CHAPTER II
LITERATURE REVIEW

1. Lactic Acid Bacteria

Lactic acid bacteria (LAB) consist of a number of bacterial genera within the phylum Firmicutes. The genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* are recognized as LAB (Ercolini et al., 2001; Jay, 2000; Holzapfel et al., 2001; Stiles and Holzapfel, 1997). Lactic acid-producing Gram-positive bacteria but belonging to the phylum Actinobacteria are genera such as *Aerococcus, Microbacterium*, and *Propionibacterium* (Sneath and Holt, 2001) as well as *Bifidobacterium* (Gibson and Fuller, 2000; Holzapfel et al., 2001). Members of LAB share the property of being Gram-positive bacteria (Fooks et al., 1999) that ferment carbohydrates into energy and lactic acid (Jay, 2000). Depending on the organism, metabolic pathways differ when glucose is the main carbon source: homofermentative bacteria such as *Lactococcus* and *Streptococcus* yield two lactates from one glucose molecule, whereas the heterofermentative (ie. *Leuconostoc* and *Weissella*) transform a glucose molecule into lactate, ethanol and carbon dioxide (Caplice and Fitzgerald, 1999; Jay, 2000; Kuipers et al., 2000). In addition, LAB produce small organic compounds that give the aroma and flavor to the fermented product (Caplice and Fitzgerald, 1999).

The taxonomy of LAB based on comparative 16S ribosomal RNA (rRNA) sequencing analysis has revealed that some taxa generated on the basis on phenotypic features do not correspond with the phylogenetic relations. Molecular techniques, especially the polymerase chain reaction (PCR) based methods, such as rep-PCR fingerprinting and restriction fragment length polymorphism (RFLP) as well as pulse-field gel electrophoresis (PFGE), are regarded important for specific characterization and detection of LAB strains (Gevers et al., 2001; Holzapfel et al., 2001). Recently, culture-independent approaches have been applied for the detection of intestinal microbiota (Zoetendal et al., 2002). Denaturing gradient gel electrophoresis (DGGE)

and temperature gradient gel electrophoresis (TGGE) analysis of faecal 16S rDNA gene and its rRNA amplicons have been shown to be powerful approaches in determining and monitoring the bacterial community in faeces (Zoetendal et al., 1998).

LAB were first isolated from milk (Carr et al., 2002; Metchnikoff, 1908; Sandine et al., 1972) and have since been found in such foods and fermented products as meat, milk products, vegetables, beverages and bakery products (Aukrust and Blom, 1992; Caplice and Fitzgerald, 1999; Harris et al., 1992; Gobbetti and Corsetti, 1997; Jay, 2000; Liu, 2003; Lonvaud-Funel, 2001; O'Sullivan et al., 2002). LAB occur naturally in fermented food (Caplice and Fitzgerald, 1999) and have been detected in soil, water, manure and sewage (Holzapfel et al., 2001). LAB exist in humans (Boris et al., 1998; Carroll et al., 1979; Eideman and Szilagyi, 1979; Elliott et al., 1991; Martín et al., 2003; Ocaña et al., 1999; Reid, 2001; Schrezenmeir and de Vrese, 2001) and in animals (Fujisawa and Mitsuoka, 1996; Fuller and Brooker, 1974; Gilliland et al., 1975; Klijn et al., 1995; Sandine et al., 1972; Schrezenmeir and de Vrese, 2001). However, some LAB are part of the oral flora which can cause dental caries (Monchois et al., 1999; Sbordone and Bortolaia, 2003). LAB can work as spoilage organisms in foods such as meat, fish and beverages (Jay, 2001; Liu, 2003). LAB have been used as a flavoring and texturizing agent as well as a preservative in food for centuries and are now added as starters in food (Caplice and Fitzgerald, 1999). LAB, such as lactobacilli, *L. lactis*, and *Streptococcus thermophilus*, inhibit food spoilage and pathogenic bacteria and preserve the nutritive qualities of raw food material for an extended shelf life (Heller, 2001; O'Sullivan et al., 2002). Recently, the use of metabolites of LAB as biological preservatives in food packaging materials has been discussed (Pirttijärvi et al., 2001; Scannell et al., 2000). LAB plays an important role in processing animal feeds like silage (Aukrust and Blom, 1992; Driehuis and Oude Elferink, 2000; Holzer et al., 2003). The antimicrobial effect of LAB is mainly due to their lactic and organic acid production, causing the pH of the growth environment to decrease (Caplice and Fitzgerald, 1999; Kuipers et al., 2000). Low pH induces organic acids to become lipid soluble and to diffuse through the cell membrane into the cytoplasm (Gottschalk, 1988). LAB also produce acetaldehyde, hydrogen peroxide, diacetyl, carbon dioxide, polysaccharides and bacteriocins

(Caplice and Fitzgerald, 1999; de Vuyst and Degeest, 1999; Rodrígues et al., 2003), some of which may act as antimicrobials.

2. Metabolic Activity of Lactic Acid Bacteria

Lactic acid bacteria are generally mesophilic but can grow at temperatures as low as 5°C or as high as 45°C. Similarly, while the majority of strains grow at pH 4.0–4.5, some are active at pH 9.6 and others at pH 3.2. Strains are generally weakly proteolytic and lipolytic and require preformed amino acids, purine and pyrimidine bases and B vitamins for growth. An overview of the lactic acid bacteria is presented in the texts edited by Wood and Holzapfel (1995) and the reader is directed to these sources for information relating to aspects such as taxonomy, biochemistry, physiology, ecology and applications.

All lactic acid bacteria produce lactic acid from hexoses and since they lack functional heme linked electron transport chains and a functional Krebs cycle, they obtain energy via substrate level phosphorylation. The lactic acid produced may be L (1) or, less frequently, D (2) or a mixture of both. It should be noted that D (2) lactic acid is not metabolised by humans and is not recommended for infants and young children, a fact exploited by the marketers of a strain of *Lb. bavaricus* in Germany for use in the production of speciality L (1) sauerkraut (Lucke, 1985).

The pathways by which hexoses are metabolized divide lactic acid bacteria into two groups, homofermentative and heterofermentative (Fig. 1). For a detailed description of these pathways the reader is referred to Axelsson (1998). Briefly, homofermenters such as *Pediococcus*, *Streptococcus*, *Lactococcus* and some lactobacilli produce lactic acid as the major or sole end-product of glucose fermentation. However, under altered growth conditions and when the initial substrate is a pentose this may change (Kandler, 1983). Homofermenters use the Embden–Meyerhof–Parnas pathway to generate two moles of lactate per mole of glucose and derive approximately twice as much energy per mole of glucose as heterofermenters. Heterofermenters such as *Weisella* and *Leuconostoc* and some lactobacilli produce equimolar amounts of lactate, CO_2 and ethanol from glucose via the hexose monophosphate or pentose pathway.

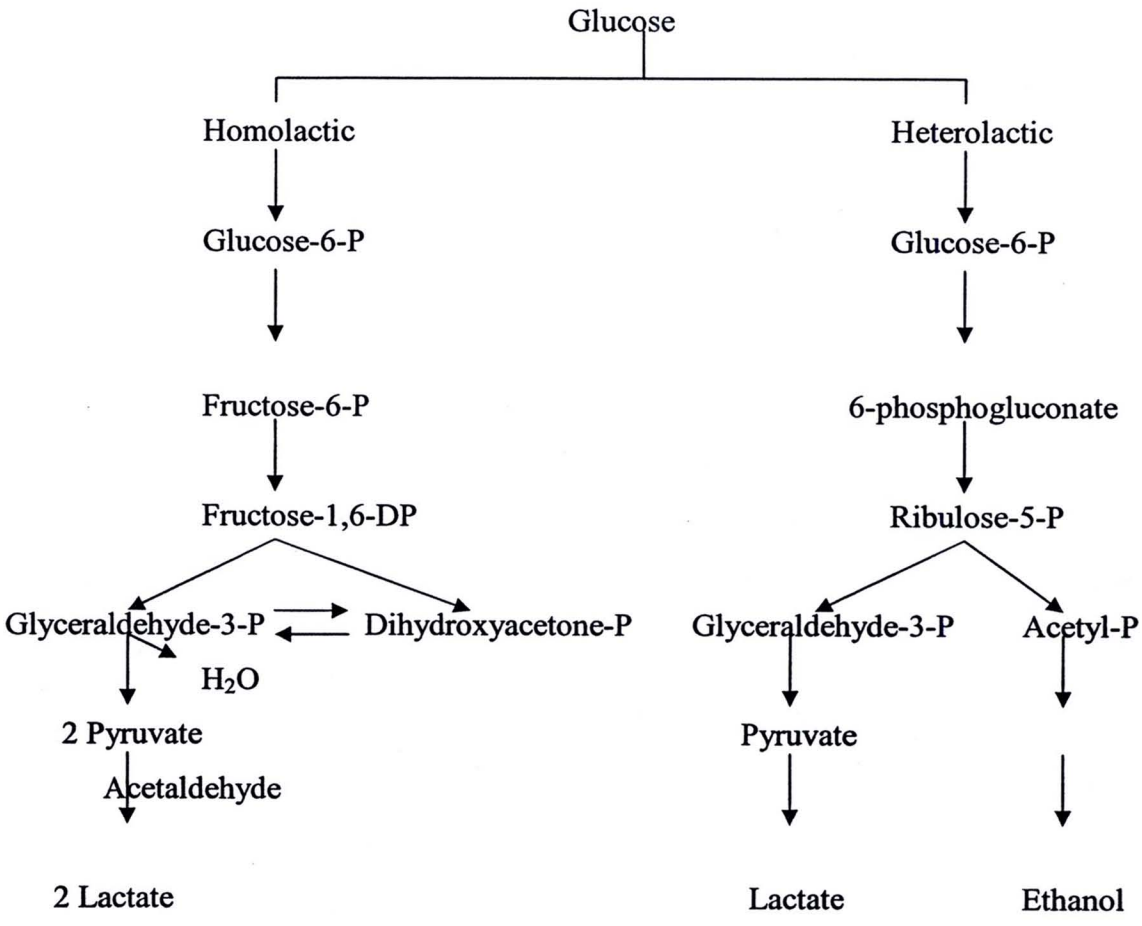

Figure 2.1 Generalized scheme for the fermentation of glucose in lactic acid bacteria.

