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**Molecular methods for detection of probiotics and intestinal microbiota, and evaluation of *Lactobacillus brevis* as a potential probiotic dietary adjunct**

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Academic Dissertation

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## Abstract

The human gastrointestinal (GI) tract harbours an extremely complex microbiota mainly composed of fastidious anaerobic organisms. Because these microbes have a profound impact on host's health, modulation of microbiota with probiotic bacteria has been proposed. However, the mechanisms of action of both the GI microbes and the proposed probiotics remain obscure. To gain more information, molecular identification methods for members of the GI microbiota and the probiotic strains are needed. Quick, robust methods are necessary for obtaining an overall image of changes in GI microbiota, and more sophisticated methods are needed for following up selected species or strains.

In this study, new methods were tested for their applicability in monitoring GI microbiota and probiotic strains. Strain-level genetic labelling without introduction of foreign DNA was demonstrated with *Lactobacillus helveticus* CNRZ32 by inserting a site containing silent mutations into the chromosomal *pepX* gene. Intact phenotypic properties of the mutated strain were confirmed with a peptidase assay. The mutated and wild-type strains could be detected from faeces and milk with the help of specific primers. Thus, the labelling method could be used for specific marking of industrial or probiotic strains in a 'food-grade' manner provided that a suitable target gene and genetic transformation tools are available.

For analysis of GI microbes and selected intestinal or dairy lactic acid bacteria, several species- or group-specific oligonucleotide primers and probes were designed and tested with three different techniques. A polymerase chain reaction – enzyme-linked immunosorbent assay (PCR-ELISA) application with simultaneous utilisation of multiple species- or group-specific oligonucleotide probes was suitable for detection of predominant members present in a mixed bacterial population. Sensitivity of the method was improved by using primers selective for the genus *Bifidobacterium*. Comparison of dot blot hybridisation and real-time PCR demonstrated the superior properties of real-time PCR for detection and quantification of bacterial ribosomal DNA targets.

The final part of this study comprised the evaluation of two *Lactobacillus brevis* strains ATCC 8287 and ATCC 14869<sup>T</sup> as supplements in dairy products. The *L. brevis* strains showed promising in vitro antagonistic properties towards selected potentially harmful microbes and were suitable as supplementary strains in yoghurt, producing no

undesirable side effects on the quality or preservation of products. A small-scale feeding study demonstrated the survival of *L. brevis* ATCC 8287 in the human GI tract, indicating, together with the favourable antagonistic properties, that this strain could be a candidate for use as a probiotic supplement in dairy products.

## List of original publications

This thesis is based on the following original articles referred to in the text by their Roman numerals.

- I Malinen, E., Laitinen, R., Palva, A. (2001). Genetic labeling of lactobacilli in food grade manner for strain-specific detection of industrial starters and probiotic strains. *Food Microbiology* 18, 309-317.
- II Laitinen, R., Malinen, E., Palva, A. (2002). PCR-ELISA I: Application to simultaneous analysis of mixed bacterial samples composed of intestinal species. *Systematic and Applied Microbiology* 25, 241-248.
- III Malinen, E., Mättö, J., Salmitie, M., Alander, M., Saarela, M., Palva, A. (2002). PCR-ELISA II: Analysis of *Bifidobacterium* populations in human faecal samples from a consumption trial with *Bifidobacterium lactis* Bb-12 and a galacto-oligosaccharide preparation. *Systematic and Applied Microbiology* 25, 249-258.
- IV Malinen, E., Kassinen, A., Rinttilä, T., Palva, A. (2002). Comparison of real-time PCR with SYBR Green I or 5'-nuclease assays and dot-blot hybridization with rDNA targeted oligonucleotide probes in quantification of selected faecal bacteria. *Microbiology* (in press).
- V Rönkä, E., Malinen, E., Saarela, M., Rinta-Koski, M., Aarnikunnas, J., Palva, A. (2002). Probiotic and milk technological properties of *Lactobacillus brevis*. *International Journal of Food Microbiology* (in press).

## Abbreviations

ATCC	American Type Culture Collection
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EBI	European Bioinformatics Institute
FISH	fluorescence in situ hybridisation
G+C	guanine-plus-cytosine
GI	gastrointestinal
GRAS	generally recognized as safe
IL	interleukin
PCR	polymerase chain reaction
PCR-ELISA	polymerase chain reaction – enzyme–linked immunosorbent assay
RNA	ribonucleic acid
rDNA	ribosomal DNA
rRNA	ribosomal RNA
SSCP	single-strand conformation polymorphism
TGGE	thermal gradient gel electrophoresis
TRF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism

## 1. Introduction

Molecular methods have facilitated culture-independent studies of gastrointestinal (GI) tract microbes. The GI microbiota is mainly composed of anaerobic organisms (Rolfe, 1997) and moreover, direct molecular approaches have confirmed the abundance of uncultivable microbes in intestinal samples (Langendijk et al., 1995; Suau et al., 1999; Leser et al., 2002). The value of molecular methods for studying GI tract microbes is therefore immense.

GI microbiota can be influenced by probiotic bacteria (Fuller, 1989; Dunne et al., 1999). The probiotics, defined as “live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance” (Fuller, 1989), have been proposed to possess several advantageous properties, such as antagonistic actions, production of antimicrobial substances, modulation of immune responses and an impact on the metabolic activities of the gut (Dunne et al., 1999). Probiotic bacteria seem to hold great promise for treatment of gastrointestinal disorders, yet further studies are required to create a more scientific basis for the probiotic action. Furthermore, as no single strain is likely to have all the aforementioned beneficial properties (Dunne et al., 1999), screening of new strains for probiotic potential is necessary.

Although several applications, such as dot blot hybridisation (Doré et al., 1998), fluorescence in situ hybridisation (FISH) (Langendijk et al., 1995), denaturing gradient gel electrophoresis (DGGE) (Millar et al., 1996) or thermal gradient gel electrophoresis (TGGE) (Zoetendal et al., 1998), and polymerase chain reaction (PCR) with species- or group-specific primers (Kok et al., 1996), are available for the detection and semi-quantitative analysis of GI microbiota, improvements are required, especially in relation to sensitivity, cost and quantification power of the methods. This is fundamental for better understanding of the GI tract microbiota and the effects of probiotic bacteria on these microbes.

## 2. Review of the literature

### 2.1. Intestinal microbiota

The human gastrointestinal (GI) tract harbours an extremely complex microbiota which has a profound impact on host's health. The normal gut bacterial population of an adult is estimated to comprise more than 400 species, with a predominance of obligate anaerobes (Rolfe, 1997). The total number of microbes present in one gram of intestinal content varies from less than  $10^3$  microbes in the stomach to  $10^4 - 10^7$  microbes in the small intestine and  $10^{10} - 10^{12}$  microbes in the colon (Goldin and Salminen, 1998; Holzapfel et al., 1998). Indeed, the quantity of microbes present in the intestine (about  $10^{14}$ ) exceeds 10-fold the total number of all human cells (Holzapfel et al., 1998). Until recently, analysis of intestinal bacteria has mainly been based on cultivation-dependent methods. Culture-independent studies have however confirmed that only a fraction of the organisms present in faeces are cultivable (Langendijk et al., 1995; Suau et al., 1999; Leser et al., 2002), therefore, the results obtained by cultivation are likely to be biased. Generally, *Bacteroides*, *Eubacterium*, *Clostridium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus*, *Bifidobacterium* and *Fusobacterium* are reported to constitute the majority of microbiota. Molecular analyses, however, suggest that most faecal bacteria belong to a few phylogenetic lineages composed of organisms from several genera (Suau et al., 1999, Sghir et al., 2000). Bacteria related to *Bacteroides*, *Prevotella* and *Porphyromonas* seem to represent one-third of bacteria present in faeces, whereas *Clostridium leptum* subgroup and *Clostridium coccoides* groups both account for approximately one-fifth of the faecal bacterial populations (Sghir et al., 2000). However, besides being an extremely diverse microbial ecosystem, the intestinal microbiota appears to be unique for each individual (Kimura et al., 1997; Tannock, 1999a).

The normal microbiota of the GI tract works as a barrier against pathogens, contributes to degradation of some food components, stimulates the host immune system, and produces certain B vitamins, enzymes and short-chain fatty acids (Holzapfel et al., 1998). The microbes can also metabolise potentially carcinogenic substances and drugs in either a beneficial or a disadvantageous way (Holzapfel et al., 1998). However, the role and action of individual microbial species or groups present in the GI tract are poorly

known. Starting at birth, the microbiota develops in a successional manner. The first colonising microbes include bifidobacteria, enterobacteria (Grönlund et al., 2000; Favier et al., 2002), clostridia, enterococci and ruminococci (Favier et al., 2002). Recent studies have suggested the importance of the type of colonising bacteria on the development of the gut immune system (Grönlund et al., 2000; Ouwehand et al., 2001a). A role in the development of food allergies has been postulated for the intestinal microbiota due to the occurrence of an adult-like bifidobacterial species composition in the intestine of allergic infants (Ouwehand et al., 2001a). Significant structural changes occur in the microbiota with age, including reduction of bifidobacteria (Hopkins et al., 2001) as well as an increase in the diversity of *Atopobium* cluster species (Harmsen et al., 2000b) present in faeces. Nutritional aspects, however, have a profound effect on the composition of the intestinal microbial population (Benno et al., 1989).

