestinal biota. However, it can not be totally excluded that a *Lactobacillus* strain detected in saliva and fecal samples is able to colonize the oral and intestinal tract and thus should be considered autochthonous to both habitats.

The detection of identical RAPD types of *L. paracasei* and *L. rhamnosus* isolates in human saliva and feces (Table 3) is not surprising, as these species are commonly associated with foods (12) and have been shown to survive the passage through the GIT (5, 11, 14). Therefore, our results indicate that food-associated lactobacilli such as *L. paracasei* and *L. rhamnosus* enter the mouth and constitute, during transit through the human digestive tract, a major part of the detectable *Lactobacillus* biota. These results are consistent with those of previous studies, where the food-associated lactobacilli were found to represent a major part of the predominant fecal *Lactobacillus* biota (7, 13, 32, 33).

In the past, studies on the ecology of lactobacilli in the oral cavity relied mainly on bacteriological culture (2, 17). However, cultivation on selective media is labour-intensive and time-consuming, and sometimes may result in underestimation of the bacterial diversity of a particular habitat. On the contrary, molecular techniques, like PCR-DGGE, allow a more complete and rapid assessment of the bacterial diversity, especially in complex ecosystems (23, 34). In this study, we successfully applied the group-specific PCR-DGGE (33) to characterize the *Lactobacillus* biota in human saliva samples. Investigation of saliva samples of three human subjects revealed the presence of *L. gasseri*, *L. paracasei*, *L. rhamnosus* and *L. vaginalis* among the predominant lactobacilli. The results were consistent with those obtained by bacteriological culture on Rogosa SL medium, demonstrating the applicability of PCR-DGGE for a fast and reliable identification of the predominant *Lactobacillus* species of the oral cavity.

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Chapter V

Inducible gene expression in *Lactobacillus reuteri* LTH5531 during type II sourdough fermentation

Abstract

Lactobacillus reuteri LTH5531 is a dominant member of the microbiota of type II sourdough fermentations. To investigate the genetic background of the ecological performance of LTH5531, *in vivo* expression technology (IVET) was used to identify promoters that show elevated levels of expression during growths of this organism in a type II sourdough fermentation. Thirty-eight sourdough induced fusions were detected, and 29 genes could be identified on the basis of the available sequence information. Four genes encoded stress-related functions (e.g. acid and general stress response) reflecting the harsh conditions prevailing during sourdough fermentation. Further eight genes were involved in acquisition and synthesis of amino acids and nucleotides, indicating their limited availability in sourdough. The remaining genes were either part of functionally unrelated pathways or encoded hypothetical proteins. The identification of a putative proteinase and a component of the arginine deiminase pathway are of technological interest, as they are potentially involved in the formation of aroma precursors. Our study allowed insight into the transcriptional response of *Lactobacillus reuteri* to the dough environment, what founds the molecular basis to investigate bacterial properties that are likely to contribute to the ecological performance of the organism and influence the final outcome of the fermentation.

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Introduction

Sourdough is an intermediate product in bread production and contains a microbiota comprised of lactic acid bacteria (LAB) and yeasts (reviewed in 16). Microbiological studies have revealed that 43 species of LAB, mostly species of the genus *Lactobacillus*, and more than 23 species of yeasts occur in this ecological niche. The metabolic activity of these microorganisms leads to an acidification of the dough and the development of aroma precursors, and is therefore of major importance for the quality of the final product. Sourdough breads are characterized for their unique flavor and texture, enhanced nutritional value, and favorable technological properties such as prolonged shelf life and delayed staling (reviewed in 15). Type II sourdoughs serve mainly as dough acidifier and are fermented for long periods (up to 5 days) at temperatures >30°C and high dough yields to permit pumping of the dough. Strains of *Lactobacillus reuteri* have been shown to be highly competitive in type II sourdough fermentations and persist over several years of continuous propagation in industrial fermentation processes (12, 22). Numerous ecological factors affect the competitiveness of lactobacilli in sourdough fermentations, i.e. temperature, ionic strength, dough yield, and microbial products such as lactate, acetate, CO_2, and ethanol as well as factors resulting from substrates present in the cereal fraction and from enzymatic reactions (6, 22). The properties and genetic background responsible for the ecological performance of *L. reuteri* in sourdough fermentation are however poorly understood.

In vivo expression technology (IVET) has proved to be a valuable tool for dissecting bacterial adaptation to various environments and in the identification of colonization determinants (13, 25, 32). IVET permits the identification of promoters that are selectively induced in a particular habitat, and has been used to identify genes of *L. reuteri* 100-23 that have elevated expression during colonization of the murine gut (42). It has been argued by Rainey (25) that genes showing greater expression in a particular ecosystem ('niche specific genes') are more likely to contribute towards ecological fitness than genes expressed equally across a range of environments. This assumption could be confirmed for a variety of *in vivo*-induced (*ivi*) genes (13, 25, 32). For example, the *ivi* gene methionine sulfoxide reductase B (MsrB) was shown to contribute to the ecological performance of *L. reuteri* strain 100-23 in the gut of mice (43).

In this paper we describe the application of IVET to investigate *in vivo* gene expression of *L. reuteri* LTH5531 during type II sourdough fermentation. This strain has been isolated from the dominant *Lactobacillus* biota of a type II sourdough (22). Our results showed that 38 promoters are selectively expressed in LTH5531 during the fermentation.

Materials and methods

Bacterial strains, plasmids and media

The *Lactobacillus* strains used in this study are listed in Table 1. Bacteria were routinely cultured in MRS medium (Difco) anaerobically or microaerobically (2% O_2, 10% CO_2, 88% N_2) at 37°C. When required, erythromycin and chloramphenicol were added to the culture media at a concentration of 100 µg/ml and 10 µg/ml, respectively. Modified MRS (mMRS) medium contained per liter: 10.0 g glucose, 5.0 g peptone, 4.0 g beef extract, 5.0 g Na-acetate, 2.0 g yeast extract, 2.0 g K_2HPO_4, 2.0 g triammonium citrate, 0.2 g $MgSO_4$, 0.05 g $MnSO_4$, 1 ml sorbitan monooleate, 0.5 g lichenan (Sigma) and 10 µg/ml chloramphenicol.

Genetic techniques

DNA manipulation methods were used according to standard protocols described by Sambrook et al. (22). Plasmid isolation, electrotransformation and *in vitro* stability of plasmids pJW100 and pJW200, were performed as described previously (32).

Construction of IVET library

A library containing DNA fragments (0.3 to 1.5 kbp) of the *L. reuteri* LTH5531 genome was constructed in the promoter trap vector pJW100 as described previously with some modifications (42). The high electrotransformation efficiency of LTH5531 allowed the direct establishment of the library in this strain, and the intermediate host (*Escherichia coli*) was omitted. The ligation reaction (pJW100 plus chromosomal DNA of LTH5531) was therefore used to electrotransform cells of LTH5531 directly. Transformants were grown microaerobically on mMRS agar at 37°C for 18 h. To determine *in vitro* promoter activity, Congo-red solution (17) was introduced beneath the agar. β-Glucanase activity (active promoter) was indicated by yellow zones (halos) surrounding the bacterial growth on an otherwise red-colored plate (36). To reduce the number of strong constitutive promoters in the IVET library, transformants showing halos smaller than 2 mm in diameter were picked and cultivated on MRS agar containing chloramphenicol. These colonies were recovered from agar plates by using saline, and cell suspensions were subjected to plasmid isolation. The pooled plasmid preparation was used to generate the IVET library in *L. reuteri* LTH5531 by electrotransformation. To determine the average insert size, 20 colonies were picked randomly for plasmid isolation from MRS agar plates. An aliquot (1 µl) of the DNA solution was subjected to PCR (see below).