The metabolism of the disaccharide lactose is of primary importance in those lactic acid bacteria used in dairy fermentations and is reviewed in Fox et al. (1996) and Axelsson (1998). Lactose may enter the cell using either a lactose carrier, lactose permease, followed by cleavage to glucose and galactose or via a phosphoenolpyruvate-dependent phosphotransferase (PTS) followed by cleavage to glucose and galactose-6-phosphate. Glucose is metabolised via the glycolytic pathway, galactose via the Leloir pathway and galactose-6-phosphate via the tagatose 6-phosphate pathway. Most *L. lactis* strains used as starters for dairy fermentations use the lactose PTS, the genes for which are plasmid located. Among some thermophilic LAB only the glucose moiety of the sugar is metabolised and galactose is excreted into the medium, although mutants of *S. thermophilus* have been described which metabolise galactose via the Leloir pathway (Hutkins, Ellefson and Kashket, 1987).

Citrate metabolism is important among *L. lactis* subsp. *lactis* (biovar *diacetylactis*) and *L. mesenteroides* subsp. *cremoris* strains used in the dairy industry, as it results in excess pyruvate in the cell. The pyruvate may be converted via α-acetolactate to diacetyl, an important flavour and aroma component of butter and some other fermented milk products (Fig. 2.1). Strategies designed to increase the carbon metabolic flux towards diacetyl production have resulted in mutants which produce large amounts of this compound (Hugenholtz and Kleerebezem, 1999).

The proteolytic system of *Lactococcus* has been investigated in detail due to its pivotal role in allowing growth in milk and the development of flavour and texture in cheese. In summary, casein is degraded by a membrane-anchored serine proteinase (PrtP) with many of the resulting oligopeptides being sufficiently small to allow them to be transported into the cell via an oligopeptide transport system (Opp), where they are further processed by a variety of intracellular peptidases. Amino acid and di- and tri-peptide transport systems also exist but there is only poor growth in milk when mutants are deficient in PrtP. Commercial culture adjuncts (mesophilic and thermophilic starter cultures) are available to promote proteolysis in cheese and can aid in the development of a consistent cheese flavour (Fox et al., 1996; Beresford et al., 2001). It is notable that over-expression of a lactococcal proteinase did not result in an acceleration of cheese ripening or in an enhancement of flavour (Law, 2001). Mutants lacking combinations of up to five peptidases have been isolated by Mierau et al. (1996) and these are currently being used to examine the contribution of these enzymes to the flavour and ripening of cheese (Daly et al., 2000).

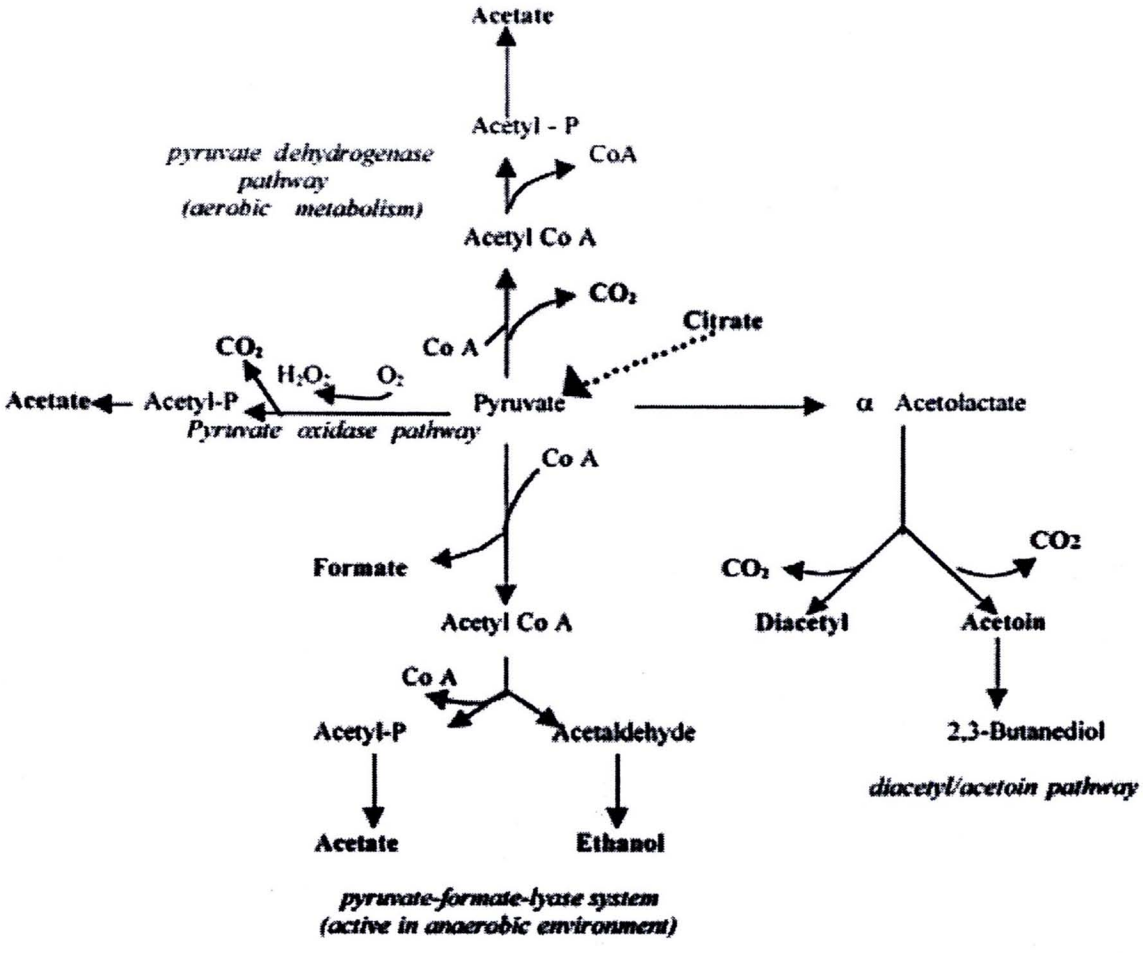

Figure 2.2 Generalized scheme for the formation of important metabolic products from pyruvate in lactic acid bacteria.

Lysis of starter cultures during cheese ripening leads to increased proteolytic activity in cheese and thus theoretically results in accelerated ripening and/ or a better cheese flavour. A good example of this is found in Swiss cheese where autolytic propionibacteria increase the amount of free proline in the cheese aiding flavour development. Morgan et al. (1997) have used bacteriocin-induced lysis of a starter to produce cheese with a higher concentration of free amino acids and decreased bitterness levels.

3. Identification of Lactic Acid Bacteria

3.1 Culture-dependent identification

Although most industrial applications and probiotic health effects of LAB depend on the specific characteristics of a particular strain, it is not always necessary to identify bacteria down to the strain level. An optimal balance has to be found between the desired taxonomic resolution of a certain application and the involved workload, speed and cost. In general, phenotypic methods are cheaper compared to genotypic methods, which have stimulated the popular use of commercially available miniaturized identification systems such as API (BioMerieux) or BIOLOG. Although the application of phenotypic techniques has proven to be useful for certain LAB, there is a general awareness that similar phenotypes displayed by strains do not always correspond to similar or even closely related genotypes. Consequently, there has been a shift towards the use of genotypic characterization methods in order to provide a more robust classification and differentiation (McCartney, 2002). Additional weaknesses of phenotypic methods include poor reproducibility, ambiguity of some techniques (often caused by complex growth conditions), extensive logistics for large-scale investigations and poor discriminatory power. However, also genotypic characterization techniques are not without limitations (cost, equipment, data bases) and thus a polyphasic, or combined approach is preferred. Table 2.1 lists a number of frequently applied identification techniques that are further discussed in the following paragraphs.

Table 2.1 List of techniques used for the identification of Lactic Acid Bacteria (L: low; M: Moderate; H: High)

Technique	Principle	Workload	Discriminatory power	Reproducibility	Reference
Phenotypic methods					
Morphological analysis	Microscopic analysis	L	Genus level or less	M	Gonzalez et al. (2000)
Physiological analysis	Growth characteristics, simple tests	M	Genus level or less	L	Corsetti et al. (2001)
Biochemical characterization	Assimilation and fermentation patterns (API, BIOLOG,...)	L	Genus or species level	M	Muyanja et al. (2003)
Protein profiling	Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis of cellular proteins	H	Species level	H	Leisner et al. (2001)
Genotypic methods					
Specific primers	PCR with group-specific primers	L	Depending on primer	H	Nomura et al. (2002)
Sequencing	Determination of gene sequences (16S rDNA...)	H	Genus to species level	H	Booysen et al. (2002)
RFLP	Restriction Enzyme Analysis (REA)	M	Species to strain level	H	Giraffa et al. (2002)
AFLP	Combination of REA and PCR amplification	H	Species to strain level	H	Giraffa and Neviani (2000)
RAPD-PCR	Randomly primed PCR	L	Species to strain level	L	Booysen et al. (2002)
Rep-PCR	PCR targeting repetitive interspersed sequences	L	Species to strain level	H	Gevers et al. (2001)
PFGE	REA and pulsed-field gel eletrophoresis	H	Strain level	H	Ventura and Zink (2002)
Ribotyping	REA and oligonucleotide probe detection	H	Species to strain level	H	Lyhs et al. (2002)
Hybridisation probes	DNA-DNA hybridization using labeled probes	H	Genus to species level	H	Manero and Blanch (2002)

3.1.1 Phenotypic methods

Especially in industrial or applied microbiology units, phenotypic tests are still being used on a routine basis for the identification of (food-associated) LAB. These methods include morphological and physiological characterization, carbohydrate fermentation patterns and protein profiling. Gonzalez et al. (2000) identified 249 LAB isolates from freshwater fish and their environment using 44 morphological and physiological tests. A high percentage (90%) of the isolates could only be identified at the genus level, demonstrating the low taxonomic resolution of this labour-intensive approach. In most cases, these physiological tests are combined with the determination of carbohydrate fermentation patterns using commercially available systems. Corsetti et al. (2001) analyzed 317 presumptive LAB isolates from sourdoughs based on morphological and physiological characteristics followed by further identification using the commercial API50CHL system (BioMerieux, France). Still, only 38% of the isolates could be identified to the species level. A similar approach was used to identify 113 LAB isolates from a Ugandan traditional fermented beverage (Muyanja et al., 2003), which often resulted in only a tentative identification to the species level. The combined use of two carbohydrate fermentation test kits could not allocate 14 LAB isolates to known species (Wijtzes et al., 1997). These studies demonstrate that phenotypic methods have their limitations because of relatively poor reproducibility and low taxonomic resolution that often only allows differentiation at the genus level. The popularity of these methods is mainly due to the fact that no specialized equipment is required to carry out most tests and because of the availability of an identification database. Noteworthy, the exact taxonomic background of these databases is sometimes unknown or poorly documented.