## **2.2. The Probiotics**

Probiotic bacteria have been defined as “live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance” (Fuller, 1989). Probiotic bacteria are increasingly utilised in human food as well as in animal feed products (Fuller, 1999; Sanders and Huis in’t Veld, 1999). However, composition of the intestinal microbiota is poorly known, which hinders understanding of the probiotic functions (Tannock, 1999b). A probiotic strain should be of host origin, non-pathogenic, technologically suitable for industrial processes, acid- and bile-fast, adhere to the gut epithelial tissue, persist in the gastrointestinal tract for short periods, produce antimicrobial substances, modulate immune responses and influence the metabolic activities of the gut (Dunne et al., 1999). The properties of the strain should be well documented (Dunne et al., 1999). Although some criteria, such as the non-pathogenic status, technological suitability and careful documentation of the probiotic effects of a microbial strain, are invariably required, no single strain is likely to carry all of the abovementioned properties (Dunne et al., 1999). Moreover, probiotic properties are considered strain-specific, and results obtained with one strain cannot therefore be claimed for another, even a closely related strain. Microbes used in probiotic products

include strains from several *Lactobacillus* and *Bifidobacterium* species, *Enterococcus faecalis* and *Enterococcus faecium*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Pediococcus acidilactici*, and *Sporolactobacillus inulinus*, *Streptococcus thermophilus*, as well as *Bacillus cereus*, *Escherichia coli*, *Propionibacterium freudenreichii*, *Saccharomyces cerevisiae* and *Saccharomyces boulardii* (Holzapfel et al., 1998). However, lactobacilli and bifidobacteria are most common in probiotic products designed for human use; probiotic properties of these bacteria are also the best studied (Tannock, 1999b).

Lactobacilli belong to the lactic acid bacteria, which comprise a diverse group of Gram-positive bacteria, most typically represented by non-sporing, catalase-negative, devoid of cytochromes, non-aerobic but aerotolerant, fastidious and acid-tolerant cocci or rods producing lactic acid as the major end-product during the fermentation of carbohydrates (Axelsson, 1998). Being of fastidious nature, lactic acid bacteria require a rich environment for growth, such as decaying plant material, food products and a mammalian gastrointestinal tract or vagina. The genus *Lactobacillus* is heterogeneous, containing species with 32-53% G+C of the chromosomal DNA arranged into three groups based on differences in sugar metabolism caused by the presence or absence of fructose-1,6-diphosphate aldolase and phosphoketolase (Axelsson, 1998). Although possessing some phenotypical features common for lactic acid bacteria, the genus *Bifidobacterium* is actually related to the Actinomycetes branch, having a high chromosomal G+C content (Axelsson, 1998). Sugar metabolism of bifidobacteria differs from that of lactic acid bacteria; the bifidobacteria lack aldolase and glucose-6-phosphate dehydrogenase, and hexose sugars are exclusively degraded by the fructose-6-phosphate pathway characterised by fructose-6-phosphate phosphoketolase (Ballongue, 1998). Bifidobacteria are predominant members of the human intestinal microbiota, with bacterial counts of  $10^9$ - $10^{11}$  per gram of stool, with *B. bifidum*, *B. longum*, *B. infantis*, *B. breve*, *B. adolescentis*, *B. angulatum*, *B. catenulatum*, *B. pseudocatenulatum*, and *B. dentium* reported as human isolates (Ballongue, 1998).

Several positive effects have been proposed for probiotic lactobacilli and bifidobacteria. Antagonism towards intestinal pathogens has been demonstrated for probiotics (Fernandes et al., 1987; Asahara et al., 2001; Fujiwara et al., 2001a, b, c).

Alleviation of diarrhoea is a well-documented characteristic of some strains (Bennet et al., 1996; Kaila and Isolauri, 1996; Arvola et al., 1999; Orrhage et al., 2000; de Roos and Katan, 2000; Van Niel et al., 2002), and particularly the ability of *Lactobacillus rhamnosus* GG to shorten the duration of acute rotavirus diarrhoea has been established (Kaila and Isolauri, 1996; de Roos and Katan, 2000). Stimulation of the gut immune system by probiotic strains has been reported (Link-Amster et al., 1994; Kishi et al., 1996; Miettinen et al., 1996 and 1998; Majamaa and Isolauri, 1997; Murosaki et al., 1998; Pessi et al., 1999, 2000; Isolauri et al., 2000; Maassen et al., 2000), and induction of cytokine profiles has been shown to be strain-dependent (Miettinen et al., 1996, 1998; Maassen et al., 2000). Alleviation of allergic reactions in the gastrointestinal tract has also been suggested for some strains: using a mouse model, Murosaki et al. (1998) showed that heat-killed *Lactobacillus plantarum* L-137 suppressed production of antigen-specific IgE by stimulating the production of IL-12. In addition, *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb-12 have been demonstrated to relieve symptoms of atopic eczema (Majamaa and Isolauri, 1997; Isolauri et al., 2000; Kalliomäki et al., 2001). Administration of *L. rhamnosus* GG has been shown to enhance the production of IL-10, acting thus as an anti-inflammatory mediator in atopic children (Pessi et al., 2000). Consumption of probiotics has also been observed to lower activities of harmful faecal enzymes (Goldin et al., 1992; Spanhaak et al., 1998). Some animal models have even suggested the ability of probiotic strains to reduce the incidence of cancer (Pool-Zobel et al., 1993; Goldin et al., 1996; Matsuzaki et al., 1996; Matsuzaki, 1998; O'Mahony et al., 2001). Finally, probiotics may have significance for alleviation of intestinal disturbances. For example, ingestion of a mixture of lactobacillar and bifidobacterial strains together with a *Streptococcus salivarius* ssp. *thermophilus* was observed to help maintain remission status in ulcerative colitis patients (Venturi et al., 1999).

Although lactobacilli and bifidobacteria are stated as GRAS (generally recognized as safe) organisms due to their long usage and non-pathogenic status, safety issues have been investigated. Isolation of these organisms from infections has been reported with a low incidence, and in most cases, bifidobacteria and lactobacilli were associated with immunocompromised patients (Brook, 1996; Saxelin et al., 1996). One major concern among lactic acid bacteria is, however, resistance to vancomycin, especially within the

genus *Enterococcus* where the resistance has been shown to be transferable (Arthur et al., 1996). Vancomycin resistance has been reported for lactobacilli by several authors (Swenson et al., 1990; Herrero et al., 1996; Charteris et al., 1998; Zarazaga et al., 1999; Felten et al., 1999; Klein et al., 2000), but it is generally considered to be an intrinsic property. Vancomycin resistance expressed by the probiotic strain *L. rhamnosus* GG has been studied in detail (Tynkkynen et al., 1998). Strain GG was not observed to transfer vancomycin resistance or receive other resistance elements from enterococci, nor were any genes resembling enterococcal vancomycin resistance genes found in *Lactobacillus* GG (Tynkkynen et al., 1998). Similarly, Klein et al. (2000) reported no indications for the presence of the *vanA* gene cluster, the *vanB* gene or the *vanC* gene from five *L. reuteri* strains or *L. rhamnosus* GG, suggesting that the vancomycin resistance of the strains studied is unrelated to the acquired resistance in the *Enterococcus* species.

### **2.3. Genetic labelling of lactic acid bacteria**

Insertion of an extra DNA label into a target strain genome provides a way to monitor the specific strain in various environments. However, introduction of foreign DNA is often required, and in many cases, genetic elements producing a phenotypic change are used to distinguish the marked strain. Genetic elements encoding resistance to an antibiotic have been utilised for labelling of lactic acid bacteria (Rush et al., 1994, Klijn et al., 1995), but the increasing spread of antibiotic resistance factors between bacterial species or genera make such approaches unsuitable for strains intended for human or animal use. Therefore, usage of various ‘food-grade’ markers has been suggested. Labelling of lactic acid bacteria with a plasmid-encoded green fluorescent protein gene placed under an inducible promoter has been reported for *Lactococcus lactis* and *Lactobacillus plantarum* (Geoffroy et al., 2000), whereas Allison and Klaenhammer (1996) suggested the use of a native *Lactobacillus* gene encoding immunity to Lactacin F as a food-grade genetic marker. A similar role has also been demonstrated for *ltnI* conferring immunity to lactacin 3147 (McAuliffe et al., 2000). Site-specific integration of desired genetic elements into bacterial chromosomes through phage attachment sites has been described for *Lactococcus lactis* (Lillehaug et al., 1997), *Lactobacillus delbrueckii* and *Lactobacillus plantarum* (Dupont et al., 1995).

Legislation as well as consumer acceptance limit the usage of genetically engineered organisms, and therefore, genetic changes introduced to target organisms must be carefully considered. Maguin et al. (1996), for example, combined insertion sequence *ISSI* with a thermosensitive replicon, enabling a high frequency of random insertion (about 1%) with *Lactococcus*, *Enterococcus* and *Streptococcus thermophilus*, while efficient excision of the plasmid generated stable mutants with no foreign markers, leaving only a single *ISSI* copy at the mutated site. However, the smallest detectable alteration introduced to a target organism is changing one or a few bases in a gene-coding sequence without affecting the amino acid sequence of the corresponding gene product. In principle, such a mutation could be caused by natural processes due to the degeneracy present in the genetic code. Silent mutations have been utilised for modification of a *Lactococcus lactis* subsp. *cremoris* strain plasmid-encoded proteinase *prtP* gene by in vitro mutation of the third positions of four adjacent codons, thus providing a genetic label with no phenotypic effects (Hertel et al., 1992). Although technically more demanding, genetic marking should, however, preferably be directed to gene replacement on a chromosomal locus to ensure maximal stability of the alterations introduced.