Inoculation and performance of sourdough fermentations

L. reuteri LTH5531 containing the IVET library and the control strains FDB200, Con1, FDB100 and Pre1 (Table 1) were cultivated on MRS agar containing chloramphenicol (and erythromycin for FDB200 and Con1) for 48 h. Cells were recovered from the agar plates using 3 ml saline and the cell suspensions were adjusted to an OD_{600} of 20. An aliquot (100 µl) of the suspension (about 5 x 10^8 cells) was used to start separated fermentations. Type II sourdough fermentations were performed as described by Meroth et al. (22), but lincomycin was added at a concentration of 0.5 g/kg dough. Briefly, batches of dough (200 g) were prepared from tap water and rye bran providing a dough yield of 367. Fermentation was started by the addition of 100 µl of inoculum (about 5 x 10^8 *L. reuteri* cells) followed by incubation at 40°C for 24 h stirred at 200 rpm. Thereafter, the dough was propagated by back-slopping of 1% of ripe dough and incubation for further 24 h. A sample of 1 g was subjected to microbial counting on agar plates supplemented with 0.1 g/l cycloheximide and the appropriate antibiotics. From the control batches, lactobacilli were recovered on MRS agar containing chloramphenicol (and erythromycin for FDB200 and Con1) incubated at 37°C for 48 h, microaerobically.

Detection of *ivi* genes during sourdough fermentation

From the batch inoculated with LTH5531 containing the IVET library, lactobacilli were grown on mMRS agar incubated for 18h at 37°C, microaerobically. Approximately 3,000 colonies were screened for ß-glucanase activity (*in vitro*-active promoter) by observing the halo size on the mMRS agar plates. Clones with reduced or no halos were subcultured and stored at –80°C. The erythromycin resistance of these ß-glucanase-negative clones was determined by comparing growth on MRS agar supplemented with chloramphenicol (10 µg/ml) and erythromycin (100 µg/ml) to that of the control culture, *L. reuteri* FDB200. Finally, to confirm *in vivo* induction of genes, putative *ivi* clones were used to inoculate small batches of sourdough (5 g) containing 0.5 g of lincomycin /kg.

Analysis of *ivi* fusions

Plasmid inserts of putative *ivi* clones were amplified by PCR using primers IVETrrnT1T2 and IVETrev as described previously (42). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced with the IRD 800-labeled primer IVETrrnT1T2-800 and IVETrev-800 using the AutoRead sequencing kit (Amersham Pharmacia Biotech). Homology searches were performed against the NCBI database using the BLASTX program (http://www.ncbi.nlm.nih.gov/BLAST). Homology searches against the genome sequence of *L. reuteri* ATCC 55730 (which is estimated to cover 90-95% of the complete genome, unpublished data) were performed using a local version of the BLASTN program.

Results

Identification of *L. reuteri* LTH5531 genes selectively expressed during sourdough fermentation

The IVET system consists of the promoter-trapping vector pJW100 (Table 1) in which genomic DNA fragments of LTH5531 were inserted upstream of two promoter-less reporter genes (42). The primary reporter gene (essential growth factor) was *'ermGT* which confers lincomycin and erythromycin resistance. Selection of active promoters was achieved by addition of lincomycin to the sourdough. The second reporter gene, *'bglM* (encoding a β-glucanase), allowed the differentiation between constitutive and *ivi* promoters. *In vitro*-active promoters could be sorted out by screening for β-glucanase activity on mMRS agar plates. This system was tested with sourdough containing lincomycin by comparing a *L. reuteri* culture containing a constitutively-expressed promoter cloned in pJW100 (FDB200) with that of a culture without a cloned promoter (FDB100). As shown in Fig. 1, FDB200 could grow in sourdough supplemented with lincomycin, whereas FDB100 could not be detected, indicating that pJW100 was suitable for use as a promoter trap vector to identify promoters that are active during sourdough fermentation.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s)	Reference(s) or source
Strain		
Lactobacillus reuteri LTH5531	Type II sourdough isolate	18
Lactobacillus reuteri FDB100	Strain LTH5531 harboring pJW100, Cm^r, Em^s, $BglM^-$	This study
Lactobacillus reuteri FDB200	Strain LTH5531 harboring pJW200, Cm^r, Em^r, $BglM^+$	This study
Lactobacillus reuteri Con1	Strain FDB100 containing an insert with *in vitro*-active promoter, Cm^r, Em^r, $BglM^+$	This study
Lactobacillus reuteri Pre1	Strain FDB100 containing an insert without *in vitro* promoter activity, Cm^r, Em^s, $BglM^-$	This study
Lactobacillus reuteri ATCC 55730	Breast milk isolate. Source of the genomic information	
Plasmid		
pJW100	Promoter trap vector containing promoter-less *bglM* and *ermGT*, Cm^r, 7.4 kb	32
pJW200	Derivative of pJW100 containing the *ldhL* promoter in multiple cloning site, Cm^r, 7.5 kb	32

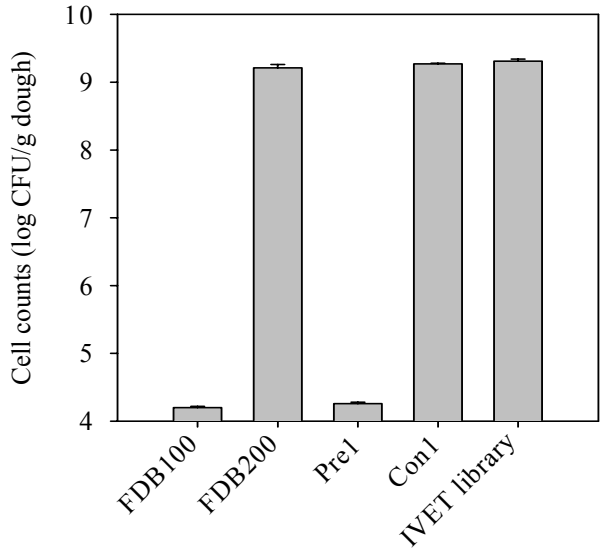

Figure 1. *Lactobacillus* population in type II sourdoughs after 48h of fermentation in the presence of lincomycin. Batches were inoculated with strains FDB200 and Con1 (positive controls), FDB100 and Pre1 (negative controls), or IVET library (LTH5531 containing the IVET library). For strain description see Table 1.