In comparison, Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) analysis of whole-cell proteins has proven to be a more reliable identification method for LAB (Pot et al., 1994). Protein profiling has been successfully used by Leisner et al. (2001) to identify 64 LAB isolates from Malaysian condiment at the species level. More recently, a collection of 355 isolates from probiotic products could be speciated using SDS–PAGE of proteins (Temmerman et al., 2003a). Despite its taxonomic reliability and reproducibility, the major drawback of this method is the rather high workload. In addition, SDS–PAGE of proteins lacks discriminatory

power on the (sub)species level in the *Lactobacillus acidophilus* group (*L. acidophilus, L. crispatus, L. amylovorus, L. gallinarum, L. johnsonii* and *L. gasseri*) (Gancheva et al., 1999) and the *L. plantarum* group (incl. *L. plantarum, L. pentosus and L. paraplantarum*) (Torriani et al., 2001). Species differentiation within these *Lactobacillus* groups is usually performed using genotypic techniques such as RAPD-PCR (Du Plessis and Dicks, 1995).

Other phenotypic techniques that have been used for identification of LAB isolates, but with limited success, include thin layer chromatography of organic acids and Fatty Acid Methyl Ester (FAME) analysis (Lee, So and Heo, 2001). In order to obtain a reliable identification of LAB at the species level, multiple phenotypic techniques are often combined along the route of a polyphasic approach. In this way, strong features of one method may compensate shortcomings of another method.

3.1.2 Genotypic methods

The past two decades have witnessed the development of a large series of DNA-based identification and detection methods. Undoubtedly, one of the main advantages of these methods is their independence of variations in the growth conditions of the microorganisms. Genotypic techniques exhibit various levels of discriminatory power, from species level to differentiation of individual strains (typing). Many genotypic methods are based on the principle of the Polymerase Chain Reaction (PCR), which enables the selective amplification of specifically targeted DNA fragments through the use of oligonucleotide primers under controlled reaction conditions (Table 2). In theory, PCR primers can be designed for amplification at any taxonomic level. Kaufmann et al. (1997) designed primers that amplify a 16S rDNA fragment specific for bifidobacteria, which enable the genus-specific detection of bifidobacterial isolates in a food matrix.

Table 2.2 List of PCR primers designed for identification and detection of LAB

Primer designation	Target organism (target molecule)	Reference
Lac1/2	*Lactobacillus/Leuconostoc/Pediococcus/ Weisella*	Walter et al. (2001)
Lm-26f /lm-3r	*Bifidobacterium* (16S rDNA)	Kaufmann et al. (1997)
Bif 164/bif662	*Bifidobacterium* (16S rDNA)	Kok et al. (1996)
InfY-BV.L/R	*Bifidobacterium infantis* (16S-23S rDNA)	Brigidi et al. (2000)
BreY-BV.R/L	*Bifidobacterium breve* (16S-23S rDNA)	Brigidi et al. (2000)
BiADO-1/2	*Bifidobacterium adolescentis* (16S rDNA)	Matsuki et al. (1998)
BiANG1/2	*Bifidobacterium angulatum* (16S rDNA)	Matsuki et al. (1998)
BiBIF1/2	*Bifidobacterium bifidum* (16S rDNA)	Matsuki et al. (1998)
BiCAtg1/2	*Bifidobacterium catenulatum* (16S rDNA)	Matsuki et al. (1998)
BiLONg1/2	*Bifidobacterium longum/infantis* (16S rDNA)	Matsuki et al. (1998)
LactV5	*Lactococcus lactis* (16S rDNA)	Ercolini, Hill, and Dodd (2003)
LeucV5	*Leuconostoc mesenteroides* (16S rDNA)	Ercolini, Hill, and Dodd (2003)
LbpV3	*Lactobacillus plantarum* (16S rDNA)	Ercolini, Hill, and Dodd (2003)
Case1	*Lactobacillus casei* (16S rDNA)	Chagnaud et al. (2001)
Ferm1	*Lactobacillus fermentum* (16S rDNA)	Chagnaud et al. (2001)
Para1	*Lactobacillus paracasei* (16S rDNA)	Chagnaud et al. (2001)
Reut1	*Lactobacillus reuteri* (16S rDNA)	Chagnaud et al. (2001)
Sal1	*Lactobacillus salivarius* (16S rDNA)	Chagnaud et al. (2001)
Aci-1/2	*Lactobacillus acidophilus* (16S-23S rDNA)	Tilsala-Timisjaevi and Alatossava (1997)
SS1-DB1	*Lactobacillus delbrueckii* (16S rDNA)	Drake, Small, Spence, and Swanson (1996)
SS2-HE1	*Lactobacillus helveticus* (16S rDNA)	Drake, Small, Spence, and Swanson (1996)
Y2-rham	*Lactobacillus rhamnosus* (16S rDNA)	Ward and timmins, (1999)
Thl/Thll	*Streptococcus thermophillus* (16S-23S rDNA)	Tilsala-Timisjarvi and Alatossava (1997)
16MAC	*Streptococcus macedonius* (16S rDNA)	Papadelli and Manolopoulou, kalantzopoulos and tsakalidou (2003)
ENT1-ENT2	*Enterococcus* (16S rDNA)	Ke et al. (1999)
AVI	*Enterococcus avium* (16S rDNA)	Manero and Blanch (2002)
ASI	*Enterococcus asini* (16S rDNA)	Manero and Blanch (2002)
CEC	*Enterococcus cecorum* (16S rDNA)	Manero and Blanch (2002)
COL	*Enterococcus columbae* (16S rDNA)	Manero and Blanch (2002)
CGF	*Enterococcus casseliflavus/gallinarum /flavescens* (16S rDNA)	Manero and Blanch (2002)
DUR	*Enterococcus durans* (16S rDNA)	Manero and Blanch (2002)
DIS	*Enterococcus dispar* (16S rDNA)	Manero and Blanch (2002)
Efs 130c	*Enterococcus faecalis* (16S rDNA)	Manero and Blanch (2002)
FMDUR	*Enterococcus faecium/durans* (16S rDNA)	Manero and Blanch (2002)
HIR	*Enterococcus hirae* (16S rDNA)	Manero and Blanch (2002)
MAL	*Enterococcus makodoratus* (16S rDNA)	Manero and Blanch (2002)
MUN	*Enterococcus mundtii* (16S rDNA)	Manero and Blanch (2002)
PSE	*Enterococcus pseudoavium* (16S rDNA)	Manero and Blanch (2002)
RAF	*Enterococcus raffinosus* (16S rDNA)	Manero and Blanch (2002)
SAC	*Enterococcus sacharolyticus* (16S rDNA)	Manero and Blanch (2002)
SOL	*Enterococcus solitarius* (16S rDNA)	Manero and Blanch (2002)

Beyond the genus level, 16S rRNA based species and group-specific primers have been designed for Bifidobacterium adolescentis, *B. angulatum*, *B. bifidum*, *B. breve*, the *B. catenulatum* group (*B. catenulatum* and *B. pseudocatenulatum*), and the *B. longum* group (*B. longum* and *B. infantis*), which are species commonly found in the human intestinal tract (Matsuki et al., 1998). A similar approach using species-specific primers for various LAB was recently used for the identification of 543 cheese isolates (Mannu et al., 2002). At the intra-specific level, the PCR-based discrimination of *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* was performed by Nomura et al. (2002). The highest discriminatory identification level was reached by Brandt and Alatossava (2003) for the specific detection of certain *Lactobacillus rhamnosus* strains using phage-related primers, and by Chagnaud et al. (2001), who designed primers directed at 6 *Lactobacillus* strains. Overall, the PCR-based detection of LAB and other bacteria mostly requires a difficult design strategy and thorough validation before a valuable set of primers is obtained. Therefore, this approach is only considered suitable if the presence or absence of well-known bacteria is to be verified.

When unknown bacterial isolates have to be identified, a powerful tool with high discriminatory power is 16S or 23S rDNA sequencing (Vandamme et al., 1996). The obtained sequence is to be compared with DNA sequences stored in online databases of previously sequenced DNA, of which the most popular ones are the EMBL and Genbank databases. Searching these databases for corresponding sequences can be performed using a search algorithm, such as BLAST or FASTA. Booysen et al. (2002) sequenced the 16S rDNA for the identification of LAB isolates from a malting process at the subspecies level, whereas fecal LAB isolates exhibiting antimicrobial action against *Clostridium difficile* were identified at the species level by means of sequencing the 16SrDNA and Internal Transcribed Spacer (ITS) regions by Lee, Yu, and Heo (2003). Sequencing analysis of these two regions was also applied to identify 317 isolates from 25 wheat sourdoughs at the species level (Corsetti et al., 2001). Although a very powerful tool, sequencing of ribosomal genes is highly dependent on the reliability and the taxonomic coverage of the available databases. Furthermore, the degree of strain and inter-operon sequence variation may in some cases yield confusing identification results (Nubel et al., 1996).