## **2.4. Utilisation of nucleic acid based methods for identification and monitoring of bacteria in population samples**

### 2.4.1. Utilisation of methods independent of prior knowledge on sequence data

#### 2.4.1.1. Sequencing analysis of 16S ribosomal DNA populations

Cloning and sequencing of the 16S ribosomal DNA (rDNA) pools in a population sample provides a method for obtaining sequence-level information on uncultivable bacteria abundantly present in various parts of the gastrointestinal tract. Suau et al. (1999) analysed the sequence of 284 16S rDNA clones derived from one faecal sample and classified the clones into 82 molecular species, using 98% similarity criteria for a species. Importantly, only 24% of the molecular species were derived from a described organism (Suau et al., 1999). Similarly, comparative 16S rDNA sequence analysis of the intestinal bacterial community in pigs revealed only a 17% fraction of previously described

organisms among a total of 375 phlotypes (Leser et al., 2002). In a large-scale study of subgingival plaque samples, including analysis of a sequence of 2522 16S rDNA clones, 347 species was observed among the clones studied, which correlated well with a previous estimate of expected species diversity in the oral cavity (Paster et al., 2001). Thus, the direct cloning approach seems to give a good idea of the total microbiota present in a complex population. It also facilitates determination of species-level differences between bacterial populations, as shown by the discovery of novel phlotypes and species not previously associated with childhood caries by comparing of the oral microbiota of a healthy subject and a subject with early childhood caries (Becker et al., 2002).

Approaches based on cloning are, however, rather tedious and are not optimal for analysis of large numbers of samples. Like all PCR-dependent methods, construction of clone libraries may be prone to biasing, possibly leading to falsification of the library structure (Bonnet et al., 2002). Nevertheless, with the help of cloning, design of phylogenetically relevant oligonucleotide probes is enabled (Suau et al., 1999).

#### 2.4.1.2. Denaturing gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE)

Denaturing gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE) have become popular methods for analysis of microbial populations present in various habitats such as water ecosystems (Overmann et al., 1999), microbial fermentations (Ampe et al., 2001), and the GI tract (Millar et al., 1996; Zoetendal et al., 1998; Simpson et al., 2000; McCracken et al., 2001; Satokari et al., 2001a, 2001b; Walter et al., 2001; Favier et al., 2002; Heilig et al., 2002; Requena et al., 2002; Zhu et al., 2002). These methods allow separation of nucleic acid molecules based on their size and sequential differences. Thus, a population of DNA molecules, such as the 16S ribosomal RNA (rRNA) or PCR-amplified 16S rDNA, can be studied and predominant members of the population identified via sequencing of isolated nucleic acid bands. Sensitivity of gradient gel electrophoresis is affected by the choice of PCR primers used. For example, utilisation of universal 16S primers limits the sensitivity to detection of 1% subpopulations (Zoetendal et al., 1998). By contrast, utilisation of species- or

group-specific primers may allow detection and identification of bacteria representing a minority of the total population (Walter et al., 2001; Heilig et al., 2002).

Drawbacks of gradient gel electrophoresis include difficulty of comparisons between individual gels, requirement of careful adjustments, and the need for sequencing to confirm identities of bands seen in a gel. Furthermore, some bacteria may remain unidentified due to low resolution of DNA bands (Ampe et al., 2001). With both DGGE- and TGGE-based methods, sequencing is required for correct identification of individual bands seen in the gel. As stated by Schmalenberger et al. (2001), intraspecies operon heterogeneities may significantly contribute to genetic profiles in microbial community analysis, as amplification of one bacterial DNA may yield several separate bands, which can then be wrongly interpreted as high microbial diversity. Depending on the 16S binding universal primer pairs used, single-strand conformation polymorphism analysis (SSCP) revealed an average of 1.7 – 2.3 bands per pure cultured bacterial organism (Schmalenberger et al., 2001).

#### 2.4.1.3. Terminal restriction fragment length polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (T-RFLP) is based on endonuclease digestion of PCR-amplified DNA and capillary electrophoresis analysis of the terminal restriction fragment (TRF) containing a fluorescent label. Terminal restriction patterns have been used to analyse marine bacterioplankton communities (Moeseneder et al., 1999, 2001) as well as faecal bacteria (Kaplan et al., 2001). The method is, however, limited by the choice of primers, which can, with their different affinities, dramatically change the patterns observed, while another problem is the TRF length overlap by phylogenetically distant bacteria (Kaplan et al., 2001).

#### 2.4.1.4. Analysis of community DNA profiles

Analysis of total bacterial community structure can be accomplished by measurement of guanosine-cytosine profiles of a population DNA sample with DNA reassociation (Griffiths et al., 1997; Ritz et al., 1997; Chatzinotas et al., 1998) and density gradient centrifugation (Holben and Harris, 1995; Apajalahti et al., 1998; Nüsslein and

Tiedje, 1998; Apajalahti et al., 2001). Density gradient fractionation of community DNA enables analysis of interesting fractions by cloning of PCR-amplified DNA pools, followed by sequencing (Nüsslein and Tiedje, 1998; Apajalahti et al., 2001). Density gradient centrifugation has been applied in analysis of intestinal microbial guanosine-cytosine profiles (Apajalahti et al., 1998, 2001). However, because this approach requires sophisticated and expensive equipment, it is out of reach for most research laboratories.

## 2.4.2. Utilisation of specific oligonucleotide primers or probes

### 2.4.2.1. Hybridisation

Hybridisation offers a means for direct semi-quantitative monitoring of population samples. A very high sensitivity for detection of DNA targets can be obtained with radioactively labelled probes (Palva, 1985). However, because of its abundance in bacterial cells, rRNA provides for a more attractive target for hybridisation studies. Indeed, hybridisation assays targeting rDNA have been verified as 10-fold less sensitive than assays for rRNA (Muttray and Mohn, 2000). Dot blot hybridisation with rRNA – targeted probes has been used for semiquantitative analysis of ruminal microbes (Forster et al., 1997) and intestinal microbiota (Doré et al., 1998; Sghir et al., 2000; Hopkins et al., 2001). Comparison of hybridisation results of a specific probe with a universal probe enables assessment of the target bacterial proportion present in a sample (Doré et al., 1998; Sghir et al., 2000; Hopkins et al., 2001; Marteau et al., 2001). At best, detection of a 0.1 – 0.01% rRNA subpopulation has been reported (Marteau et al., 2001), corresponding to approximately  $10^7$  target cells if  $10^{11}$  bacteria are considered to be present in a gram of faeces. Use of radioactive isotopes, especially in large-scale and long-term analyses, is, however, complicated by the short half-lives of labels. Non-radioactively labelled probes are more convenient, but their sensitivity is limited. Pre-enrichment of the target DNA by polymerase chain reaction can be used to enrich the target DNA. For example, 0.1 pg of *B. distasonis* or *B. thetaiotaomicron* DNA (approximately 10-20 cells) or 0.01 pg of *B. vulgatus* DNA (1-2 cells) applied to a PCR was sufficient to produce a positive hybridisation signal with specific digoxigenin-labelled probes (Kreader, 1995).

An additional factor of concern for filter hybridisations is the need to label each probe separately and to perform hybridisation reactions after optimisations in separate vessels. However, Ehrmann et al. (1994) described a reverse dot blot hybridisation with several membrane-bound oligonucleotide probes for identification of lactic acid bacteria present in mixed populations. Similarly, Becker et al. (2002) used simultaneous detection of PCR-amplified rDNA of 23 oral bacterial species or groups with oligonucleotide probes.

In the fluorescence in situ hybridisation (FISH) method, the bacterial cell samples to be studied are immobilised on microscope slides and made permeable for fluorescently labelled oligonucleotides with subsequent microscopic observation of the hybridisation signal intensities. FISH has been used for monitoring faecal microbiotas (Langendijk et al., 1995; Franks et al., 1998; Harmsen et al., 2000a, 2000b, 2002; Jansen et al., 1999; Schwiertz et al., 2000), and detection of a 1 – 0.1% bacterial subpopulation has been obtained (Schwiertz et al., 2000; Harmsen et al., 2002). Automated analysis of fluorescent signals has also been utilised to facilitate objective interpretation of results (Jansen et al., 1999). The major problem with FISH applications is the different penetration of probes in bacteria with various cell wall types, resulting in a possible underestimation of Gram-positive bacteria (Langendijk et al., 1995; Jansen et al., 1999).

#### 4.4.2.2. Polymerase chain reaction (PCR)

Amplification of target nucleic acids with PCR is an effortless method for detection of target DNA from various samples. In principle, the detection limit for a PCR assay, based on usage of one or two oligonucleotide primers specific for the target bacteria, is the presence of one copy of the amplified DNA region. Although achievement of such an extremely sensitive assay is unlikely, the PCR remains a very powerful detection method, typically requiring a minimum of 10-20 target copies for successful amplification. The high sensitivity is also somewhat problematic, as a simple aerosol contamination may lead to false amplification.

PCR-based detection of rRNA genes has been used for direct detection of various bacteria present in faeces (Kok et al., 1996; Wang et al., 1996, 1997; Lawson et al., 1998; Matsuki et al., 1999; Bernhard and Field, 2000; Saito et al., 2002) and pathogens from

clinical samples (Harper-Owen et al., 1999). Partial or full amplification of the 16S rDNA with primers specific for a genus or a few closely related genera has also been reported (Kok et al., 1996; Kaufmann et al., 1997; Walter et al., 2001; Heilig et al., 2002). In addition, utilisation of 16S-23S intergenic sequences as targets of species-specific primers for different lactic acid bacteria has been described (Tilsala-Timisjärvi and Alatossava, 1997). Good sensitivities have been reported for the PCR detection assays of faecal bacteria. For example, PCR detection sensitivity of five ruminococcal species with species-specific primers from faecal samples spiked with the target species did not markedly differ from the observed detection limit of 4-100 cells from pure cultures (Wang et al., 1997).