Construction and screening of the IVET library

Transformation of *L. reuteri* LTH5531 with DNA of pJW100 and pJW200 revealed efficiencies of about 10^6 transformants per μg of plasmid DNA. This high transformability enabled the construction of the IVET library in *L. reuteri* LTH5531. Analysis of 20,000 transformants revealed that 9% exhibited a detectable β-glucanase activity on mMRS agar plates, indicating the presence of a cloned *in vitro*-active promoter. As clones containing strongly expressed constitutive promoters might overgrow *ivi* promoters in the ecosystem, clones exhibiting a halo size of > 2 mm on mMRS agar were discarded. One clone exhibiting a large halo (> 4 mm; Con1) and one with a small halo (< 2 mm; Pre1) were retained as control cultures (Table 1). A plasmid pool was derived from the remaining 18,600 clones which subsequently constituted the IVET library in *L. reuteri* LTH5531. PCR analysis of plasmid DNA of 20 randomly picked clones revealed an average insert size of about 700 bp (range 0.2 to 1.2 kbp).

Detection of selectively induced genes during sourdough fermentation

To identify *L. reuteri* LTH5531 promoters induced during sourdough fermentation, a batch of dough was inoculated with 30,000 LTH5531 transformants. After 48 h of fermentation, the *Lactobacillus* population was comparable in size to that of batches inoculated with cultures FDB200 and Con1 (Fig. 1), whereas negative control cultures FDB100 and Pre1 did not grow in the sourdough. Screening of 3,000 clones for *in vitro* β-glucanase activity detected 180 that were erythromycin sensitive and had reduced or no β-glucanase activity. To confirm that they contained *ivi* promoters, each clone was tested for growth during sourdough fermentation in the presence of lincomycin. One hundred and seventy three of these clones grew in the sourdough and achieved populations of about 2 x 10^9 CFU/g dough.

Characterization of sourdough *ivi* promoters and genes

Sequence analysis of the plasmid inserts in the 173 *ivi* clones revealed putative *Lactobacillus* promoters (–35 region, **TT**GAC**A**; –10 region, **TA**TAA**T**; 21) in the correct orientation to induce expression of the two reporter genes. Thirty eight different promoters were detected, most on more than one occasion. In 29 of these sequences a ribosomal binding site and an open reading frame (ORF) were located downstream of the promoter. Fourteen ORFs could be annotated by alignment of sequences with those in public databases and the results were confirmed by considering the corresponding sequences of complete ORFs available from the genome of *L. reuteri* ATCC 55730. Further thirteen *ivi* genes could be identified by comparison of the ORFs with the genome sequence of ATCC 55730. Finally, two ORFs were annotated by homology search in public databases but could not be found in the genome of ATCC 55730. The 29 *ivi* genes were grouped according to the standard Clusters of Orthologous Groups (COG) classification (35) and are listed in Table 2. The genes were involved in amino acid transport and metabolism (4 ORFs), translation (3 ORFs), nucleotide transport and metabolism (4 ORFs), cell envelope biogenesis and outer membrane (3 ORFs), intracellular trafficking and secretion (1 ORF), energy production and conversion (1 ORF), inorganic ion transport and metabolism (2 ORFs), transcription (2 ORFs), general functions (4 ORFs), unknown functions (5 ORFs). In the case of the remaining 9 sequences, neither any ORF downstream of the promoter nor any match with the genome sequence of *L. reuteri* ATCC 55730 was found.

Discussion

Application of the IVET library constructed from the genome of *L. reuteri* LTH5531 allowed the detection of promoters that are specifically induced during type II sourdough fermentation. Sequence analysis of the downstream located ORFs revealed 29 *ivi* genes (Table 2) which are likely to influence the ecological performance of LTH5531 in sourdough. Two of these genes (*ivi64* and *ivi121*) as well as nine insert sequences missing any ORF were not present in the genome of *L. reuteri* ATCC 55730, which is a human isolate. As the available sequence is estimated to cover 90-95% of the complete genome, the *ivi* genes or sequences may be located in the unsequenced regions. On the other hand, they may not be present in the genome of the human isolate and therefore are of special interest, since they could regulate expression of unique proteins of the sourdough isolate LTH5531 which are of ecological importance in the sourdough fermentation.

As type II sourdoughs are used as dough acidifier, the fermentation aims to accumulate high amounts of lactic and acetic acid, accounting for a harsh environment requiring high acid tolerance of the bacteria. In the fermentation type used in this study the final pH drops below 3.8 and the total titratable acids reach values of up to 80 (22). Strain LTH5531 responded to the acid stress by expressing the arginine deiminase (ADI) pathway as indicated by the detection of the *ivi* gene *arcD* (Table 2, *ivi40*). This is consistent with the observation that *Lactobacillus sanfranciscensis*, which is highly competitive in type I sourdoughs, also induces the ADI pathway during fermentation (8). Moreover, Rollan et al. (27) recently demonstrated that arginine metabolism in the sourdough isolate *L. reuteri* CRL 1098 contributes to the survival at acid conditions and that the ADI enzymes are triggered by adaptation to low pH and/or energy depletion in the stationary phase of growth. Degradation of arginine through this pathway leads to formation of ammonia which increases the acid tolerance of the organism by neutralization of the environment. Furthermore, the *guaA* gene encoding a guanosine monophosphate synthase was found to be induced in strain LTH5531. Rallu et al. (26) showed that alteration in the guanine nucleotide pool is responsible for increased heat and acidic stresses resistance in *Lactococcus lactis*.

Table 2. *L. reuteri* genes that were induced during sourdough fermentation.

Clusters of Orthologous Groups (COG)	*Ivi* clone	Redundancy	Product or function [gene]	Accession number (corresponding ORF in strain ATCC 55730)
Amino acid transport and metabolism	*ivi2*	25	Asparagine synthase (glutamine-hydrolyzing) [*asnB*]	AJ937232 (AY970987)
	ivi40	3	Arginine/ornithine antiporter [*arcD*]	AJ937236 (AY970978)
	ivi48	7	Branched-chain amino acid transport protein [*azlC*]	AJ937238 (AY971000)
	ivi59[1]	2	Cysteine desulfhydrase/selenocysteine lyase	AJ937255 (AY970980)
Translation	*ivi78*[1]	6	Threonyl-tRNA synthetase	AJ937256 (AY970981)
	ivi105	1	Peptidyl-tRNA hydrolase [*pth*]	AJ937241 (AY970976)
	ivi143	1	Seryl-tRNA synthetase [*serS*]	AJ937246 (AY970998)
Nucleotide transport, and metabolism	*ivi19*	2	ADP-ribose pyrophosphatase	AJ937234 (AY970983)
	ivi33[1]	3	Adenine-specific methyltransferase	AJ937250 (AY970995)
	ivi52[1]	2	GMP synthase (glutamine-hydrolyzing) [*guaA*]	AJ937252 (AY970977)
	ivi113	7	Inosine-uridine preferring nucleoside hydrolase [*iunH*]	AJ937242 (AY970992)
Cell envelope biogenesis, outer membrane	*ivi11*	5	Extracellular protein	AJ937233 (AY970993)
	ivi42	8	Undecaprenyl pyrophosphate synthetase [*uppS*]	AJ937237 (AY970997)
	ivi119[1]	2	UDP-N-acetylglucosamine 1-carboxyvinyltransferase [*murA1*]	AJ937259 (AY971001)
Intracellular trafficking and secretion	*ivi124*	1	Protein translocase [*yajC*]	AJ937244 (AY970986)
Energy production and conversion	*ivi138*[1]	1	NADH dehydrogenase	AJ937260 (AY970990)
Inorganic ion transport and metabolism	*ivi97*[1]	1	Arsenate reductase family protein	AJ937258 (AY970996)
	ivi94[1]	1	Manganese-binding protein	AJ937257 (AY970985)
Transcription	*ivi5*[1]	9	Transcriptional regulator, LytR family	AJ937248 (AY970974)
	ivi64[2]	2	Transcriptional regulator	AJ937239
General function predicted only	*ivi36*	1	Phosphohydrolase	AJ937235 (AY970989)
	ivi38[1]	6	Metalloproteinase	AJ937251 (AY970991)
	ivi58[1]	7	Putative regulatory protein	AJ937254 (AY970975)
	ivi126	1	HIT family protein	AJ937245 (AY970999)
Function unknown	*ivi6*[1]	8	Unknown conserved protein	AJ937249 (AY970988)
	ivi57[1]	1	Unknown unconserved protein	AJ937253 (AY970979)
	ivi82	8	LemA like protein	AJ937240
	ivi121[2]	3	Unknown conserved protein	AJ937243
	ivi149	2	Veg protein, bacterial lipocalin	AJ937247 (AY970994)