Total DNA or amplicons resulting from a selective PCR reaction can also be digested by restriction enzymes, resulting in a mixture of fragments different in size. This technique is commonly referred to as Restriction Fragment Length Polymorphism (RFLP) analysis and is the prototype of a DNA fingerprinting method mostly used to identify isolates at the intra-specific level. Giraffa et al., (2002) applied RFLP analysis of protein-coding genes (β-galactosidase, lactose permease, and proline dipeptidase) for molecular typing of 35 *Lactobacillus delbrueckii* subsp. *lactis* and *L. delbrueckii* subsp. *bulgaricus* isolates used as starter cultures for dairy products. Furthermore, 46 LAB isolates from wine were analysed by RFLP to indicate the presence of *Oenococcus oeni*, a specie showing no strain diversity in its RFLP patterns (Sato et al., 2000). The discriminatory power of these methods is very high (i.e. strain level) making them very useful for typing LAB starter cultures, of which the strain-specific properties are crucial to the production process. Pulsed Field Gel Electrophoresis (PFGE) employs an alternating field of electrophoresis to allow separation of the large DNA fragments obtained from restriction digests with rare-cutting enzymes. Crucial to PFGE is the extraction of intact chromosomal DNA, which may render the technique more time consuming than other fingerprinting strategies. However, since PFGE analyses large DNA fragments, representing the whole genome, this technique has superior discriminatory power, with excellent subspecies differentiation for a large number of microorganisms (McCartney, 2002). Multiple strains of the species *Lactobacillus johnsonii* were analysed with PFGE by Ventura and Zink (2002). They demonstrated a pronounced heterogeneity among all *L. johnsonii* isolates that was potentially linked to their origin of isolation. A more advanced fingerprinting technique that combines PCR amplification with double restriction digest is Amplified Fragment Length Polymorphism (AFLP) (Janssen et al., 1996). Originally developed for plant breeding purposes, AFLP has been found to be a very useful fingerprinting technique for bacteria, allowing both species resolution and strain differentiation. AFLP has mostly been employed in epidemiological studies and in investigations aiming to distinguish virulence markers in food-borne pathogens. Within LAB, species-level discrimination has been documented for the phylogenetically closely related species *L. pentosus*, *L. plantarum* and *L. pseudoplantarum* (Giraffa and Neviani, 2000).

DNA fingerprinting techniques that solely rely on PCR include Randomly Amplified Polymorphic DNA (RAPD) and repetitive genomic element (rep)-PCR. RAPD analysis utilizes short arbitrary primers and low-stringency conditions to randomly amplify DNA fragments, which are separated electrophoretically to produce a fingerprint. Booysen et al. (2002) recently reported the use of RAPD to identify LAB isolates from a malting process. The great flexibility in primer choice offered by this method implies that it can be applied to differentiate LAB at different taxonomic levels ranging from genus to intra-specific level. However, because RAPD primers are not directed against a specific sequence, the reproducibility of the technique over a longer study period has often posed a problem (Olive and Bean, 1999). In contrast, rep-PCR specific primers amplify repetitive bacterial DNA elements such as ERIC, BOX or (GTG) 5 (Versalovic et al., 1994). In recent studies, (GTG) 5-PCR was proven to be useful for the differentiation of lactobacilli and bifidobacteria at the species, sub species and potentially on the strain level (Gevers, Huys, and Swings, 2001; Masco et al., 2003). Ribotyping combines an enzymatic restriction digest with the detection of the resulting fragments by means of rDNA probes. Lyhs, Korkeala, and Bjorkroth (2002) applied ribotyping for the identification of 296 LAB isolates from vacuum-packed trout. To a large extent, the discriminatory power of this technique depends on the number and type of restriction enzymes and probes used. Either fluorescent or radioactively labeled probes can be used to hybridize with specific DNA sequences (Table 2.2). For instance, enterococci were identified using a dot blot hybridisation method using species-specific probes (Manero and Blanch, 2002), whereas LAB isolates from wine have been identified using total genomic DNA probes (Sohier and Lonvaud-Funel, 1998). The authors also applied the same probes directly on wine samples, that were previously bound to a membrane, in which case the technique is referred to as Fluorescent In Situ Hybridisation (FISH). A technique similar to the use of probes is the application of fluorescently labeled monoclonal antibodies directed against a specific species or strain, as demonstrated by Yuki et al. (1999) for the identification of the probiotic strain *Lactobacillus casei Shirota*. DNA–DNA hybridization is a method in genetics that provides a higher resolution than 16S rDNA sequencing, and has been the cornerstone for species description. Various approaches exist such as the nitrocellulose filter methods, free-solution methods, and more recently the use of microaray technology (Cho and Tiedje, 2001). As a general rule, strains are

considered to belong to the same species when they have approximately 70% or more DNA–DNA relatedness, with 5°C or less difference in melting temperature. However, major drawbacks such as the labor-intensity, use of isotopes and impossibility to create a central database have negatively influenced the techniques' popularity. DNA–DNA hybridization was used to select probiotic species out of 297 *Lactobacillus* isolates (du Toit et al., 1998).

Finally, because many LAB strains harbour plasmids varying in size and number, plasmid profiling is sometimes used for strain-specific identification as demonstrated for LAB isolates from Malaysian condiment (Leisner et al., 2001), and from fermented dry sausages (Gevers et al., 2003). However, as not all LAB strains actually harbour plasmids, this method is not universally applicable. Moreover, plasmids can be acquired or lost during horizontal gene transfer events. To increase the reliability of a genotypic identification, a polyphasic combination of different techniques is sometimes desirable. Ventura and Zink (2002) characterized a collection of *Lactobacillus johnsonii* strains using a combination of multiplex PCR, rep-PCR, PFGE, AFLP and RAPD.

3.2 Culture-independent identification

The identification methods described in the previous section rely on the ability to isolate and cultivate LAB isolates from a given food or environmental sample. Because these culture-dependent approaches have shown limitations in terms of recovery rate, the set of obtained isolates may not always truly reflect the microbial composition of the sample (Ampe et al., 1999; Ercolini et al., 2001). More specifically, detection of bifidobacteria by culture-dependent techniques suffers from a poor reproducibility because of the lack of suitable selective isolation media (Roy, 2001). As a result, culture-independent methods have been developed to circumvent the limitations of conventional cultivation for analysis of microbial communities (Vaughan et al., 2002). For instance, during the production of yoghurts or probiotic products, in which bacterial starter cultures are often joined by additional LAB strains to improve the organoleptic or functional properties, the slightest quantitative or qualitative shift in bacterial composition may compromise the end product quality. The microbiological screening at different steps in the process line of a food product can, however, be a very laborious task when only culture-dependent techniques are available. Clearly, on-time interventions in the production process are only possible

when complex LAB ecosystems can be analyzed in a reliable and fast culture-independent way.

The fastest culture-independent approach for the genus, species or strain specific detection of LAB in a food matrix is the use of specific primers for the PCR-based detection of the target organisms in bacterial DNA extracted from the sample (Table 2.2). However, with an increasing degree of microbial complexity of the sample, several PCR primers are needed in order to detect different LAB taxa or strains, thereby substantially increasing the workload. Perhaps the main disadvantage of this approach is the fact that only 'expected' microorganisms will be detected, making such PCR assays of limited value in the analysis of highly complex ecosystems or samples showing a variable or unknown species composition. Tilsala-Timisjarvi and Alatossava (1997) designed a set of six species-specific primerpairs targeting the 16S–23S intergenic rDNA region of the probiotic species *L. paracasei, L. rhamnosus, L. delbrueckii, L. acidophilus, L. helveticus* and *S. thermophilus*. Using a Bifidobacterium-specific set of PCR primers, Kaufmann et al. (1997) facilitated genus-specific detection of bifidobacteria in food and fecal samples.

Probing techniques are based on the hybridisation of synthetically designed oligonucleotides to specific target sequences in bacterial DNA. Unlike conventional PCR primer, these probes are linked to a radioactive or fluorescent label which enables the visual detection of the target after hybridisation under controlled conditions. The specificity of the probe is largely dependent on the target sequence, although the stringency of the hybridisation and washing conditions are also critical (O'Sullivan, 1999). Labeled oligonucleotide probes are employed in a number of assays including colony, dotblot and in situ hybridisations (Giraffa and Neviani, 2000). The most frequently applied method using probes is Fluorescent In Situ Hybridisation (FISH) making use of fluorescence microscopy for the counting of fluorescently labeled bacteria. Using specific probes, Sohier and Lonvaud-Funel (1998) reported on a method to monitor the bacterial population in wine at different stages of vinification during storage, and to identify wine spoiling LAB. A more advanced FISH application used an array of genus-specific primers targeting *Bifidobacterium*, Bacteroides, *Lactobacillus/ Enterococcus* and *Clostridium* to monitor the fecal flora of infants (Kirjavainen et al., 2001). A method that allows both quantitative and qualitative analysis of samples is Flow Cytometric Analysis. Bacteria

in a liquid sample or suspension are fluorescently labeled using one or more specific dyes or probes after which the labeled solution is run through a flow cytometer or cell sorter, determining the identity and quantity of the bacteria (Bunthof and Abee, 2002). The current disadvantage of these probe-based methods is their high workload, preventing fast analysis. In addition, the use of primers and probes restricts the number of possible applications because of the limited number of bacteria targeted.

3.3 The future trends of identification of lactic acid bacteria

Currently, a great number of mostly molecular techniques are available for the identification of LAB, for instance in industrial processes and food products. For each specific type of research or analysis, a well-considered choice has to be made of the methodology to be applied, in relation to taxonomic resolution, workload and cost. It is important to realize that every technique cannot be used for any purpose. For instance, RFLP and PFGE are only suitable for typing purposes, but will generally not yield a species identification. In the course of safety assessments, it is crucial to use multiple techniques working on the strain level in order to obtain a detailed fingerprint of individual isolates. However, most situations only demand identification to the species level, for which techniques such as biochemical characterization, 16S rDNA sequencing or DNA–DNA hybridisation are used. If species-specific primers or probes are available, these offer a very fast way to detect the target organism(s). For routine quality control, commercial easy-to-perform phenotypic tests may provide satisfying results if performed under standardized conditions. Analyses of food fermentations involving pure cultures will benefit from the use of culture-dependent techniques, although most food fermentations in fact involve more complex bacterial communities. At present, DGGE is one of the few techniques allowing a fast microbial analysis of these communities. Undesirable changes in the bacterial population caused by fluctuation of ingredients or false fermentation conditions can be rapidly detected using PCR-DGGE, facilitating subsequent adjustments by means of technological measures. However, at the moment quantitative data can only be obtained using culture-dependent methods, which are also required for live/dead discrimination at the cellular level.