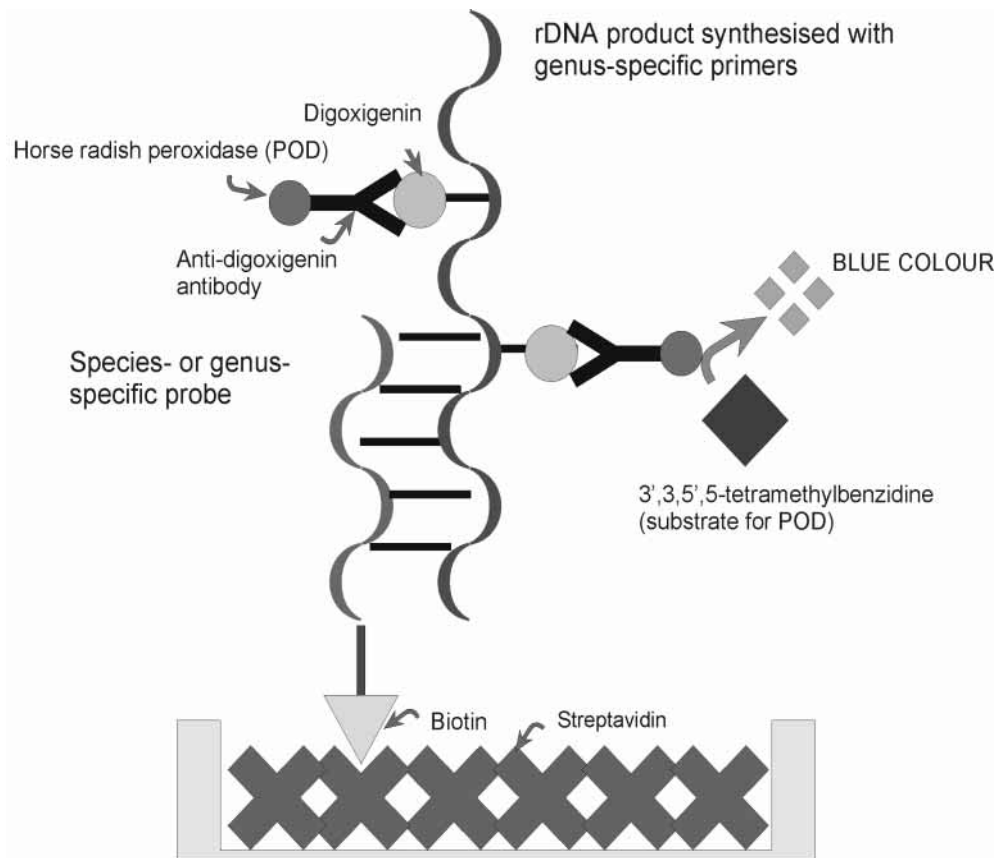
Although a highly potent detection method, results obtained by conventional end-point PCR should not be considered to be directly quantitative. PCR may lead to differential amplification of target templates originally present in equal amounts (Reysenbach et al., 1992). In addition, very low template concentrations may generate random fluctuations in priming efficiency of population DNA samples with universal primers, leading to a bias in the end-product concentration (Chandler et al., 1997). Knowledge of the rRNA gene copy numbers and genome sizes of bacteria in a mixed DNA sample has been observed to be insufficient in predicting the final product ratio of a PCR amplification (Farrelly et al., 1995). Suzuki and Giovannoni (1996) also noticed that the accumulation of end products during mixed-template PCR caused biasing of the various end-product ratios towards a 1:1 situation, which was hypothesised to be caused by an increase in the homologous template hybridisation, decreasing the efficiency of primer annealing and subsequent amplification.

Some improvements in the reliability of PCR quantification have been obtained by competitive PCR approaches. Hahn et al. (1995) created a quantitative PCR assay based on post-amplification differentiation of the internal standard and the sample DNA by selective restriction analysis and digoxigenin-based colorimetric detection. Quantitative detection of *Mycobacterium tuberculosis* PCR products was performed by enzyme-linked immunosorbent assay by comparison of hybridisation results with two probes and two IS6110 elements derived from either an internal control or a modified template (Kox et al., 1996). With co-amplification of an internal standard, detection of

*Clostridium proteoclasticum* was linear between  $1 \times 10^4$  and  $5 \times 10^1$  cells, with a detection limit of 50 fg or 25 cells (Reilly and Attwood, 1998), whereas detection of *Oxalobacter formigenes* from human faecal samples by competitive PCR was shown to be linear over a range of six logarithmic units, with a detection limit of approximately 100 genomes (Sidhu et al., 1999).

#### 2.4.2.3. Polymerase chain reaction – enzyme-linked immunosorbent assay (PCR-ELISA)

Polymerase chain reaction – enzyme-linked immunosorbent assay (PCR-ELISA) combines utilisation of polymerase chain reaction for efficient multiplication of the target DNA and hybridisation with a detection probe to ensure the specificity of the reaction. Figure 1 summarises the concept of PCR-ELISA detection. The PCR-amplified products are labelled with digoxigenin during or after the amplification reaction and hybridised with the specific biotinylated detection probe. The probe is immobilised in streptavidin-coated microtitre plate wells, and hybridised DNA products are detected via digoxigenin-targeted antibodies linked with an enzyme capable of producing a colorimetric or fluorimetric signal when brought together with a substrate. Usage of PCR-ELISA hybridisation for detection of single pathogens of clinical importance and spoilage bacteria of food has been described (Hahn et al., 1995; Kox et al., 1996; Denis et al., 1997; Gutiérrez et al., 1997; Nelson et al., 1997; Bazzichi et al., 1998; Fletcher et al., 1998; Gutiérrez et al., 1998; Löffler et al., 1998; Beyer et al., 1999; González et al., 1999; Fischer-Romero et al., 2000; Garcia et al., 2001; Sails et al., 2001). In comparison with filter hybridisation, PCR-ELISA provides more easily standardised reaction conditions by utilisation of commercially supplied microtitre plates. Other advantages of PCR-ELISA compared with several other methods are its relative simplicity and low costs.



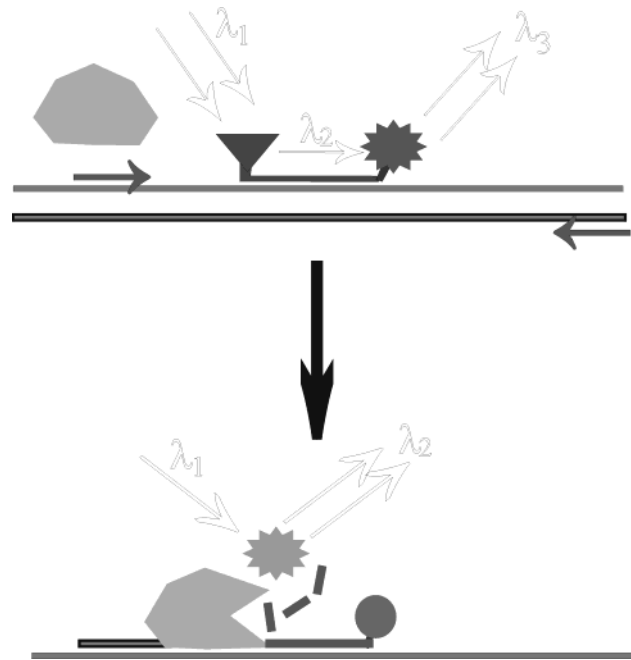
**Figure 1. PCR-ELISA detection.** The PCR-amplified, digoxigenin-labelled target molecule is hybridised with a specific probe captured into a microtitre well via biotin-streptavidin linkage. After removal of unbound DNA by washing, the hybridised molecules are visualised with the help of anti-digoxigenin antibodies conjugated with horse radish peroxidase (POD). With the application of 3',3',5',5'-tetramethylbenzidine (a substrate for POD), a blue colour is produced by the peroxidase. The reaction is terminated with sulphuric acid, after which the colour produced can be measured at 450 nm.

Good sensitivities have been reported for PCR-ELISA assays, and the results can in some cases be interpreted quantitatively. The limit for detection of *Bordetella pertussis* was 100 target organisms, starting from appliance of different target DNA amounts to PCR reaction (Nelson et al., 1997). Less than ten Epstein-Barr virus genome copies added to 750 ng of background DNA were required for a positive PCR-ELISA result (Bazzichi et al., 1998). Similarly, a sensitivity of 5 cfu/ml blood was obtained for detection of *C. albicans* and *A. fumigatus* cells, corresponding to the sensitivity of Southern blotting using digoxigenin-labelled oligonucleotides (Löffler et al., 1998).

Fletcher et al. (1998) could detect *Aspergillus fumigatus* quantitatively by a PCR-ELISA method on a log-scale between 100 and 1 pg of target DNA. Detection of *Escherichia coli* in oysters was reported to be quantitative in the range of  $10\text{-}10^5$  cfu/g (González et al., 1999). Detection of *Campylobacter jejuni* and *C. coli* with PCR-ELISA was shown to be 10- to 100-fold more sensitive than a gel-based PCR method using the same primers, the smallest amount of *C. jejuni* template DNA giving a positive signal in the assay being 1.5 fg (Sails et al., 2001). Some of the described PCR-ELISA applications can be considered to be quantitative competitive PCR approaches (Hahn et al., 1995; Kox et al., 1996; Denis et al., 1997).