[1] Available ORF sequence in the insert was of insufficient length to give a match in the public database. Predicted function based on the corresponding ORF in the *L. reuteri* ATCC 55730 genome.

[2] Insert sequences without any match in the *L. reuteri* ATCC 55730 genome.

Identification of other *ivi* genes associated with bacterial stress response illustrates further that type II sourdough produced with rye bran constitutes a harsh habitat for *L. reuteri*. The ADP ribose pyrophosphatase (ADPRase) belongs to the hydrolases of the nudix family (3). Genes encoding the nudix hydrolases are considered "housecleaning", because their function is to cleanse the cell of potentially deleterious endogenous metabolites and to modulate the accumulation of intermediates in biochemical pathways during the cell cycle or during periods of stress (3). Up to 30 nudix hydrolase genes are represented in the genome of *Bacillus* species (44), indicating their potential importance in cell function. Furthermore, ADPRase has been shown to be a tellurite resistance factor in *Rhodobacter sphaeroides* (9). However, no data are available about contamination of rye bran by this metal. Gene *ivi149* encoded a protein with 48% identity to the *Lactobacillus johnsonii* NCC 533 homologue of von Ebner's gland protein (Veg; 30), a member of the lipocalin family. Lipocalins are a diverse, poorly understood family of proteins composed, in the main, of extracellular ligand-binding proteins displaying high specificity for small hydrophobic molecules (5). In gram-negative bacteria, such as *E. coli*, these proteins are anchored to the outer membrane, where they are thought to serve a starvation response function (4). However, sequence analysis of *ivi149* did not reveal the presence of a signal peptide which would have indicated an extracellular localization of the protein. Two *ivi* genes (*uppS* and *murA1*) were detected which are involved in the biosynthesis of peptidoglycan (PG), a major component of the cell wall of Gram positive bacteria. An increased expression of these genes may be the response of strain LTH5531 to the harsh conditions of the sourdough environment. Recently, Piuri et al. (24) showed that modifications occur in the cell wall of *Lactobacillus casei* during osmotic stress. However, no data are available about a connection between cell wall modifications and acid stress in sourdough lactobacilli. To date, for lactobacilli only changes in the membrane composition have been observed (2, 31) and, in general, changes in the lipid profile of cell membranes have been reported to play a key role in the response of bacteria to environmental stresses (1, 11).

The induction of some *ivi* genes permits to conclude over the availability of nitrogen sources in rye bran sourdoughs. Gene *azlC* encodes a transporter for branched chain amino acids (BCAA) indicating the availability of BCAA during type II sourdough fermentation. This is consistent with the finding that free amino acids can accumulate during rye dough fermentation (18, 19, 37). However, *L. reuteri* LTH5531 also induced gene *asnB*, which is involved in the synthesis of asparagine, thus indicating a deficiency of this amino acid in type II sourdough. Such a deficiency may be explained by the preference of sourdough lactobacilli

to acquire amino acids via peptide transport (14, 20, 37), which may result in a bias of uptake of particular amino acids. To make use of the acquired peptides, a highly competitive sourdough strain should possess an active peptide hydrolase system. This assumption is consistent with the identification of *ivi38* that encodes a metalloproteinase. Recently, Rollán and Font de Valdez (28) showed that the sourdough isolate *L. reuteri* CRL 1098 possesses an active peptide hydrolase system consisting of several metalloenzymes. Thus, the metalloproteinase that we have detected may be part of a peptide hydrolase system in LTH5531. Such a system could enable LTH5531 to make use of peptides as nitrogen source. Proteolysis during sourdough fermentation would also be of importance in the generation of amino acids that are of relevance for flavor development in the baked goods (38). A further example of an aroma relevant *ivi* fusion is *arcD*, which belongs to the ADI pathway. This pathway leads to the formation of ornithine, a precursor of the 'roasty' aroma compound 2-acetyl-pyrroline, formed during baking (33). We have observed (unpublished result) that degradation of arginine by *Lactobacillus pontis* by the ADI pathway led to accumulation of ornithine in the dough and, upon baking, 2-acetyl-pyrroline in the crust, thus improving the sensory quality of wheat breads.

Two transcription regulators were found to be induced during sourdough fermentation. One of these belongs to the LytR family (*ivi*5) whose members are involved in regulation of toxin, bacteriocin and exopolysaccharide (EPS) production (23, 45). Sourdough isolates of *L. reuteri*, including strain LTH5531 (unpublished results), were recently shown to produce EPS during sourdough fermentation (39). Up to now, no results have been published investigating the regulation of *L. reuteri* genes involved in EPS production. However, EPS production has been shown to be affected by environmental factors like temperature and pH, e.g. in *Lactobacillus helveticus* ATCC 15807 (40, 41) and *Streptococcus thermophilus* 1275 (46). Therefore, it is tempting to speculate that *L. reuteri* LTH5531 may synthetize EPS in sourdough under control of the transcriptional regulator that we have detected. *In situ* EPS production is of technological relevance since it affects dough rheology and bread texture (39). Moreover, EPS produced by sourdough lactobacilli, such as *L. sanfranciscensis*, has shown to exert a prebiotic effect by selectively supporting the growth of bifidobacteria (7).

Lactobacilli have been used for centuries in food preservation and are used with increasing intensity for specific industrial food fermentation processes. The results of this study show that IVET can be used to gain insight into the transcriptional response of lactobacilli during a food fermentation process. *Ivi*-genes were detected that have not previously been functionally

characterized (e.g. the Veg protein) but might be essential for the ecological performance of lactobacilli in the food environment. In addition, genes (*arcD*, *ivi38* and *ivi5*) were identified with potential to influence the quality of the final bread in relation to aroma and nutritional value. Knowledge of the complex regulatory processes that occur in starter organisms during food fermentations will provide a molecular basis on which improved starter strains could be developed for industrial exploitation.