Current research is focussed on the further optimisation of the DGGE technique, including the coupling of DGGE to real-time and Reverse Transcriptase

PCR, which may allow the culture-independent quantitative analysis of investigated microbial communities. An additional area of interest for the near future is the evaluation of new techniques for (routine) analyses of LAB (food) samples. The rapid evolution in total genome sequencing may open new possibilities for identification or detection, when implemented in microarray technology (Wang et al., 2002). Also, a number of non-genomic whole-cell analysis techniques with high discriminatory power are in full expansion, such as those based on mass spectra (Reid et al., 2002). It is clear from the above discussion that a careful consideration of numerous factors has to be made in order to find the most suitable technique for a certain purpose. Some studies will benefit optimally from the use of a fast, inexpensive, though less discriminating method, whereas other applications will require multiple high-resolution techniques. Although in the future high-tech approaches will make some of the currently used methods redundant, it is expected that both culture-dependent and culture-independent approaches will remain necessary in many studies targeting the speciation or fingerprinting of LAB.

4. Molecular Techniques Involved in Identification of Lactic Acid Bacteria

Molecular techniques are major tools for the analysis of microorganisms from food and other biological substances. The techniques provide ways to screen for a broad range of agents in a single test (Field and Wills, 1998). It has truly come of age and its range of application is perceived to broaden in the near future. The food industries, water processors, and analytical laboratories have taken up the latter method; for rapid differentiation of species, strain identification and definition of strain relatedness from infected samples. Molecular methods vary with respect to discriminatory power, reproducibility, ease of use, and ease of interpretation (Lasker, 2002).

4.1 Polymerase Chain Reaction (PCR)

PCR methods have been described in more details by Hoelzel and Green (1998). Saiki et al. (1985) published the first experimental data on PCR, and ever since PCR technique (Mullis and Faloona, 1987) has tremendously influenced research in diverse areas of biological sciences leading to an unprecedented understanding of microorganisms. Using PCR, it is now possible to make virtually

unlimited copies of a fragment of DNA (Field and Wills, 1998). The organism of interest can be detected directly through PCR assays in a much shorter time than conventional culture takes. *Campylobacter*, the most common cause of acute bacterial gastroenteritis in the developed world, has been detected from meat by PCR (Cloak et al., 2001). PCR assays also allow the identification of *Lactobacillus curvatus*, *L. graminis*, and *L. sake* (Berthier and Ehrlich, 1998). Brooks et al. (1992) used PCR to amplify specific rDNA sequences of *Carnobacterium* spp. in purified DNA extracts, crude cell lysates, and food samples. An analogous PCR method has been designed for the identification of genetically engineered *L. curvatus* in raw sausage. Heilig et al. (2002) developed a *Lactobacillus* group-specific PCR primer, which selectively amplify 16S ribosomal DNA (rDNA) from lactobacilli and related lactic acid bacteria, including members of the genera *Leuconostoc*, *Pediococcus*, and *Weissella*. The sequences of *Leuconostoc* species (Heilig et al., 2002) retrieved have only been detected in fermented food products and never in gastrointestinal tract samples. Theron et al. (2001) also developed a sensitive seminested PCR method for the detection of *Shigella* in spiked environmental water samples. These workers had earlier detected toxigenic *Vibrio cholerae* from environmental water samples by an enrichment broth, semi-nested PCR procedure (Theron et al., 2000). Sea water and organic material analysed (Alam et al., 2003) to determine *Vibrio parahaemolyticus*, a potentially pathogenic bacterium, showed over 22% of samples positive for *V. parahaemolyticus* than the conventional (Most Probable Number) MPN culture technique could detect. PCR has already proven valuable in the screening of rhizobacteria for 1-amino-cyclopropane-1-carboxylic (ACC) deaminase (Babalola et al., 2003). Taylor et al. (2001) described the detection of *Erwinia amylovora* (pectolytic bacteria) in plant material using PCR. Similarly, Sánchez-Contreras et al. (2000) developed and tested four primers that recognize homologous conserved regions in the *Sinorhizobium meliloti* genome by PCR. The method was used to establish a collection of *S. meliloti* strains from soils polluted with polychlorinated biphenyls and/or polycyclic aromatic hydrocarbons a process, which could have been otherwise timeconsuming. Moreover, the PCR approach has identified the bacterium from nodules of *Medicago* sp. plants collected from field samples. The results are useful for identification of *S. meliloti*, especially when high numbers of other bacteria are expected to be present in nodules.

The development of multiple assays such as multiplex PCR means that several bacterial species can be identified in a single assay (Field and Wills, 1998). For example, a single multiplex PCR has been used to detect *Salmonella*, *Campylobacter*, *Shigella* species and *E. coli* in faecal samples (QIAGEN, 2001).

4.2 DNA Amplification Fingerprinting (DAF) and Random Amplified Polymorphic DNA (RAPD)

DAF and RAPD are amplification-based nucleic acid fingerprinting techniques (concurrent detection of multiple loci without assignment of a genotype) that use an in vitro enzymatic reaction to specifically amplify a multiplicity of target sites in one or more nucleic acid molecules (Micheli et al., 1994). The amplification reaction is generally driven by short synthetic oligonucleotides of arbitrary or semi-arbitrary sequence that produce a collection of amplified products of largely non-allelic nature. DAF uses a single primer (5-10 bp) to amplify genomic DNA at random. *Salmonella enterica* serotype Typhimurium, obtained from human, animal (clinical), and food sources, were typed by DAF (Daly et al., 2000). Data from DAF and their other studies indicate a remarkable degree of homogeneity at a molecular level among contemporary isolates of *S. enterica* serotype Typhimurium DT104. The RAPD technique was first employed by Williams et al. (1990) to examine human DNA samples from anonymous individuals. Since then several authors have reported on the application of RAPD technique in microorganisms (e.g. Babalola, 2002). It uses random primers (Williams et al., 1990) and can be applied to any species without requiring any information about the nucleotide sequence. The amplification products from this analysis exhibit polymorphism and thus can be used as genetic markers. The presence of an RAPD band, however, does not allow distinction between hetero- and homozygous states. The fragments are scored as dominant Mendelian elements, and the protocols are relatively simple. Nowrouzian et al. (2001) designed an RAPD typing method for the identification of *E. coli* strains in the normal human intestinal microflora. The band pattern generated in the analysis represents genome characterization of a particular bacterial strain (Welsh and McClelland, 1990). In addition, the method has the potential for analyzing phylogenetic relationships among closely related species (Williams et al., 1990) and can distinguish between strains within species.

4.3 Restriction Fragment Length Polymorphisms (RFLPs)

The procedures involve isolation of DNA, digestion of DNA with restriction endonucleases, size fractionation of the resulting DNA fragments by electrophoresis, DNA transfer from electrophoresis gel matrix to membrane, preparation of radiolabelled and chemiluminiscent probes, and hybridisation to membrane-bound DNA. RFLP fingerprinting technique is regarded as the most sensitive method for strain identification and several bacterial strains have been widely studied using this technique. Kabadjova et al. (2002) established a rapid PCR-RFLP-based identification scheme for four closely related *Carnobacterium* species (*C. divergens*, *C. piscicola*, *C. gallinarum*, and *C. mobile)* that are of interest to the food industry. Three isolates previously incorrectly identified as *C. divergens* (INRA 508, INRA 586, and INRA 515) were reclassified as *C. piscicola*. Similarly, four isolates deposited as *C. piscicola* (INRA 545, INRA 572, INRA 722, and ENSAIA 13) were reclassified as *C. divergens* based on the patterns obtained by the 16S-23S ISR-RFLP methods. Wang et al. (2000) and Penrose et al. (2000) proved the role of PCR and Southern hybridisation in assessing the effect of introducing 1-aminocyclopropane-1-carboxylic acid deaminase genes on disease-suppressive capabilities of *Pseudomonas fluorescens* strain CHAO. One of their results suggested that the constructed stains could be developed as biosensors for the role of ethylene in plant diseases. Manceau and Horvais (1997) used RFLP analysis of rRNA operons to assess phylogenetic diversity among strains of *Pseudomonas syringae* pv tomato. They successfully established the close relationships existing between *P. syringae* and *P. viridiflava* species. However, the findings of Lu et al. (1996) suggested that PCR-based multiple-loci marker techniques (RAPD, AFLP, microsatellite and inter-SSR PCR) could replace RFLP in the estimation of genetic diversity.

4.4 Amplified Fragment Length Polymorphisms (AFLPs)

Amplified fragment length polymorphism (AFLP) analysis was developed by a team led by Marc Zabeau at Keygene N.V., Wageningen, The Netherlands (Vos et al., 1995; Zabeau and Vos, 1993). Vos et al. (1995) had described the principle of AFLP fingerprinting technique. AFLP is a variation of RAPD, able to detect restriction site polymorphisms without prior sequence knowledge using PCR amplification for detection of restriction fragment (Blears et al., 1998; Mueller and

Wolfenbarger, 1999; Vos et al., 1995; Zabeau and Vos, 1993). Here, the template for a PCR reaction is a restriction enzyme digested genomic DNA. The primers contain the restriction enzyme recognition site as well as additional 'arbitrary' nucleotides that extend beyond the restriction site. The fixed portion gives the primer stability and the random portion allows it to detect many loci. Amplified products are resolved by polyacrylamide gel electrophoresis. AFLP analysis is one of the robust multiple-locus fingerprinting techniques among genetic marker techniques that have been evaluated for genotypic characterization (Koeleman et al., 1997). Restrepo et al. (1999) used AFLP to characterize the genetic relationships between *X. axonopodis* pv Manihotis strain. The study of Janssen et al. (1996) revealed extensive evidence for applicability of AFLP in bacterial taxonomy through comparison of the newly obtained data with results previously obtained by well-established genotypic and chemotaxonomic methods such as DNA-DNA hybridization and cellular fatty acid analysis.