#### 2.4.2.4. Real-time PCR

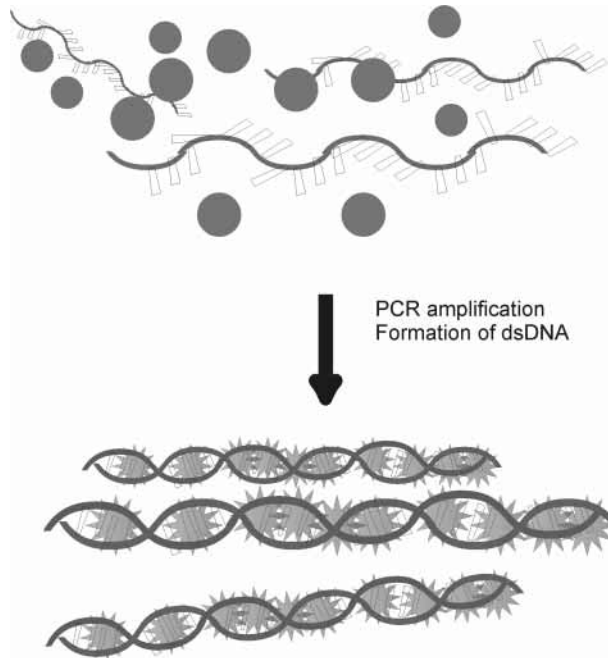
Real-time PCR is based on on-line measurement of the amplification reaction, enabling quantification of the product during the logarithmic phase of PCR. The first and so far most commonly used real-time PCR approach is the 5'-nuclease (TaqMan) assay introduced by Holland et al. (1991). The original assay operates using a radioactively labelled probe that hybridises in the PCR template region to generate a specific, detectable signal from the amplification reaction. The detection probe becomes annealed to one of the DNA strands during the amplification and is cleaved by the *Thermus aquaticus* DNA polymerase 5'-exonuclease activity during the primer extension step (Holland et al., 1991). Lee et al. (1993) further extended the 5'-nuclease assay by utilisation of doubly labelled oligonucleotide probes for fluorescent measuring of formation of the specific PCR product during primer extension (Figure 2). Introduction of an automated detection method enabled real-time monitoring of the PCR product formation during the exponential amplification phase (Heid et al., 1995). Since the advent of real-time PCR, several techniques have been introduced, including primers with fluorescent dyes, molecular beacons, dual probes and intercalating dyes such as SYBR Green I. Real-time PCR is a superior technique for quantification of nucleic acids. While competitive PCR has been demonstrated to be as reproducible and accurate as real-time PCR, the latter has the benefit of an easier methodology, reducing the need for sample DNA treatment (Desjardin et al., 1998; Wall and Edwards, 2002).



**Figure 2. 5'-nuclease assay.** A dual-labelled oligonucleotide probe is designed to hybridise near the 3'-end of one of the primers used in the amplification reaction. An intact probe contains two fluorescent molecules, and at its 5'-end the reporter dye is quenched due to its near vicinity to the quencher dye at the 3'-end. Upon primer annealing, the probe is digested by the 5'-nuclease activity of the DNA polymerase. This results in separation of the two fluorescent molecules. Measurement of light ( $\lambda_2$ ) emitted by the reporter is now enabled.

Although real-time PCR is a relatively new technique, several detection or quantification assays targeting various bacteria have already been described. Target bacteria include carious dentine bacteria (Martin et al., 2002), *Desulfotomaculum* from soil (Stubner, 2002), faecal bifidobacterial species (Requena et al., 2002), *Helicobacter hepaticus* (Ge et al., 2001), *Staphylococcus aureus* (Hein et al., 2001), *Borrelia burgdorferi* sensu lato (Rauter et al., 2002), *Campylobacter jejuni* (Nogva et al., 2000a), *Listeria monocytogenes* (Nogva et al., 2000b), *Rhodococcus coprophilus* (Savill et al., 2001) and *Mycoplasma genitalium* (Yoshida et al., 2002). Some applications for determination of larger bacterial groups have also been published (Lyons et al., 2000; Suzuki et al., 2000; Bach et al., 2002; Nadkarni et al., 2002). Typically, sensitivities of 1 to 100 target genomes per reaction and linearity ranges of 4 to 8 logarithmic units are obtained in assays targeted to specific bacteria. Real-time PCR is also utilised as a rapid

diagnostic tool for detection of pathogenic bacterial species or strains present in a sample. With pathogenic bacteria, the target of choice for real-time PCR is generally a gene associated with the pathogenic traits (Ke et al., 2000; Bellin et al., 2001; Corless et al., 2001; Ge et al., 2001; Hein et al., 2001; Bélanger et al., 2002; Rauter et al., 2002; Tondella et al., 2002).



**Figure 3. SYBR Green I –based measurement of amplification products.** SYBR Green I is an intercalating dye with virtually no fluorescence unless bound to double-stranded DNA. During PCR-amplification formation of double-stranded DNA molecules can be measured as an increase in SYBR Green I fluorescence.

Detection of PCR amplicons with specific probes is often favoured over usage of intercalating dyes due to the former's better sensitivity and lack of detection of falsely primed products. However, SYBR Green I (Figure 3) has become popular because of the possibility to use this intercalating dye in virtually any assay. Real-time quantitative PCR with SYBR Green I has been reported to be 10-fold less sensitive than a corresponding TaqMan assay due to the formation of non-specific products in reactions starting with small amounts of template DNA (Hein et al., 2001). On the other hand, SYBR Green I has great potential in situations where a diverse target population is to be detected with

PCR (Bach et al., 2002). A probe-based methodology requires a binding site for the probe in the vicinity of one of the primers; however, such a conserved site is likely to be missing from a degenerate target DNA population. This should be taken into account, especially when a bacterial population containing several hitherto unknown species is studied.

### 3. Aims of the study

The aims of this study were to develop molecular methods for analysis and monitoring of faecal bacterial populations and putative probiotic strains as well as to characterise the technological properties of two *Lactobacillus brevis* strains. The following goals were set:

1. To create a genetic label by introducing silent mutations to adjacent amino acid codons of a genomic peptidase gene of a selected *Lactobacillus* strain and to confirm the unchanged phenotype of the strain with an available peptidase assay.
2. To develop oligonucleotide PCR primers or probes targeting the 16S ribosomal DNA as species- or group-specific detection tools.
3. To exploit a PCR-ELISA application with multiple oligonucleotide capture probes for analysis of artificial mixed DNA samples or faecal DNA preparations.
4. To test suitability of real-time PCR for quantification of selected intestinal or probiotic bacteria in faecal samples.
5. To evaluate the applicability of two *Lactobacillus brevis* strains as supplementary strains with potential probiotic actions in dairy products.

## **4. Materials and methods**

### **4.1. Microbial strains, plasmids, human cell lines and culture conditions**

The microbial strains, plasmids and human cell lines used in this study have been described in detail in the respective original publications I-V. Briefly, *Lactobacillus helveticus* CNRZ32 was chosen as a model organism for demonstration of genetic labelling (I). Plasmids pUC19 (Yanisch-Perron et al., 1985) and pSA3 (Dao and Ferretti, 1985) were used to construct plasmids pKTH5052 and pKTH5053, respectively (I). The PCR-ELISA application with universal primers targeting 16S and 23S rDNA was tested with a set of bacteria including 25 species, that represented type strains of lactobacilli, bifidobacteria and other bacteria reported as members of human intestinal microbiota, or lactobacilli species used in dairy fermentations (II). Extension of the PCR-ELISA method with bifidobacteria-targeted 16S rDNA amplification was tested with a set of ten bifidobacterial species belonging to the human intestinal microbiota, suggested as potentially harmful oral microbes, or used in dairy products (III). With real-time PCR, six bacterial strains, each representing a bacterial species or group, present in the human intestinal tract or dairy products, were used (IV). Dairy technological and probiotic properties of two lactobacilli strains, *L. brevis* GRL1 (ATCC 8287) and *L. brevis* GRL62 (ATCC 14869<sup>T</sup>), were determined using dairy starters, positive or negative control strains or target strains for testing the antagonistic properties (V). The human intestinal cell lines Intestine-407 and Caco2 were used in adhesion studies (V). Culturing of microbes and human cell lines was carried out using media and conditions as outlined in the Materials and methods sections of original publications (I-V).

### **4.2. Basic DNA techniques**

Rapid isolation of genomic DNA from pure cultured bacteria was performed by cell mill disruption of bacterial samples in the presence of glass beads, followed by phenol-chloroform extraction and ethanol precipitation (I, II, III, V). For rapid and effective purification and isolation of DNA from faecal material, a method described in Study I was used (I, III). A large-scale DNA isolation method described by Apajalahti et

al. (1998) was used to produce a sufficient amount of template DNA for real-time PCR (IV).

Wizard Minipreps (Promega, USA) were used for isolation of plasmid DNA from *E. coli* clones (I), whereas the Qiagen Plasmid Protocol Kit (Qiagen, Hilden, Germany) was exploited for isolation of plasmid DNA from *L. brevis* (V).

Restriction enzyme digestions and ligations were carried out according to the enzyme manufacturer's recommendations (Promega). The transformations of *L. helveticus* cells were performed as described by Bhowmik and Steele (1993). Dot blot hybridisation was executed using standard methods (IV). DNA concentrations were measured with a Versafluor fluorometer (Bio-Rad). DNA sequencing was performed with an ABI310 DNA sequencer (Applied Biosystems) using BigDye Terminator chemistry (I).