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Chapter VI

Inducible gene expression in *Lactobacillus reuteri* LTH5531 during the transit through the gastrointestinal tract of mice

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Lactobacilli have been detected in diverse environments and have been the subject of considerable research due to their commercial use in the food industry (reviewed in Hammes and Hertel, 2003). They are used in the production of foods prepared by means of lactic acid fermentation (dairy products, fermented vegetables, fermented meats, and sourdough bread) (reviewed in Hammes and Hertel, 2003). Lactobacilli are also associated with the body of humans and other animals and are considered to benefit the health of the consumer when ingested as probiotics (Mitsuoka, 1992; Vaughan et al., 1999). Several *Lactobacillus* species are commonly detected in both fermented food and the gastrointestinal tract (GIT), but the genetic background for this ecological versatility is poorly understood.

Lactobacillus reuteri is both a dominant member of the microbiota of type II sourdough fermentations (Meroth et al., 2003, Gänzle and Vogel, 2003) and the proximal GIT of rodents (Salzman et al., 2002; Tannock, 1997), environments with distinctly different ecological features. The predominance of *L. reuteri* in the GIT is due to the ability of these bacteria to adhere to the epithelial surface of the rodent forestomach and to form a biofilm-like layer of bacterial cells (Tannock, 1997, Walter et al., 2004). Recently, IVET has been used to identify genes of *L. reuteri* 100-23 (Wesney and Tannock, 1979) that have elevated expression during colonization of the GIT of mice (*ivi* genes) (Walter et al, 2003). One product of the detected *ivi* gene, the methionine sulfoxide reductase B (MsrB), was found to contribute to the ecological performance of *L. reuteri* strain 100-23 in the GIT of mice (Walter et al., 2005).

Strains of *L. reuteri* have been shown to be highly competitive in type II sourdough fermentations and persist over several years of continuous propagation in industrial fermentation processes (Meroth et al., 2003, Gänzle and Vogel, 2003). To investigate the genetic background of this ecological performance, we recently used an *in vivo* expression technology (IVET) to identify *L. reuteri* promoters that show elevated levels of expression

during growths in a type II sourdough fermentation (Dal Bello et al., 2005; see Chapter V). Investigations were performed using strain LTH5531, which had previously been isolated as a dominant member of type II sourdough microbiota (Meroth et al., 2003). On the basis of the available sequence information, thirty-eight sourdough induced fusions were detected, and 29 genes could be identified, allowing conclusions to be made over the transcriptional response of LTH5531 to the dough environment and its contribution to the final outcome of the fermentation (see Chapter V). Recent studies revealed that at least some strains of *L. reuteri* that have been isolated from sourdough are able to colonize the GIT of reconstituted *Lactobacillus*-free (RLF) mice (Tannock et al., 1988; Gänzle, 2004). The aim of this work was to investigate the potential of strain LTH5531 to colonize the murine GIT and, additionally, to apply IVET to detect genes highly expressed during the colonization.

To compare the potential of *Lactobacillus* strains to colonize the murine gut, cell suspensions of the sourdough isolate LTH5531, as well as of the autochthonous rodent isolates *L. reuteri* 100-23 and *Lactobacillus gasseri* 21 (Bateup et al., 1995), were prepared from MRS agar plates and administered by intragastric gavage to RLF mice (about 5×10^8 cells per animal; 3 mice per bacterial strain). Approval for the animal experimentation was obtained from the University of Otago Animal Ethics Committee. Mice were killed 14 days after inoculation and lactobacilli were enumerated in forestomach, jejunal, and caecal samples on Rogosa SL agar plates after anaerobic incubation at 37°C for 2 days (Walter et al., 2003). Results of bacteriological counts (Table 1) revealed that strain LTH5531 is able to colonize the GIT of RLF mice at levels comparable to those of autochthonous strains.

Table 1. Colonization of gut of mice by *Lactobacillus* strains.

Strain	Log_{10} lactobacilli per gram of organ[1]		
	Forestomach	Jejunum	Cecum
L. reuteri LTH5531	8.8 (0.1)	6.1 (0.4)	7.1 (0.3)
L. reuteri 100-23	8.0 (0.2)	7.3 (0.2)	8.0 (0.2)
L. gasseri 21	8.8 (0.2)	8.2 (0.2)	8.2 (0.3)

[1] Each value represents the mean from the values obtained from 3 different mice. The standard deviation is given in parenthesis.

In order to identify genes of strain LTH5531 specifically induced during the colonization of the GIT of RLF-mice, the same IVET procedure described by Walter et al. (2003) for strain 100-23 was used. Briefly, *L. reuteri* LTH5531 containing the IVET library was administered by intragastric gavage to anaesthetized RLF mice (about 5 x 10^8 cells per animal). The library used was that used for the sourdough IVET application (Dal Bello et al., 2005; see Chapter V). Lincomycin was added to the drinking water in three different antibiotic treatment (ABT) regimens: ABT 1, lincomycin treatment (19 mg/L) was started 3 days before inoculation of the mice with lactobacilli and was continued throughout the experiment; ABT 2, lincomycin treatment (19 mg/L) was started 24 h after inoculation of the mice with lactobacilli and was continued throughout the experiment; ABT 3, ABT 2 but using 9.5 mg of lincomycin per litre (Walter et al. 2003). Two animal experiments were performed. In the first experiment, three groups of mice were subjected to the different antibiotic treatments (group 1, 3 mice using ABT 1; group 2, 3 mice using ABT 2; group 3, 2 mice using ABT 3). The mice were killed after an appropriate time (3 days for group 1; 4 days for groups 2 and 3) and lactobacilli were recovered from the forestomach and caecum on Rogosa SL agar (Difco) containing 10 µg/ml chloramphenicol. The second experiment was conducted in the same manner, but the animals of group 3 were killed after 5 days. Colonies obtained on Rogosa SL agar plates after 2 days of anaerobic growth at 37°C were patched on both PHB agar (Heng et al. 1997) supplemented with lichenin and chloramphenicol, and on MRS agar containing chloramphenicol (10 µg/ml) and erythromycin (100 µg/ml). The plates were incubated anaerobically at 37°C for 18 and 48 h, respectively. From the two experiments, about 30,000 colonies were investigated and found to possess strong *in vitro* activity of both reporter genes, thus indicating a strong *in vivo* selection of active promoters. This provides the necessary indication that the selection of strains that contain fusions to promoters that are transcriptionally active *in vivo* had taken place. However, hypothetical *ivi* clones were not detected among the colonies cultured from the GIT of mice.

Remarkably, IVET with the genomic library that was successfully used in the sourdough study (Dal Bello et al., 2005; see Chapter V) did not detect *ivi* promoters when LTH5531 inhabited the GIT of mice. This result was surprising, as it indicates that the sourdough strain LTH5531 does not respond by specific gene expression to the ecological conditions in the GIT. With IVET, active promoters are selected by expression of an "essential growth factor" (in this study the erythromycin resistance mediated by ErmGT) that allows the organism to colonize and/ or grow in the ecosystem (Rainey, 1999, Walter et al., 2003). Expression of *ivi* promoters in particular ecosystems must therefore be permanent and strong in order to allow

comparable growth rates of *ivi* clones and clones bearing constitutive promoters, especially in the GIT, where inactive bacteria are rapidly washed out. Our findings indicate that *L. reuteri* LTH5531 does not possess strongly expressed "GIT inducible" genes, while possessing 38 ones specifically induced in sourdough.