5. Lactic Acid Bacteria Benefiting Health

LAB has been cited to be part of human (Fuller, 1991; Goldin, 1990; Holzapfel et al., 2001; Reid, 2001; Schrezenmeir and de Vrese, 2001; Sghir et al., 2000) and animal (Batt et al., 1991; Benno et al., 1992; Fujisawa and Mitsuoka, 1996; Perdigón et al., 2001; Rodríguez et al., 2003; Schrezenmeir and de Vrese, 2001) microbiota. The neonates receive their microbiota primarily in labor and later from the environment (Edwards and Parrett, 2002; Fuller, 1989; Fuller and Gibson, 1998; Metchnikoff, 1908). LAB and bifidobacteria dominate the microbiota of the full-term neonate (Hall et al., 1990), especially when breast-fed (Edwards and Parrett, 2002; Lönnerdal, 2000) with a healthpromoting effect on the child (Arici et al., 2004; Boris et al., 1998; Edwards and Parrett, 2002). Heikkilä and Saris (2003) isolated LAB from human milk. Martín et al. (2003) detected *Lactobacillus gasseri* from breast-feeding mothers and children in pair and observed coccoid LAB sharing identical randomly amplified polymorphic DNA (RAPD) patterns. Although it is difficult for microbes to establish themselves in an already colonized ecosystem (Tannock, 1990), the health impact of microbiota consisting of LAB is well documented in humans (Bezkorovainy, 2001; Fooks et al., 1999; Majamaa and Isolauri, 1997; Reid et al., 2003) and in animals (Bezkorovainy, 2001; Ehrmann et al., 2002; Fujisawa and

Mitsuoka, 1996; Nurmi and Rantala, 1973). Gut bacteria are anticipated to interact with the host, encompassing direct interaction between bacteria and host epithelial cells (de Vos et al., 2004).

LAB are regarded as a major group of probiotic bacteria (Collins et al., 1998; Metchnikoff, 1908; Schrezenmeir and de Vrese, 2001; Tannock, 1998). The probiotic concept has been defined by Fuller (1989) to mean "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Salminen et al. (1999) proposed that probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host. Several lactobacilli, lactococci and bifidobacteria are held to be health-benefiting bacteria (Rolfe, 2000; Tuohy et al., 2003), but little is known about the probiotic mechanisms of gut microbiota (Gibson and Fuller, 2000). LAB constitute an integral part of the healthy gastrointestinal (GI) microecology and are involved in the host metabolism (Fernandes et al., 1987).

Table 2.3 Selected health-promoting lactic acid bacteria, their impacts and mechanisms

Health effect	Mechanisms	Strain example	Reference
Relieve lactose intolerance symptoms	Hydrolysing lactose into glucose and galactose and forming the physical appearance of milk into a thick substance, such as yogurt, that passes through the GI tract slowly, reducing the lactose pulse in the colon.	*Lactobacillus rhamnosus* GG	Drouault and Corthier, 2001 Heyman, 2000 Hove et al., 1999
Control viral, bacterial and antibiotic-associated diarrhea in humans and animals	Reinforcing the local immune defence through specific IgA response to rotavirus and pathogens.	*L. rhamnosus* GG *L. reuterii* *Enterococcus faecium*	Ehrmann et al., 2002 Heyman, 2000 Majamaa and Isolauri, 1997 Oksanen et al., 1990 Vahjen and Männer, 2003
Prevention of allergies and atopic eczema	Prevention is partially due to serum antibodies IgG and secretory IgA and IgM immune response enhanced by probiotics.	*L. rhamnosus* GG *Bifidobacterium lactis* Bb-12	Cross, 2002 Link-Amster et al., 1994 Perdigón et al., 1999 Majamaa and Isolauri, 1997 Isolauri et al., 2000
Prevention of intestinal bacterial enzymes involved in the synthesis of colonic carcinogens	Enhancing host's immune response, binding and degrading carcinogens, producing antimutagenic compounds, alteration of metabolic activities of intestinal bacteria and alteration of physiochemical conditions in colon might work to prevent cancer.	*B. bifidum* *B. infantis* *B. longum* *L. acidophilus* *L. paracasei*	Hirayama and Rafter, 2000 Rolfe, 2000 Sanders, 1998
Inactivation and reduction of pathogenic bacteria	Production of antimicrobic substances and Competitive Exclusion (CE).	*Lactococcus lactis* *Pediococcus acidilactici*	Elliason and Tatini, 1999 Nurmi and Rantala, 1973 O'Sullivan et al., 2002
Direct stimulation of the immune system on the gut mucosal surface	Adherence to mammalian extracellular matrix. Stimulation via localized GI tract lymphoid cell foci.	*L. crispatus* strains JCM1132, ST1, A33 and 134mi *L. gasseri* CT5 *L. reuteri* CT7	Edelman et al., 2002 Toba et al., 1995

Fermentation has been specified as a mechanism of probiotics (Gibson and Fuller, 2000; Metchnikoff, 1908). LAB along with other gut microbiota ferment various substrates like lactose, biogenic amines and allergenic compounds into short-chain fatty acids and other organic acids and gases (Gibson and Fuller, 2000; Gorbach, 1990; Jay, 2000). LAB synthesize enzymes, vitamins, antioxidants and bacteriocins (Fernandes et al., 1987; Knorr, 1998). With these properties, intestinal LAB constitute an important mechanism for the metabolism and detoxification of foreign substances entering the body (Salminen, 1990). The health-promoting effects of LAB are strain specific and result in different mechanisms to produce beneficial health impacts (Table 2.3).

LAB have been found to control intestinal disorders, partially due to serum antibodies IgG, and secretory IgA and IgM enhancing immune response (Cross, 2002; Grangette et al., 2001; Kimura et al., 1997; Link-Amster et al., 1994; Perdigón et al., 1999). Certain strains of LAB can intermittently translocate across the intestinal mucosa without causing infection (Berg, 1995), thus influencing systemic immune events (Cross, 2002). Evidence has been presented that some lactobacilli can directly stimulate the immune system on the gut mucosal surface via localized GI tract lymphoid cell foci (Perdigón et al., 1999). Morishita et al. (1971) demonstrated that intestinal origin LAB established in the digestive tract of germ-free chickens better than did non-intestinal LAB strains. Several reports have been made on LAB surviving the GI tract of humans and animals (Drouault et al., 1999; Klijn et al., 1995; Yuki et al., 1999). A number of mechanisms work to prevent harmful bacteria from growing on and attaching to the intestinal epithelium: production and secretion of antimicrobial agents such as bacteriocins and organic acids (Fooks et al., 1999; Reid, 2001), adherence via competition for the binding sites and steric hindrance (Bezkorovainy, 2001; Boris et al., 1998; Schrezenmeir and de Vrese, 2001) and barriers interfering with pathogens and hence promoting the elimination of harmful bacteria (Boris et al., 1998). Boris et al (1998) reported vaginal LAB strains being able to self-aggregate in a process mediated by surface proteins or lipoproteins, depending on the strain. In addition, strains adhered to vaginal epithelial cells, interferred with other bacteria and coaggregated with tested pathogens *in vitro*. Both aggregation and adhesion may favor the vaginal epithelium through the formation of a

bacterial film contributing to the exclusion of pathogens from the vaginal mucosa (Boris et al., 1998). *L. rhamnosus* strain GG and *L. reuteri* ING1 have been shown to exhibit disease-specific adhesion to intestinal tissue (Ouwehand et al., 2003). Additionally, reports have been published on bacteriocin production by some probiotic bacteria targeting pathogenic bacteria *in vitro* (Elliason and Tatini, 1999; O'Sullivan et al., 2002; Ziemer and Gibson, 1998). Reutericyclin, an antibiotic produced by *Lactobacillus reuteri* LTH2584, has recently been discovered to inhibit a broad range of bacteria (Gänzle et al., 2000). Its biological activity is comparable to that of nisin. The colonized *L. reuteri* LTH2584 cells were recovered from the intestine of reconstituted lactobacilli-free (RLF) mice in high cell counts. This strain is proposed to be a valuable tool for studying the role of antibacterial agents in intestinal habitats (Gänzle, 2004).

However, the validity of the probiotic concept has been questioned (Shanahan, 2003; Tannock, 2003). The adequate information by which the consumer and health professional can judge the efficacy and safety of retailed probiotics is lacking. Probiotic products have not been subjected to large scale trials of efficacy that are used in the pharmaceutical industry (Tannock, 2003). As an example, no direct evidence of LAB suppressing colon cancer has yet been put forward (Hove et al., 1999). In addition, the difficulty of *in vivo* studies poses problems in further showing the complete effects of probiotics (Shanahan, 2003). Much remains to be done to understand the full effect of probiotics, given the extreme complexity of the biological systems of humans and their interactivity (Klaenhammerand Kullen, 1999). Studies conducted on bacteria beneficial to health cover only a segment of the human ecosystem (Klaenhammer and Kullen, 1999; Shanahan, 2003). *In vivo* sampling in humans also raises ethical issues limiting the scope of physiological and clinical testing. For this reason, alternative methods are implied, such as performing mucosal biopsies on specific parts of the GI tract (Shanahan, 2003) and detecting selective bacteria from faecal samples (Simmering and Blaut, 2001). The stablity of probiotic LAB in the GI tract is another concern. Heilig et al (2002) showed that GI tract bacterial biota altered during first five months of an infant's life, while the composition of the *Lactobacillus* community remained more stable over a two-year

study of adults, with individual differences. *L. lactis* may survive in human (Klijn et al., 1995) and in the mouse (Drouault et al., 1999) GI tract.

6. Mechanisms of Antibiosis Mediated by Lactic Acid Bacteria

The specific antimicrobial mechanisms of lactic acid bacteria exploited in the biopreservation of foods include the production of organic acids, hydrogen peroxide, carbon dioxide, diacetyl, broad-spectrum antimicrobials such as reuterin and the production of bacteriocins (De Vuyst and Vandamme, 1994). Many of these factors are thought to play a role in the inhibitory effect of Microguard (Al-Zoreky et al., 1991; Lyon et al., 1993). It is produced by the fermentation of skimmed milk by *Propionibacterium freudenreichii* ssp. *shermanii*, which is subsequently pasteurised and added as a Generally Recognised as Safe (GRAS) food preservative to much of the cottage cheese production in the U.S. to prevent the growth of gram-negative bacteria and moulds.