#### **4.3. Design of oligonucleotide primers and probes**

Oligonucleotide primers and probes were designed with the help of published sequence data available in sequence databanks (I, II, III, IV). Generally, the online Internet tools ClustalW (Thompson et al., 1994) and Fasta3 (Pearson and Lippman, 1988) provided by the European Bioinformatics Institute (EBI) were used for identifying the primers and probes with desired specificity towards intended target DNA. Oligonucleotides required for creation of the silent mutation site (I) were planned by utilising the sequence of the *L. helveticus* CNRZ32 *pepX* gene (Accession number U22900). While creating the mutations, major changes in the codon usage frequency were avoided to reduce the likelihood of changes in the expression level of the target gene. The Ribosomal Database Project (Maidak et al., 2001) was utilised for designing the primers and probes used in Study IV. In addition, signature oligonucleotides published previously by others were utilised or modified when necessary (II, III, IV).

#### **4.4. Polymerase chain reaction (I-V)**

For end-point detection or production of DNA templates, polymerase chain reactions were carried out in reaction conditions recommended by the manufacturer of Dynazyme

DNA polymerase (Finnzymes). In Study I, the PCR conditions used in detection of wild-type and mutant strains were as follows: the reaction mixture consisted of 50 mM Tris-HCl pH 9.0, 15 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.1% Triton X-100, 1.8 mM  $\text{MgCl}_2$ , 360  $\mu\text{M}$  of each deoxynucleotide triphosphate, 1  $\mu\text{M}$  of each primer and 0.02 U/ $\mu\text{l}$  Dynazyme EXT<sup>TM</sup> Polymerase (Finnzymes, Finland), and 1  $\mu\text{l}$  of template or water. Each sample was subjected to a primary denaturation cycle at 95°C for 2 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 1.5 minutes. The reaction was terminated at an elongation step for 5 minutes at 72°C and followed by incubation at 4°C. In Study IV, gradient PCR was used to select optimal annealing temperatures for PCR primer pairs. Oligonucleotides were synthesised by commercial suppliers.

#### 4.4.1. PCR-ELISA (II, III)

PCR-ELISA was carried out as described in Studies II and III. Briefly, the PCR products were labelled with the DIG-High Prime Kit (Boehringer Mannheim, Germany) according to the instructions of the manufacturer, and concentrations of the PCR products were measured with the Versafluor fluorometer. For calculation of molecular quantities in the labelled end products, the amount of DNA (ng/ $\mu\text{l}$ ) was divided by the length of the PCR product (base pair) multiplied by the average weight of a base pair (ng/base pair). The hybridisations were performed in commercial streptavidin-coated microtiter plates (Labsystems, Finland). Results were analysed by measuring absorbance at 450 nm (iEMS Reader MF, Labsystems, Finland).

#### 4.4.2. Real-time PCR (IV)

Real-time PCR was tested with two different chemistries (5'-nuclease- and SYBR Green I assays) as described in Study IV. Quantitative PCR was performed with an iCycler iQ (Bio-Rad, USA) and iCycler Optical System Interface software version 2.3 (Bio-Rad, USA). All PCR reactions were performed in triplicate, in a volume of 25  $\mu\text{l}$ , using 96-well optical grade PCR plates and optical sealing tape (Bio-Rad, USA).

Optimal concentrations for various reaction components were tested for each primer set and chemistry with a dilution series of genomic DNA from the target test species. With SYBR Green I chemistry, effect of the polymerase type on PCR product formation was tested with a standard polymerase, Dynazyme II (Finnzymes, Finland), and hot-start polymerases AmpliTaq Gold® DNA Polymerase (Applied Biosystems, USA), BlueTaq (Euroclone Ltd., UK) and FastStart Taq DNA Polymerase (Roche). The TaqMan assays were performed successfully with the Dynazyme II enzyme and therefore switching to the hot-start enzyme was not considered.

#### **4.5. Peptidase activity assays (I)**

Activity measurement of the PepX was determined from both the wild-type and mutated *L. helveticus* strains according to the method of El Soda and Desmazeaud (1982).

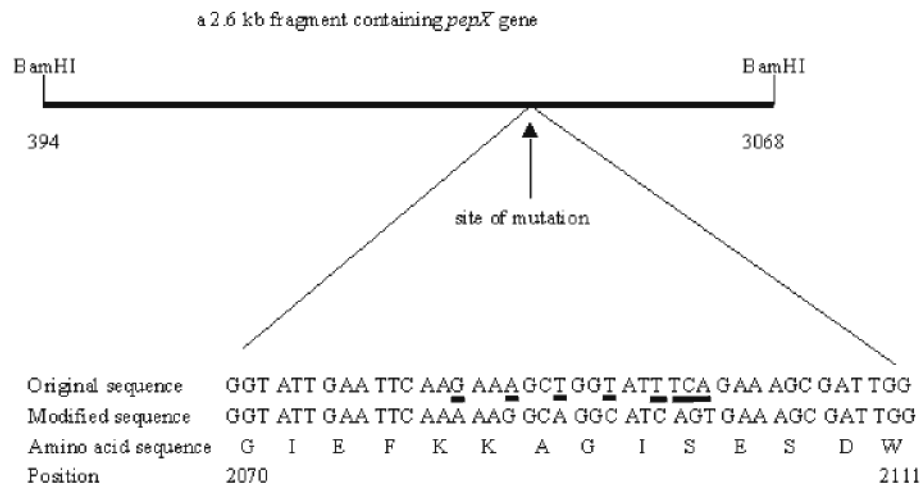
#### **4.6. Evaluation of *L. brevis* strains as dairy adjuncts**

In vitro tolerance of the *L. brevis* strains to low pH, bile salts and pancreatic fluid was examined as described in Study V. Antimicrobial properties towards selected food spoilage bacteria and dairy lactic acid bacteria starters were evaluated by following the microbial growth in MRS broth supplemented with 10% filter-sterilized *L. brevis* GRL1 or GRL62 supernatant at 37°C for two days with an automated turbidometer Bioscreen C Analysing System (Labsystems, Finland). Adherence to Caco-2 and Intestine-407 cell lines was studied as described in Study V. Resistance to selected antibiotics was tested with disc diffusion and microdilution methods, modified from instructions given by the antibiotic disc manufacturer (A/S Rosco, Denmark) and NCCLS. In vivo feeding trials with four healthy volunteers were performed to see whether the *L. brevis* strains could survive the GI passage. Technological suitability of the strains for dairy processes was also evaluated as described in Study V.

## 5. Results

### 5.1. Strain-specific genetic labelling of *Lactobacillus helveticus* CNRZ32 (I)

Silent mutation positions were introduced in adjacent codons of the *Lactobacillus helveticus* CNRZ32 chromosomal *pepX* gene without altering the amino acid sequence of the gene product, creating a strain-specific tag which enabled direct nucleic-acid level identification of the changed strain (Figure 4). The mutation site was introduced into the *pepX* gene with a PCR primer containing designed mismatched nucleotides. The target gene was amplified in two separate reactions to obtain a conserved fragment and a mutation site-containing fragment, which were ligated together to form a single mutation site-containing fragment. The mutant gene was first cloned with pUC19 to *E. coli* to create plasmid pKTH5052 and further subcloned to pSA3, resulting in the plasmid pKTH5053, which was then used to transform *L. helveticus* CNRZ32.



**Figure 4. Schematic representation of the silent-mutation site –containing *pepX* gene.**

A *L. helveticus* CNRZ32 transformant strain GRL1021 containing the plasmid pKTH5053 was grown at a restrictive temperature (45°C) under antibiotic selection to find clones with the thermosensitive plasmid integrated into the bacterial chromosome through homologous recombination between the wild-type and mutant forms of *pepX*. An erythromycin-resistant strain, observed to contain the integrated plasmid, was grown at permissive conditions (37°C) for approximately 100 generations to enable a second

homologous recombination event between the two *pepX* genes, resulting in excision of the plasmid and one of the *pepX* genes. Samples were plated out and colonies that had lost antibiotic resistance were screened with PCR for replacement of the wild-type *pepX* with the mutated *pepX*. A strain with the mutated gene was found and designated as GRL1023. Sequencing was used to confirm the correctness of the integration construct.

The intact phenotypic properties of the GRL1023 were verified by measuring the activity of the target gene product PepX by utilising its ability to break down L-glycyl-L-prolyl-*p*-nitroanilide, with production of colour. End-point PCR with PCR primers targeting the mutated or original *pepX* sequence was successfully used to detect the wild-type and mutant strains added to faeces or milk.

## **5.2. Suitability of PCR-ELISA for analysis of mixed DNA populations (II, III)**

### **5.2.1. PCR-ELISA with universal primers targeting the 16S and 23S genes (II)**

A PCR-ELISA method was tested with 16 oligonucleotide probes to simultaneously detect under standardised conditions selected intestinal bacteria, lactobacilli and bifidobacteria. For this purpose, species- or group-specific oligonucleotide probes for lactobacilli and selected intestinal bacteria were designed or adapted from the literature. Specificity of the probes was tested with 25 species. The 16S and 23S region of these test species was first PCR-amplified with universal eubacterial primers, followed by digoxigenin-labelling of the PCR products and addition of  $2 \times 10^{10}$  labelled molecules to each hybridisation reaction. For hybridisation, the oligonucleotide probes to be tested were bound to the microtiter plate wells via biotin-streptavidin linkage. Under the hybridisation conditions used, the specificity level expected was obtained with most of the oligonucleotide probes chosen. Only the differentiation of closely related *L. casei*, *L. paracasei* and *L. rhamnosus* was partly unsatisfactory. Furthermore, in addition to its intended target DNA, the *F. nucleatum* probe, fuso16S, also recognized *E. biforme*. Sensitivity of the PCR-ELISA with the probes tested varied between detection of  $6.77 \times 10^7$  and  $1.29 \times 10^9$  PCR-amplified molecules in a hybridisation reaction. The sensitivity obtained was somewhat lower than expected,

which was at least partly due to compromises that had to be made in the hybridisation conditions when using several probes simultaneously.