Ivi genes are more likely to contribute to the ecological performance of an organism in a specific environment than genes expressed equally in a broad range of habitats (Rainey, 1999, Gal et al, 2003, Walter et al., 2005). Therefore, traits encoded by *ivi* genes are likely to be adaptive and the extent of their expression would be shaped by natural selection to improve ecological fitness. It has been shown in laboratory experiments that adaptation to an ecological niche is often accomplished by phenotypic innovations which arise through changes to regulatory rather than structural genes, leading to changes in gene expression (as reviewed by Kassen and Rainey, 2004). For example, Riehle et al. (2003) showed that the adaptation of three lineages of *Escherichia coli* to high temperatures (41.5˚C) over 2,000 generations resulted in an increased fitness at high temperatures combined with significant changes in the expression of heat-inducible genes. These findings demonstrate that bacterial adaptation result in increased expression of inducible genes that contributed to ecological performance. Such genes should be detectable by applying IVET. A strain that is allochthonous to an ecosystem would not have developed strong transcriptional responses to the prevailing conditions, and the detection of *ivi* genes would be unlikely. The presence of 38 "sourdough specific" *ivi* fusions in *L. reuteri* LTH5531 probably reflects the long term adaptation of LTH5531 to the sourdough environment, just as *ivi* genes detected in strain 100-23 reflect adaptation of this GIT isolate to the rodent GIT (Walter et al., 2003; Walter et al., 2005). LTH5531 was isolated from an experimental sourdough that had been inoculated with an industrial starter. This industrial starter has been propagated over several years, giving the organisms present sufficient time to adapt. In accordance with this, by using RAPD-PCR analysis, Meroth et al. (2003) showed that strain LTH5531 was present in a commercial type II sourdough starter collected 10 years prior isolation of LTH5531, thus indicating that this strain had the chance to adapt to the sourdough environment for at least 10 years. Even for an allochthonous organism one would expect that some environmental signals are generic (such as stress response and nutrient limitation) and would lead to increased gene expression compared to expression in laboratory media. Such non adaptive responses are probably weak in comparison to responses shaped by adaptation. Techniques that pick up transient gene expression such as R-IVET (resolvase-based IVET) have been shown to identify *ivi* genes in

allochthonous organisms and have the potential to detect generic environmental responses (Bron et al., 2004).

From a food technology perspective, lactobacilli have been used for centuries in food preservation and are used increasingly in industrial food fermentation processes (as reviewed in Hammes and Hertel, 2003). Knowledge of gene expression and metabolic activities of bacteria during food fermentations can be obtained with IVET (Dal Bello et al., 2005; see Chapter V) and provides an important molecular basis on which improved starter strains can be developed for industrial exploitation. Our results show the importance of working with highly adapted, autochthonous strains in studies of microbial ecology in order to reveal the adaptive interactions responsible for the ecological success of these bacteria in their natural environment or during food fermentations.

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Chapter VII

Summary

Among the bacteria inhabiting the human gut, lactobacilli have received considerable attention, due to their putative health promoting effects (Reid, 1999; Vaughan et al., 1999). Cultivation of lactobacilli is considered to be reliable and numerous studies using plating on selective media have been performed to investigate these bacteria in intestinal ecosystems (Tannock, 1995; Reuter, 2001). Recently, the application of PCR-DGGE in combination with primers specific for lactic acid bacteria (LAB) detected species which are not considered to be intestinal inhabitants but food-associated, such as *Lactobacillus curvatus*, *Lactobacillus sakei*, *Leuconostoc mesenteroides* and *Pediococcus pentosaceus* (Walter et al., 2001; Heilig et al., 2002). Remarkably, these species could not be recovered by traditional bacteriological culture on Rogosa SL agar (Walter et al., 2001). In Chapter III, different cultivation media, as well as new incubation conditions were applied to overcome these difficulties. Human faecal samples were plated on selective and non-selective media and incubated under standard condition (37°C, anaerobiosis) for faecal LAB as well as alternative condition (30°C, 2% O_2). PCR-DGGE analyses of resuspended bacterial biomass (RBB) obtained from agar plates revealed that the species composition of the recovered LAB was affected stronger by the incubation condition than by the used medium. It was observed that food-associated LAB such as *L. sakei* and *Lc. mesenteroides*, hitherto not described as intestinal inhabitants, are more easily selected when the alternative incubation condition is used. Identification of randomly picked colonies grown under the alternative condition on Rogosa SL agar showed that *L. sakei* is one of the predominant food-associated LAB species in faecal samples, reaching counts of up to 10^6 CFU per gram faeces. Comparison of the results of bacteriological culture with those obtained by PCR-DGGE analysis of the RBB showed that investigation of RBB is a fast and reliable method to gain insight into the species composition of culturable LAB in faeces.

Examination of the faecal *Lactobacillus* populations over longer periods has revealed marked variation in the complexity and stability of these populations among human subjects (Vanhoutte et al., 2004, Walter et al., 2001). Ecological studies indicate that most *Lactobacillus* species found in the human gastrointestinal tract (GIT) are likely to be transient (allochthonous), originating from either the oral cavity or food (reviewed in Bibiloni et al., 2004). In order to investigate if oral lactobacilli constitute a part of the faecal *Lactobacillus* biota, the *Lactobacillus* biota of saliva and faeces of three human subjects were investigated

and compared at two time-points in a three months interval (Chapter IV). The species composition of the *Lactobacillus* biota of human saliva and faeces was found to be subject-specific and fluctuated to some degree, but the species *Lactobacillus gasseri*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus* and *Lactobacillus vaginalis* were detected at both time-points in saliva and faecal samples of individual subjects. RAPD-PCR analysis indicated that several strains of these species were present both in the oral cavity and in the faecal samples of the same subject. Oral isolates of the species *L. gasseri* and *L. vaginalis* showing identical RAPD types were found to persist over time, suggesting that these species are autochthonous to the oral cavity. The results of Chapter IV, together with recently published data (reviewed in Bibiloni et al., 2004), give strong evidence that some lactobacilli found in human faeces are allochthonous to the intestine and originate from the oral cavity.