6.1 Organic acids, acetaldehyde and ethanol

The direct antimicrobial effects of organic acids including lactic, acetic and propionic which may be produced by lactic acid bacterial fermentation of foods, are well known (Davidson, 1997). The antagonism is believed to result from the action of the acids on the bacterial cytoplasmic membrane which interferes with the maintenance of membrane potential and inhibits active transport (Sheu et al., 1972; Eklund, 1989; De Vuyst and Vandamme, 1994a), and may be mediated both by dissociated and undissociated acid (Cherrington et al., 1991). The antimicrobial activity of each of the acids at a given molar concentration is not equal. Acetic acid is more inhibitorier than lactic acid and can inhibit yeasts, moulds and bacteria (Blom and Mortvedt, 1991). Propionic acid inhibits fungi and bacteria and is present in Microguard^{R} as described above and also in another commercial product, Bioprofit^{R} where the use of a *Propionibacterium freudenreichii* strain along with *Lactobacillus rhamnosus* increases inhibitory activity against fungi and some gram positive bacteria (Mayra-Makinen and Suomalainen, 1995). The contribution of acetaldehyde to biopreservation is minor since the flavour threshold is much lower than the levels that are considered necessary to achieve inhibition of microorganisms (Kulshrestha and Marth, 1974). Similarly, although ethanol may be produced by lactic cultures, again

the levels produced in food systems are so low that the contribution to antibiosis is minimal.

6.2 Hydrogen peroxide

Lactic acid bacteria lack true catalase to break down the hydrogen peroxide generated in the presence of oxygen. It is argued that the H_2O_2 can accumulate and be inhibitory to some microorganisms (Condon, 1987). Inhibition is mediated through the strong oxidising effect on membrane lipids and cell proteins (Morris, 1976; Lindgren and Dobrogosz, 1990). Hydrogen peroxide may also activate the lactoperoxidase system of fresh milk with the formation of hypothiocyanate and other antimicrobials (Reiter and Harnulv, 1984; Pruitt et al., 1986; Condon, 1987; De Vuyst and Vandamme, 1994a). However, because of the ability of other enzyme systems such as flavor proteins and peroxidases to breakdown H_2O_2 it is not clear what, if any the in vivo contribution of H_2O_2 is to antibacterial activity (Nagy et al., 1991; Fontaine et al., 1996).

6.3 Carbon dioxide

Carbon dioxide, formed from heterolactic fermentation, can directly create an anaerobic environment and is toxic to some aerobic food microorganisms through its action on cell membranes and its ability to reduce internal and external pH (Eklund, 1984; DeVuyst and Vandamme, 1994a). At low concentration, it may be stimulatory to the growth of some bacteria (Lindgren and Dobrogosz, 1990). Production of CO_2 resulting from the use of lactate by propionibacteria in Swiss cheese manufacture is responsible for the characteristic ''eyes'' of the finished product.

6.4 Diacetyl

Diacetyl is a product of citrate metabolism (Lindgren and Dobrogosz, 1990; Cogan and Hill, 1993) and is responsible for the aroma and flavour of butter and some other fermented milk products. Many lactic acid bacteria including strains of *Leuconostoc*, *Lactococcus*, *Pediococcus* and *Lactobacillus* may produce diacetyl although production is repressed by the fermentation of hexoses (Jay, 1982; Cogan, 1986). Gram-negative bacteria, yeasts and moulds are more sensitive to diacetyl than gram-positive bacteria and its mode of action is believed to be due to interference with the utilisation of arginine (Jay, 1986; Motlagh et al., 1991; De Vuyst and

Vandamme, 1994a). Diacetyl is rarely present in food fermentations at sufficient levels to make a major contribution to antibacterial activity.

6.5 Reuterin

Reuterin is produced during the stationary growth phase by the anaerobic growth of *Lactobacillus reuteri* on a mixture of glucose and glycerol or glyceraldehyde. It has a general antimicrobial spectrum affecting viruses, fungi and protozoa as well as bacteria (Axelsson et al., 1989; Chung et al., 1989). Its activity is thought to be due to inhibition of ribonucleotide reductase (Dobrogosz et al., 1989).

7. Safety of Lactic Acid Bacteria

The use of LAB as a probiotic requires a safety assessment. The functional properties of the strains should be well studied and documented (Holzapfel et al., 2001). Generally recognized health-promoting properties are non-pathogenic behavior, the ability to persist within the GI tract and adhesion, and the ability to modulate immune responses (Dunne et al., 2001; Gibson and Fuller, 2000; Holzapfel et al., 2001; Reid et al, 2003). Gibson and Fuller (2000) pointed out the importance of considering the possible side effects of probiotics on the consumer, e.g. bloating or blocking the normal functional gut transit. Ishibashi and Yamazaki (2001) pursued the research of bacteria converting food components or biological secretions into secondary substances harmful to the host.

Lactobacilli and lactococci commonly hold a GRAS status. Japan legally recognizes functional foods (Foods for Specified Health Use, FOSHU) (Sanders, 2003). Lethal dose 50% (LD_{50}) of LAB was measured for mice by oral administration and found to be $> 10^{11}$ cfu/kg, depending on the strain (Ishibashi and Yamazaki, 2001). The safety of two *Bifidobacterium longum* strains of human origin was evaluated on healthy adult volunteers: no side effects were reported and the immune parameters measured remained without undesirable changes (Mäkeläinen et al., 2001). However, some enterococci such as *E. faecalis* and *E. faecium* are classified in risk group II as pathogens (Anonymous, 2004c). Special concern has been expressed on the potential risk arising from the existence of antibiotic transferable genes among lactobacilli (Lindgren, 1999). Some species of LAB (*L. acidophilus*, *L. reuteri*, *L. rhamnosus*, *Leuconostoc* spp.) commonly used in the food industry or naturally occuring in raw food materials are resistant to glycopeptide antibiotics such as

teicoplanin and vancomycin (Felten et al., 1999; Goldstein et al., 2000; Tynkkynen et al., 1998; Vescovo et al., 1982). Antibiotic resistance encoding genes may transfer into a susceptible strain via a mobile genetic element (Noble et al., 1992; Shlaes et al., 1989), such as plasmids (Leclercq et al., 1987; Teuber et al., 1999; Vescovo et al., 1982) and transposons (Arthur et al., 1993; Hill et al., 1985) to produce new resistant bacterial strains (Danielsen and Wind, 2003). Conjugative transposons are commonly found in enterococci and streptococci as well as in some *Lactococcus lactis* strains reported to contain a chromosomally located transposon (Immonen et al., 1998; Rauch and de Vos, 1992). Plasmids of LAB do not commonly carry transmissible antibiotic resistance genes but can take in conjugative transposons and plasmids. Some plasmids, such as those with bacteriocin immunity genes, can integrate into the chromosome (Rauch and de Vos, 1992; Steele and McKay, 1989). Plasmid-linked antibiotic resistance therefore poses a hazard (Lindgren, 1999).

Resistance to glycopeptides in clinical isolates is classified as high-level resistance as well by inducibly and constitutively low-level resistance (Quintiliani et al., 1993). Vancomycin resistance in enterococci is associated with the presence of nucleotide sequences related to *vanA* (Dutka-Malen et al., 1990), *vanB* (Hayden et al., 1993) and *vanC* (Quintiliani et al., 1993). Use of feeds containing antibiotics and antibiotics for promoting growth in animals, such as fluoroquinolones for poultry, were shown to correlate with antibiotic-resistant bacteria in the animals (Teuber et al., 1999; Witte, 1998). Several *Enterococcus* strains and some of *Lactobacillus* spp. (*L. casei, L. plantarum, L. rhamnosus*) with transferable vancomycin resistance have been isolated from clinical samples (Cooper et al., 1998; Leclercq et al., 1989; Shlaes et al., 1989), indicating that antibiotic medication may be involved in such cases (Shlaes et al., 1989; Witte, 1998). Lactobacilli appear to be sensitive to penicillins but less so to oxacillin, cefoxitin, ceftriaxone, metronidazole, cephalothin and imipenem (Danielsen and Wind, 2003; Goldstein et al., 2000). Low sensitivity to ampicillin and piperacillin has been fully observed as well (Goldstein et al., 2000). *L. acidophilus* and *L. reuteri* as well as the genus *Enterococcus* are examples of probiotic bacteria (Benyacoub et al., 2001; Vescovo et al., 1982) resistant to some degree to vancomycin (Arthur et al., 1993; Leclercq et al., 1989; Vescovo et al., 1982).

Table 2.4 The probiotic effect of lactic acid bacteria (LAB) in human and animal health.

Medical target	Example strain	Reference
Prevent food allergy	*L. rhamnosus* GG	Sütas et al., 1996
Block formation of biogenic amines	*L. lactis* ESI 561 *E. faecalis* INIA 4-07 *E. faecalis* EFS 2	Joosten et al., 1996
Overcome lactose intolerance	*L. acidophilus*	Gilliland and Kim, 1984
Prevent diarrhea (antibiotic-induced, rotavirus, travellers, community acquired, *Clostridium difficile* colitis)	LAB *L. rhamnosus* GG *L. acidophilus* LB	Fooks et al., 1999 Heyman, 2000 Oksanen et al., 1990 Simakachorn et al., 2000 Sanders, 2003
Reduce intestinal disorders and pouchitis	LAB *L. rhamnosus* GG	Gionchetti et al., 2000 Kuisma et al., 2003
Suppress side effects of *Helicobacter pylori* medication with antibiotics.	*L. acidophilus*	Canducci et al., 2000
Treat Crohn's disease, ulcerative colitis and imflammatory bowel disease (IBD)	*L. rhamnosus* GG *B. infantis* UCC35624 LAB	Gupta et al., 2000 Von Wright et al., 2002 Marteau et al., 2002
Stimulate anticarcinogenic activity	LAB *L. acidophilus*	Goldin, 1990 Hirayma and Rafter, 2000
Treat coronary heart disease and anticholesterolaemic effects	*L. acidophilus*	Schaafsma et al., 1998 Gilliland et al., 1985
Control of human urinary tract infection and vaginosis	*L. rhamnosus* GG *L. rhamnosus* GR-1	Kontiokari et al., 2001 Reid, 2001 Reid, 2002
Prevent kidney stones	*L. acidophilus* *L. plantarum* *L. brevis* *S. thermophilus* *B. infantis*	Campieri et al., 2001
Treat atopic disease	*L. rhamnosus* GG	Kalliomäki et al., 2001
Prevent caries formation	*L. rhamnosus* GG	Näse et al., 2001
Protection against tetanus toxin	*L. plantarum*	Grangette et al., 2001
Treat chronic fatigue syndrome	LAB	Logan et al., 2003
Inhibit pathogens causing bovine mastitis	*L. lactis* DPC3147	Ryan et al., 1998
Feed supplement for growth promotion in animals	*L. brevis* C10	Jin et al., 1998
Reduce pathogens in chickens by Competitive Exclusion (CE)	Undefined faecal cultures	Nurmi and Rantala, 1973
Inhibit enteropathogens in small intestine of animals	*L. acidophilus* LA1	Bernet-Camard et al., 1997

LAB = Lactic acid bacteria species not specified.