PCR biasing was studied by combining genomic DNA from different test species into two mixed sample pools, each with seven DNA targets, prior to PCR amplification. PCR was also performed using the pools in the same amplification. The amplification products were hybridised with 14 different probes. Most of the probes tested performed well and could detect their target DNA from mixed samples of both seven and fourteen species, but the probes for *B. adolescentis* (ado440, b162), *L. brevis* (lab86), *L. paracasei* (par160), *L. plantarum* (pla448) and *L. curvatus* (cur150) failed to recognize their target DNAs. Failure in the detection of *B. adolescentis*, *L. paracasei* and *L. plantarum* in particular could have been due to either bias during multi-template PCR, where some templates are amplified more efficiently as a result of differences in the specificity of eubacterial primers, or bias by chance. Changing of the total template concentrations tested in the PCR amplification was not observed to affect the hybridisation results.

#### 5.2.2. PCR-ELISA with bifidobacterial primers targeting the 16S genes (III)

The PCR-ELISA method was extended for detection of the most common *Bifidobacterium* species in humans and applied to a feeding trial including administration of *Bifidobacterium lactis* Bb-12 and galacto-oligosaccharide -containing syrup as probiotic and prebiotic preparations, respectively. Oligonucleotide probes based on 16S rDNA sequences were designed and tested for specificity and sensitivity with nine different bifidobacterial species, followed by analysis of faecal samples. Faecal bifidobacteria were monitored for fluctuations during and after the feeding trial. Although the bifidobacterial populations present in faeces were generally consistent between samples taken from different time points, PCR-ELISA results suggested that *Bifidobacterium longum* was partly replaced by *B. lactis* Bb-12 during the probiotic feeding. This proposition could not, however, be verified with statistical methods.

The occurrence of bifidobacterial species or groups in human faecal samples is summarised in Table 1. Generally, the bifidobacterial species composition of individual

subjects was consistent and comprised 3-4 bifidobacterial species or groups. The predominant species were *Bifidobacterium longum* and *B. adolescentis*.

**TABLE 1. Distribution of different bifidobacterial species in human subjects.**

Species	No. of positive subjects	% of total
<i>Bifidobacterium adolescentis</i>	23	82.1
<i>B. bifidum</i>	12	42.9
<i>B. breve</i>	4	14.3
<i>B. catenulatum group</i> <sup>1)</sup>	11	39.3
<i>B. denticolens</i>	0	0
<i>B. dentium</i>	0	0
<i>B. lactis</i> <sup>2)</sup>	0	0
<i>B. longum</i>	28	100
Combinations of		
one species	1	3.6
two species	9	32.1
three species	13	46.4
four species	5	17.9

<sup>1)</sup> *B. catenulatum* group consists of *B. catenulatum* and *B. pseudocatenulatum*

<sup>2)</sup> Results are based on analysis of pre-feeding samples (0-samples)

### **5.3. Comparison of real-time PCR and dot blot hybridisation for quantification of the 16S ribosomal DNA of target bacteria (IV)**

Real-time PCR was tested with two chemistries: SYBR Green I and TaqMan probes. Six detection assays were tested for their sensitivity in detecting target bacterial DNA from pure and mixed samples with both chemistries. The same target DNAs were also quantified with dot blot hybridisation probes. Real-time PCR was shown to have sensitivity superior to dot blot hybridisation. The linear range of amplification of pure cultured target DNA varied between 0.1-1 pg and 1-10 ng of specific target genome, which corresponds to approximately 20-400 to  $2 \times 10^5$ – $4 \times 10^6$  genomes, depending on the genome size of the target species. Furthermore, a 1 pg addition of target DNA to 20 ng of mixed DNA samples could be detected reliably with both real-time PCR chemistries. A

reconstruction assay with addition of  $10^9$ ,  $10^8$ ,  $10^7$ ,  $10^6$  or  $10^5$  target bacterial cells to faecal samples confirmed that real-time PCR could detect addition of  $10^6$  cells to one gram of faeces, provided that the target bacterium was not included in the original faecal sample. Addition of  $10^9$  cells of *Bifidobacterium longum* to faeces resulted in no difference compared with control sample, which had no added bacterial cells, due to the pre-existence of *B. longum* in the original sample.

The two chemistries used in real-time PCR were equally sensitive; however, successful usage of the intercalating dye SYBR Green I required applying of a hot start polymerase to prevent formation of primer dimers, whereas with 5'-nuclease chemistry, a similar sensitivity level was reached with a conventional DNA polymerase. Furthermore, the 5'-nuclease chemistry was faster to perform because of its more straightforward incubation protocol, one run taking approximately 80 min.

#### **5.4. Suitability of *L. brevis* strains as dairy supplements (V)**

Two *Lactobacillus brevis* strains, ATCC 8287 and ATCC 14869<sup>T</sup>, were evaluated for their applicability as putative probiotics in dairy products. The strains tolerated well low pH, bile acids and pancreatic fluid under in vitro conditions. They also expressed good in vitro adherence to human Caco-2 and Intestine-407 cells. Adherence of the *L. brevis* strains was especially strong to the small intestinal cell line, Intestine 407. In antimicrobial activity assays, strain ATCC 8287 showed inhibitory properties towards selected potentially harmful micro-organisms, particularly against *Bacillus cereus*. Antimicrobial resistance tests revealed that both *L. brevis* strains were resistant to vancomycin as well as to several other antibiotics, which is, however, typical for the genus *Lactobacillus*. The *L. brevis* strains were unable to acidify milk to yoghurt but were suitable as supplement strains in yoghurts. This was demonstrated by producing a set of yoghurt products and analysing their rheological and sensory properties during a cold storage period of 28 days. Despite its human origin, *L. brevis* ATCC 14869<sup>T</sup> did not survive the gastrointestinal tract, whereas *L. brevis* ATCC 8287 was detected in faecal samples taken during and immediately after ingestion of the strain. In conclusion, *L. brevis* ATCC 8287 was considered a promising candidate for use as a probiotic adjunct in dairy products.

## 6. Discussion

Molecular biology tools are needed for better understanding of the composition and functions of the intestinal microbiota. In this study, different levels of detection were developed for lactic acid bacteria and faecal microbiota.

Probiotic studies require careful documentation of the proposed effects. For better monitoring of a potential strain, strain-specific discrimination could be beneficial in, for example, feeding studies. Furthermore, specific recognition of industrial strains with commercial value would help in monitoring the usage of these strains. Therefore, *Lactobacillus helveticus* CNRZ32 was chosen as a model organism for demonstration of genetic labelling. Although the strain in question does not fulfil the criteria set for a probiotic, selection of this organism was considered justified for several reasons. The strain is a starter strain used in the dairy industry, its proteolytic system has been studied extensively (Dudley et al., 1996; Yüksel and Steele, 1996; Fenster et al., 1997; Shao et al., 1997; Chen and Steele, 1998; Pederson et al., 1999) and methods for its transformation and gene disruption have been developed (Bhowmik and Steele, 1993; Chen and Steele, 1998). Various 'food-grade' markers for lactic acid bacteria have been suggested, often with introduction of new phenotypic properties (Allison and Klaenhammer, 1996; Geoffroy et al., 2000). However, creation of a label without introduction of any phenotypic changes into the target strain would be more easily accepted by consumers and legislators. Hertel et al. (1992) labelled a *Lactococcus lactis* strain by introducing silent mutations into a plasmid-encoded gene. By contrast, to ensure the stability of the created tag, we used a chromosomal gene for labelling of *L. helveticus* CNRZ32. As demonstrated in Study I, a silent mutation site is effortlessly detected from different matrices, such as faeces or milk, by PCR. Alternatively, hybridisation probes could be utilised for detection (Hertel et al., 1992).

Direct detection of target nucleic acids present in population samples removes the need for cultivation steps. Here, PCR-ELISA, dot blot hybridisation and real-time PCR were compared for their suitability for analysis of population samples. Similar to PCR-ELISA method described in this paper, DGGE- and TGGE-based applications have been found to detect approximately 1-10% subpopulations with PCR-amplified 16S rDNA fragments when studying faecal samples (Zoetendal et al., 1998), suggesting their use for

analysis of predominant microbes of population samples. By using DGGE with group-specific 16S rDNA primers, however, characterisation of diversity of smaller subpopulations has been reached (Overmann et al., 1999; Walter et al., 2001; Heilig et al., 2002). Similarly, the bifidobacteria-specific PCR-ELISA could successfully be used for species-level analysis of faecal bifidobacteria; previous DGGE analysis results of the same samples by Satokari et al. (2001b) generally support results obtained with PCR-ELISA. One drawback of PCR-ELISA identification has, however, been uncovered. When results were compared with PCR-ELISA analysis, it soon became evident that ado440 (Yamamoto et al., 1992), intended to be specific for *B. adolescentis*, also recognized *B. ruminantium* observed in the faeces of some volunteers (Satokari et al., 2001b). New sequence alignments confirmed that the ado440 probe had no mismatches with *B. ruminantium* 16S rDNA. While the advantage of DGGE is the possibility for further sequence analysis and identification of emerging new amplicons in DGGE profiles, PCR-ELISA seems to be slightly more sensitive to changes in the relative amounts of target species.