Lactobacilli have been detected in diverse environments and have been the subject of considerable research due to their commercial use in the food industry (reviewed in Hammes and Hertel, 2003). Several *Lactobacillus* species are commonly detected in both fermented food and the human GIT, but the genetic background for this ecological versatility is poorly understood. *Lactobacillus reuteri* is a dominant member of the microbiota of type II sourdough fermentations (Meroth et al., 2003) and is considered one of the truly autochthonous *Lactobacillus* species in humans (Reuter, 2001). The *in vivo* expression technology (IVET) developed by Walter et al. (2003) was used to identify genes (so-called *ivi* genes) of the sourdough isolate *L. reuteri* LTH5531 that show elevated levels of expression during growth of this organism in a type II sourdough fermentation (Chapter V) and during passage through the GIT of mice (Chapter VI). Thirty-eight induced fusions were found to be highly expressed during the sourdough fermentation (Chapter V), and 29 genes could be identified on the basis of the available sequence information. Four genes encoded stress-related functions (e.g. acid and general stress response) reflecting the harsh conditions prevailing during sourdough fermentation. Further eight genes were involved in acquisition and synthesis of amino acids and nucleotides, indicating their limited availability in sourdough. The remaining genes were either part of functionally unrelated pathways or encoded hypothetical proteins. The identification of a putative proteinase and a component of the arginine deiminase pathway are of technological interest, as they are potentially involved in the formation of aroma precursors.

Remarkably, IVET with the genomic library that was successfully used in the sourdough study (Chapter V) did not detect *ivi* promoters when LTH5531 inhabited the GIT of mice

(Chapter VI). With IVET, active promoters are selected by expression of an "essential growth factor" (in our system the erythromycin resistance mediated by ErmGT) that allows the organism to colonize and/ or grow in the ecosystem (Rainey, 1999, Walter et al., 2003). Expression of *ivi* promoters in particular ecosystems must therefore be permanent and strong in order to allow comparable growth rates of *ivi* clones and clones bearing constitutive promoters, especially in the GIT, where inactive bacteria are washed out. The findings of Chapter V and VI indicate that *L. reuteri* LTH5531 does not possess strongly expressed "GIT inducible" genes, while possessing 38 ones specifically induced in sourdough. *Ivi* genes are more likely to contribute to the ecological performance of an organism in a specific environment than genes expressed equally in a broad range of habitats (Rainey, 1999, Gal et al, 2003, Walter et al., 2005). Therefore, traits encoded by *ivi* genes are likely to be adaptive and the extent of their expression would be shaped by natural selection to improve ecological fitness. The presence of thirty-eight "sourdough specific" *ivi* fusions in *L. reuteri* LTH5531 probably reflects the long term adaptation of LTH5531 to the sourdough environment, just as *ivi* genes detected in strain 100-23 reflect adaptation of this GIT isolate to the rodent GIT (Walter et al., 2003). Indeed, LTH5531 was isolated from an experimental sourdough that had been inoculated with an industrial starter. This industrial starter has been propagated over several years, giving the organisms present sufficient time to adapt. In accordance with this, by using RAPD-PCR, Meroth et al. (2003) showed that strain LTH5531 was present in a commercial type II sourdough starter collected 10 years prior isolation of LTH5531, thus indicating that this strain has adapted to the sourdough environment for at least 10 years.

The results of Chapter V clearly demonstrated that knowledge of gene expression and metabolic activities of bacteria during food fermentations can be obtained by applying IVET. The results collected provide an important molecular basis on which improved starter strains can be developed for industrial exploitation. Moreover, the results of Chapter VI show the importance of working with highly adapted, autochthonous strains in studies of microbial ecology in order to reveal the adaptive interactions responsible for the ecological success of these bacteria in their natural environment or during food fermentations.

Zusammenfassung

Laktobazillen haben unter den Bakterien, die den menschlichen Darm bewohnen, eine ansehnliche Beachtung aufgrund ihres positiven Einflusses auf das menschliche Wohlbefinden erlangt. Die Kultivierung dieser Bakterien gilt als zuverlässig, und so wurden zahlreiche Studien unter Anwendung von Kultivierungstechniken mit selektiven Medien durchgeführt, um die Laktobazillen in intestinalen Ökosystemen zu untersuchen (Tannock, 1995; Reuter, 2001). Vor kurzem führte die Anwendung der PCR-DGGE in Kombination mit Milchsäurebakterien (MSB)-spezifischen Primern zum Nachweis von Spezies, die nicht zu den klassischen intestinalen MSB gehören, sondern vielmehr Lebensmittel-assoziiert sind, z.B. *Lactobacillus curvatus*, *Lactobacillus sakei*, *Leuconostoc mesenteroides* and *Pediococcus pentosaceus* (Walter et al., 2001; Heilig et al., 2002). Interessanterweise konnten diese Spezies nicht durch Kultivierung auf Rogosa SL Agar erhalten werden (Walter et al., 2001). Das Kapitel III beschreibt die Anwendung unterschiedlicher Kultivierungsmedien und neuer Inkubationsbedingungen, um diese Schwierigkeiten zu überwinden. Menschliche Stuhlproben wurden auf selektive und nicht-selektive Agarplatten ausplattiert, und die Platten wurden unter den klassischen Bedingungen (37°C, anaerob) für intestinalen MSB sowie unter alternativen Bedingungen (30°C, 2% O_2) inkubiert. Die Analyse von bakterieller Zellmasse, die von Agarplatten abgeschwemmt wurde, mittels PCR-DGGE brachte hervor, dass die Zusammensetzung der MSB-Spezies stärker von den angewandten Inkubationsbedingungen als von den Medien beeinflusst wurde. Es konnte beobachtet werden, dass Lebensmittel-assoziierte MSB wie *L. sakei* und *Lc. mesenteroides*, die bisher nicht als intestinale Bewohner beschrieben worden waren, leichter durch Einsatz der alternativen Inkubationsbedingungen kultiviert werden können. Eine Identifizierung zufällig ausgewählter Kolonien, die unter den alternativen Bedingungen auf Rogosa SL Agar gewachsen waren, zeigte, dass *L. sakei* einer der dominierenden Lebensmittel-assoziierten MSB in menschlichen Fäzesproben ist und dort in Keimzahlen von bis zu 10^6 KbE pro Gramm vorkommen kann. Ein Vergleich der kulturtechnischen Ergebnisse mit denen der PCR-DGGE-Analyse von Bakterienmassen auf Agarplatten zeigte außerdem, dass die Untersuchung der Bakterienmassen eine schnelle und zuverlässige Methode darstellt, um einen Einblick in die Spezieszusammensetzung der kultivierbaren MSB in Fäzes zu erhalten.

Die Untersuchungen der intestinalen Laktobazillenpopulation in menschlichen Stuhlproben über einen längeren Zeitraum zeigte eine hohe Variabilität in der Komplexität und Stabilität der Spezieszusammensetzung (Vanhoutte et al., 2004; Walter et al., 2001). Ökologische

Studien brachten hervor, dass die meisten *Lactobacillus*-Spezies im menschlichen Gastrointestinaltrakt (GIT) wahrscheinlich transient (allochthon) sind und entweder von Lebensmitteln oder aus der Mundhöhle stammen (Biblioni et al., 2004). Um zu untersuchen, inwieweit die oralen Laktobazillen einen Teil der fäkalen Laktobazillen ausmachen, wurde die *Lactobacillus*-Biota sowohl im Speichel als auch in Stuhlproben von drei Probanden untersucht und an zwei Zeitpunkten mit dreimonatigem Abstand verglichen (Kapitel IV). Die Zusammensetzung der *Lactobacillus*-Spezies im menschlichen Speichel und Fäzes war Individuum-spezifisch und fluktuierte in einem gewisse Maße, dennoch konnten die Spezies *Lactobacillus gasseri*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus* und *Lactobacillus vaginalis* an beiden Zeitpunkten sowohl im Speichel als auch Fäzes der Probanden nachgewiesen werden. Durch RAPD-PCR-Analyse konnte gezeigt werden, dass mehrere Stämme dieser Spezies im Speichel und in Fäzes desselben Probanden vorhanden waren. Stämme von *L. gasseri* und *L. vaginalis* mit identischen RAPD-Mustern konnten aus beiden Speichelproben isoliert werden, was darauf hinweist, dass diese Spezies in der Mundhöhle autochthon sind. Die Ergebnisse dieses Kapitels gemeinsam mit kürzlich publizierten Daten stellen ein starkes Indiz dafür dar, dass einige Laktobazillen, die aus Stuhlproben isoliert werden können, aus der Mundhöhle stammen und somit im Intestinum allochthon sind.

Laktobazillen sind in unterschiedlichen Ökosystemen gefunden worden und aufgrund ihrer kommerziellen Verwendung in der Lebensmittelindustrie Gegenstand umfassender Forschung gewesen (Hammes und Hertel, 2003). Einige *Lactobacillus*-Spezies lassen sich häufig sowohl in fermentierten Lebensmitteln als auch im menschlichen GIT nachweisen, jedoch ist der genetische Hintergrund für diese ökologische Vielseitigkeit noch weitgehend unbekannt. *Lactobacillus reuteri* ist ein dominantes Mitglied in der Mikrobiota von Typ II Sauerteigfermentationen (Meroth et al., 2003) und gilt als einer der echten autochthonen *Lactobacillus*-Spezies bei Menschen (Reuter, 2001). In Kapitel V und VI wurde die von Walter et al. (2001) entwickelte "*in vivo* expression technology (IVET)" angewandt, um bei dem Sauerteigisolat *L. reuteri* LTH5531 Gene (sogenannte *in vivo* induzierte (*ivi*)-Gene) zu identifizieren, die während des Wachstums in einer Typ II Sauerteigfermentation (Kapitel V) bzw. während der Passage durch den GIT einer Maus (Kapitel VI) eine erhöhte Expression zeigen. Während der Sauerteigfermentation wurden 38 induzierte und stark exprimierte Genfusionen gefunden (Kapitel V), die auf der Basis der verfügbaren Sequenzen eine Identifizierung von 29 Genen erlaubten. Vier Gene kodierten für Stress-verwandte Funktionen (z.B. Säure- und allgemeine Stressantwort) und spiegeln somit die harschen Bedingungen in der Sauerteigfermentation wider. Weitere 8 Gene kodierten für Proteine, die in Transport und

Synthese von Aminosäuren und Nukleotiden involviert sind, was eine limitierte Verfügbarkeit beider Komponenten während der Sauerteigfermentation anzeigte. Die restlichen Gene waren entweder Teil von Stoffwechselwegen, die in keiner Korrelation zum Ökosystem standen, oder kodierten für hypothetische Proteine. Die Identifizierung einer putativen Proteinase und einer Komponente des Argininedeiminase-Stoffwechsels ist von technologischer Bedeutung, da beide dahinter stehenden Systeme potenziell an der Bildung von Aromavorläufern beteiligt sein können.

Bemerkenswerterweise wurden bei Anwendung der IVET mit der Genombibliothek, die bereits erfolgreich bei der Sauerteigstudie eingesetzt wurde (Kapitel V), keine *ivi*-Promotoren während der Passage von *L. reuteri* LTH5531 durch den GIT einer Maus identifiziert (Kapitel VI). Mit Hilfe der IVET werden durch die Expression eines "essentiellen Wachstumsfaktors" (in unserem System die Erythromycinresistenz vermittelt durch ErmGT) aktive Promotoren selektioniert, da diese Wachstum und/oder Kolonisierung des Organismus im Ökosystem erlauben (Rainey, 1999; Walter et al., 2001). Deshalb muss die Expression eines *ivi*-Promotors im Ökosystem permanent erfolgen und hoch genug sein, um ein vergleichbares Wachstum von *ivi*-Klonen und Klonen mit konstitutiven Promoter zu erhalten, insbesondere im GIT, wo langsam wachsende Bakterien sonst ausgewaschen werden. Die Ergebnisse aus Kapitel V und VI deuten darauf hin, dass *L. reuteri* LTH5531 keine stark exprimierten und "GIT induzierbaren" Gene besitzt, obwohl der Stamm 38 im Sauerteig spezifisch induzierbare Gene besitzt. *Ivi*-Gene sind wahrscheinlich eher für die Wettbewerbsfähigkeit bzw. das ökologische Verhalten eines Organismus in einem spezifischen Ökosystem verantwortlich, als Gene, die in unterschiedlichen Ökosystemen gleich stark exprimiert werden (Rainey, 1999; Gal et al., 2003; Walter et al., 2005). Somit sind Eigenschaften, die von *ivi*-Genen kodiert werden, eher für die Adaption verantwortlich und das Ausmaß ihrer Expression würde durch natürliche Selektion in der Art gestaltet werden, dass die ökologische Fitness verbessert wird. Die Identifizierung von 38 Sauerteig-spezifischen *ivi*-Genfusionen in *L. reuteri* LTH5531 spiegelt die lange Adaptation von LTH5531 an das Ökosystem Sauerteig wider, ebenso wie die *ivi*-Gene von *L. reuteri* 100-23, der aus Ratten isoliert wurde, die Adaption dieses Keim an den GIT von Ratten widerspiegelt (Walter et al., 2003). In der Tat wurde der Stamm LTH5531 aus einem Sauerteig isoliert, der mit einem industriellen Starter inokuliert wurde. Dieser industrielle Starter wurde über mehrere Jahre propagiert, so dass die enthaltenen Keime genug Zeit hatten sich zu adaptieren. In Übereinstimmung damit stehen die Ergebnisse von Meroth et al. (2003), die unter Anwendung der RAPD-PCR zeigten, dass der Stamm LTH5531 bereits in einem

kommerziellen Typ II Sauerteigstarter enthalten war, der 10 Jahre vor der Isolierung des Stammes Gegenstand von Untersuchungen war. Das deutet darauf hin, dass sich der Stamm LTH5531 an das Ökosystem Sauerteig seit mindestens 10 Jahren angepasst hat.

Die Ergebnisse aus Kapitel V zeigen deutlich, dass die IVET eine geeignete Methode ist, die Erkenntnisse über die Genexpression und metabolische Aktivität von Bakterien während Lebensmittelfermentationen zu erweitern. Die gesammelten Ergebnisse stellen eine wichtige molekulare Grundlage dar, auf deren Basis verbesserte Starterorganismen für die Nutzung in der Lebensmittelindustrie entwickelt werden können. Darüber hinaus zeigen die Ergebnisse von Kapitel VI die Notwendigkeit in ökologischen Studien hoch-adaptierte, autochthone Stämme zu verwenden, um Kenntnisse über adaptive Wechselwirkungen zu erlangen, die für den ökologischen Erfolg dieser Bakterien in ihrem natürlichen Ökosystem sowie während der Lebensmittelfermentation verantwortlich sind.

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