Strain-level studies have confirmed the complexity, uniqueness and stability of bifidobacterial populations in the GI-tract (McCartney et al., 1996; Kimura et al., 1997; Mangin et al., 1999). PCR-ELISA results were in accord with earlier reports. The observed species distribution correlated well with previous European studies (Mangin et al., 1994; Satokari et al., 2001a), and species distribution usually remained unchanged within the samples of each subject. However, reduction of intensities of *B. longum* - specific probes was observed frequently in the groups that ingested the probiotic *B. lactis* Bb-12, although this could not be verified with statistical analysis, mainly due to the small size of the test groups. This suggests that *B. lactis* Bb-12 may have partly replaced *B. longum* during the ingestion period.

Hybridisation allows direct semi-quantitative monitoring of population samples and thus avoids the problems caused by PCR bias. Dot blot hybridisation with rRNA-targeted oligonucleotide probes is a well-established method for studying faecal microbes (Doré et al., 1998; Sghir et al., 2000; Hopkins et al., 2001; Marteau et al., 2001). Ribosomal RNA is abundantly present in the bacterial cells, thus increasing the sensitivity of the dot blot hybridisation to a reasonable level. However, radioactively

labelled probes are generally needed for successful detection of nucleic acid targets from population samples. Here, dot blot hybridisation was performed with rDNA-targeted oligonucleotide probes to quantify the same starting material as with the real-time PCR applications tested. Dot blot hybridisation could, at its best, detect a 3% DNA subpopulation from mixed DNA samples. A similar sensitivity level has been reported by Muttray and Mohn (2000) for measurement of rDNA:rRNA ratios. Thus, the result was as expected, being approximately 10-fold less sensitive than that obtained with rRNA-targeted oligonucleotides (Sghir et al., 2000). Real-time PCR was tested as an alternative for a sensitive detection of target bacteria in faeces. The sensitivity level obtained for the real-time PCR assays was 200-400 target bacteria in pure cultures or mixed DNA samples, depending on the genome size of a target bacterium. Results obtained from artificial DNA mixtures were confirmed by a reconstruction assay; addition of  $10^6$  bacterial cells to faecal samples containing approximately  $10^{11}$  bacteria could be detected with real-time PCR in a quantitative manner. The acquired level, providing a means for quantifying a 0.01% subpopulation in a DNA sample, was considered sufficient for studying the gastrointestinal tract ecology.

Although nucleic acid based detection methods offer a means to avoid bias caused by cultivation or phenotypic testing of bacterial isolates, these techniques are not totally devoid of problems. The main issues for detection of nucleic acids in mixed samples are isolating DNA from different sources with constant efficiency, minimising the occurrence of false-positive reactions due to contamination or poor planning of the primers or hybridisation probes used, and avoiding negative reactions due to inhibiting substances. Also, any oligonucleotides used as detection probes or primers should be considered specific only in relation to the current sequence data. Several methods for faecal sample treatment for isolation of DNA have been described (Biswas et al., 1994; Klijn et al., 1995; Shames et al., 1995; Wang et al., 1996; Lou et al., 1997; Monteiro et al., 1997; Apajalahti et al., 1998). Nevertheless, this step remains somewhat problematic because of the presence of PCR-inhibiting substances in the faeces and the overall complexity of faecal microbial populations. Although over 90% recovery of the total bacterial DNA present in faeces has been claimed (Apajalahti et al., 1998), non-selective isolation of bacterial DNA from population samples is difficult to achieve or

demonstrate. Commercial kits have recently become available for isolation of faecal DNA. However, McOrist et al. (2002) observed differences in the relative efficacy of extraction of bacterial DNA from faeces with four commercial kits. Furthermore, the relative sensitivities could not be extrapolated from DNA extractions performed directly from pure cultures (McOrist et al., 2002).

Evaluation of possible probiotic properties of a bacterial strain requires intensive and carefully planned testing of the strain both in vivo and in vitro. Although *L. brevis* is not typically used as a probiotic, Kishi et al. (1996) have shown that oral administration of live *L. brevis* ssp *coagulans* strain significantly stimulated the host immunity system by increasing IFN- $\alpha$  production in human subjects. This result cannot, however, be directly extrapolated to the *L. brevis* strains studied here. Adherence of lactic acid bacteria to various components of human intestine has been studied with the human colon adenocarcinoma Caco-2 cell line (Greene and Klaenhammer, 1994; Lehto and Salminen, 1997; Jacobsen et al., 1999; Kimoto et al., 1999), Intestine-407 cells derived from human embryonic jejunum and ileum (Sarem et al., 1996), and intestinal mucus (Ouwehand et al., 1999a, and b, 2001b). Both Caco-2 and Intestine-407 cells were used in the adhesion assays for evaluation of the adhesion ability of *L. brevis*. These cell lines offer complementary models for adhesion studies (Sarem et al., 1996). When compared with the probiotic *L. rhamnosus* GG, adherence of the *L. brevis* strains was especially strong to the small intestinal cell line, Intestine 407, suggesting that the small intestine could be affected with the strains studied. *L. brevis* GRL62 has also been shown to have intermediate adhesion properties to human intestinal mucus (Ouwehand et al., 2001b).

The two *L. brevis* strains studied were antagonistic towards some of the potentially harmful micro-organisms, while they did not significantly inhibit the growth of yoghurt-starter bacteria. In a previous study of Koga et al. (1998), *L. brevis* GRL1 (ATCC 8287), included in a panel of 41 *Lactobacillus* strains belonging to *L. gasseri*, *L. reuteri*, *L. casei*, *L. helveticus*, *L. brevis*, *L. fermentum* and *L. plantarum*, weakly inhibited two *Vibrio cholerae* strains of the eight strains tested. In the present study, *L. brevis* GRL1 was shown to strongly inhibit *B. cereus* and to some extent also the growth of *S. aureus* and other harmful micro-organisms chosen for the assay.

In vitro adhesion assays and testing for tolerance of low pH, bile acids and pancreatic fluids have often been considered to be good indicators of the survival of a bacterial strain through the GI tract. In this study, both *L. brevis* strains performed well in the in vitro tests, and survival through the stomach could therefore be suggested for both strains, especially when simultaneous ingestion of the bacteria with food, resulting in a higher pH in the stomach, is taken into account. However, *L. brevis* GRL62 failed to survive under in vivo conditions. This was surprising as the strain has originally been isolated from human faeces (Orla-Jensen, 1919). In contrast, *L. brevis* GRL1, originally isolated from fermented olives, could tolerate the gastrointestinal conditions and was found to persist in the human gut.

Although the strains studied were not suitable for fermenting yoghurt, supplementation of yoghurt with either of these strains had no negative effects on yoghurt taste, appearance, or preservation. Therefore, *L. brevis* GRL1, which was able to survive in the GI tract, could be considered for use as a supplementary strain in yoghurt or other dairy products. To date, several commercial probiotics are already available and many others are being studied, but no single strain is likely to possess all of the beneficial properties suggested for probiotics. *L. brevis* GRL1, shown to be antagonistic towards *Bacillus cereus* (this study) and *Vibrio cholerae* (Koga et al., 1998), could be a valuable addition to the probiotics field. The surface (S)-layer of this strain is well-characterized and potential applications for the S-layer have been suggested (Vidgrén et al., 1992; Kahala et al., 1997; Savijoki et al., 1997; Kahala and Palva, 1999, Hynönen et al., 2002). Recently, the S-layer has been shown to mediate the ability of the *L. brevis* GRL1 strain to adhere to human intestinal, urinary bladder and endothelial cells (Hynönen et al., 2002). Good adhesion properties could assist in the competitive exclusion of potentially harmful microbes by *L. brevis* GRL1, and usage of this strain as an effective mucosal vaccination tool because of its binding properties may be warranted. More studies are, however, required to confirm these hypotheses.

## 7. Conclusions

Creation of a strain-specific nucleic acid tag by labelling of a chromosomal target gene with a silent mutation and utilisation of the label for strain-specific detection of the target strain was demonstrated using *Lactobacillus helveticus* CNRZ32. Although the strain in question is not considered to be a probiotic, the method described here could be used for probiotics. However, this requires the pre-existence or development of suitable transformation tools as well as sequence data of a suitable target gene. From the viewpoint of current legislation and consumer acceptance, the silent labelling method should be the least unacceptable method for making genetic alterations to a bacterial strain used in food processing, especially as the phenotypic properties of the labelled strain were demonstrated to remain unchanged, thus attesting to the safety of the method.

PCR-ELISA with universal primers was shown to be suitable for detection of predominant bacteria present in a mixed population sample. Specificity of the PCR step proved to be critical for the sensitivity of the method; application of bifidobacterial-specific 16S primers enabled species- or group-specific detection of bifidobacteria present in human faeces. The overall sensitivity of PCR-ELISA matched DGGE approaches, with similar PCR primer specificities. Furthermore, being technically a rather simple method, PCR-ELISA is easily transferred between different laboratories, and could, in principle, be used in a similar manner as DGGE for analysis of large sample numbers.

Real-time PCR was established to be a superior method for detection of single bacterial species or larger groups from mixed DNA samples or faeces. Compared with dot blot hybridisation with radioactive probes, real-time PCR provided an improved sensitivity for detection, a means to avoid usage of radioactive labels, increased speed and volume of sample analysis, an easier methodology and better reproducibility. Detection of target bacteria added to faeces prior to the DNA isolation and purification steps was demonstrated. In future, multiplexing with different labels and improvements in hardware and software to allow simultaneous monitoring of a large number of PCR reactions are likely to make real-time PCR an even more potent tool for population analyses.

*L. brevis* GRL1 could be considered to be a potential probiotic strain. Although several probiotics are already on the market, new strains with beneficial properties are continually being sought. The strain in question has a well-characterized S-layer, and offers promise as a potential mucosal vaccination strain.

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