The antibiotic resistance genes serving as selective markers in LAB have been replaced by food-grade cloning systems (de Vos, 1999) based on i.e., nisin immunity (Takala and Saris, 2002), complementation of deficiency in lactose utilization (Takala et al., 2003), and suppression of nonsense mutation (Sorensen et al., 2000) for positive selection of transformants. The term food-grade can be used when the modified

microorganism contains such elements not harming the consumer when present in foods. Food-grade cloning systems need to be based on DNA from LAB or other microbes with a long history of safe use in the food industry (de Vos, 1999). Genetically modified LAB can in future be utilized as improved starters in food fermentation and for the safe production of metabolites used as food additives (de Vos, 1999).

The isolation of LAB from clinical samples has raised debate over the safety of probiotic bacteria and whether or not the bacteria are actually infectious (Adams and Marteau, 1995; Felten et al., 1999; Donohue et al., 1998; Ishibashi and Yamazaki, 2001). Some LAB have been implicated in local systemic infections including septicemia and endocarditis (Antony et al., 1995; Husni et al., 1997; Ishibashi and Yamazaki, 2001; Soleman et al., 2003) as well as liver abscesses (Rautio et al., 1999). In most cases of infection, the organisms were shown to be of host origin. Some cases have been linked to the consumption of probiotics (Salminen et al., 2002; Salminen et al., 2004). Except for enterococci and streptococci, the clinical significance of LAB is low (Boulanger et al., 1991), *L. rhamnosus* being the most frequently isolated LAB from clinical samples (Felten et al. 1999). The isolation of LAB from infections is likely to be the result of opportunist pathogens on an immunosuppressed host (Ishibashi and Yamazaki, 2001; Salminen et al., 2002). Many factors may promote translocation of intestinal bacteria, such as intestinal mucosal injury, immunodeficiency of the host, an abnormal intestinal bacterial microbiota (Berg, 1995), previous antibiotic treatment, complications from Acquired Immunodeficiency Syndrome (AIDS) and prior hospitalization and surgery (Antony et al., 1996; Cooper et al., 1998; Husni et al., 1997).

The development of novel approaches in food (de Vos et al., 1997; Luoma et al., 2001) and in pharmaceutoclinical therapies (Grangette et al., 2001; Saavedra, 2001; Steidler, 2002) allow broadening the potential for using lactic acid bacteria in food and pharmacology (Kuipers et al., 2000; Mollet, 1999; Renault, 2002). The nature of genetic modifications can be divided into three groups: 1) one-step genetic events like deletions, gene amplifications, plasmid insertions and losses, 2) multi-step genetic rearrangements with DNA of the same species, and 3) trans-species genetic modifications (Mollet, 1999). Kuipers et al. (2000) has emphasized the effective use

of gene manipulated LAB in the battle against food spoilage and pathogenic bacteria. As examples, genetically modified LAB have been utilized to improve cheese ripening (Luoma et al., 2001), produce phageresistant starter strains (Moineau, 1999), and protect against tetanus toxin (Grangette et al., 2001) and bovine rotavirus (Enouf et al., 2001). It can be used to treat Shiga toxigenic *Escherichia coli* infections and dysentery in humans (Paton et al., 2000), prevent dental caries (Hillman, 2002) and treat inflammatory bowel disease (Steidler et al., 2003). Netherwood et al., (1999) studied spontaneous gene transfer in the GI tract and observed that *in vivo* transfer rate in the gut was 0.03 transconjugants per recipient cell.

All new ingredients and genetically modified organisms (GMO) in foods fall under the Novel Foods Regulation of the EU legislation (Feord, 2002; Lindgren, 1999). No GMO has yet been authorized as a feed additive in Europe (Anonymous, 2001). Renault (2002) discussed the use of genetically engineered LAB in foods, emphasizing the value of risk assessment in correlation with the expected benefits of modified strains. The objective of risk assessment is to identify and evaluate the potential adverse effects of GMOs. The cumulative and long-term effects on human health and the environment have also to be taken into account. Assessment focuses on GM development and the possible genetransfer to host microbiota (Renault, 2002).

8. Fermentation of Meat

8.1 Dry sausage manufacturing process

Dry sausage is made from a mixture of frozen pork, beef and pork fat (Buckenhuskes, 1993). In addition, it contains sugars, salt, nitrite, and nitrate, ascorbates and spices. The raw sausage is stuffed into casing of variable diameters and hung vertically in fermentation and ripening chambers for several weeks. Salt acts as one of the first hurdles against the growth of unwanted microorganisms. It also induces the solubilisation and diffusion of myofibrillar proteins from muscle forming a gel between meat and meat as well as meat and fat particles of the raw sausage material.

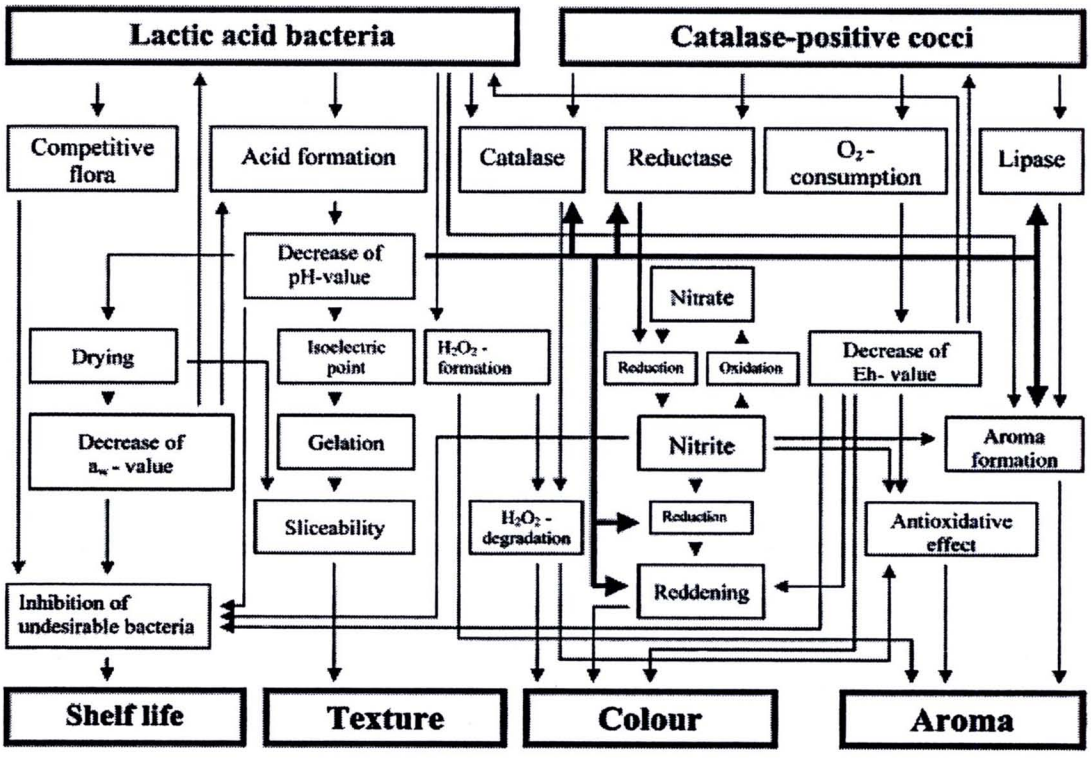

Figure 2.3 Interactions during the fermentation of sausages caused by the action of lactic acid bacteria and catalase-positive cocci (Buckenhuskes, 1993).

Salt (NaCl 2.5–3.0% (w/w), initial value) is also an important flavour component of the end product (Lucke, 1985). Nitrite acts as other hurdle against the growth of pathogens which may be introduced with the raw meat material. It also contributes to the formation of the typical cured meat colour. Ascorbates enhance the colour formation (Puolanne, 1977). Spices, such as pepper, cardemum and garlic, have an impact on flavour and they may also have antioxidative and antimicrobial effects (Hammer, 1977). Furthermore, smoke, consisting of phenols, carbonyls and different organic acids, contributes to inhibition of different bacteria on the surface of the sausages (Toth and Blaas, 1972).

Sugars are added as fermentable substrates for LAB (inoculation of 6–7 log cfu/g) and staphylococci (6 log cfu/g) used as starter cultures (Fig. 6). Catalases produced by staphylococci degrade hydrogen peroxide produced by LAB (Katsaras and Leistner, 1988). In addition, staphylococci reduce nitrate into nitrite (Niinivaara,

1955; Pohja and Niinivaara, 1957; Nurmi, 1966) and have an impact on flavour (Selgas et al., 1988; Comi et al., 1992; Berdague′ et al., 1993; Stahnke, 1994; Montel et al., 1993; 1996). LAB decrease the pH of the sausage close to pH-value 5.0 in first few days, which acts as a hurdle for several Gram-negative bacterial species (Leistner, 1995). While the pH of the sausage (i.e. salt–meat mixture) decreases and approaches the isoelectric point, the water holding capacity of the sausage decreases (Hamm, 1962). This favours the drying and consequently the weight losses of sausage, which result in the firm texture and sliceability of the end product (Buckenhuskes, 1993).

8.2 Lactic acid bacteria currently used in the fermentation of meat

According to the definition of Hammes (1996), meat starter cultures are ''preparations which contain living or resting microorganisms that develop the desired metabolic activity in the meat''. As a rule, they are facultatively heterofermentative strains, which produce lactic acid from hexoses, such as glucose and lactose, as their only metabolic product (via glycolysis). As there is no sufficient glucose in meat to markedly reduce the pH, glucose is added at 0.4–0.7% (w/w) to the sausage matrix. For lactose fermenting LAB, such as *L. sakei*, lactose may also be used (0.5–1.0%) (Pyrcz and Pezacki, 1981; Wirth, 1984). However, not all LAB can easily ferment lactose and especially some probiotics, such as *Lactobacillus rhamnosus* GG, are not able to utilize lactose. Thus, the starter culture properties have to be taken into account prior to planning new applications. From pentoses, such as arabinose and xylose, meat starter LAB produce both lactic acid and acetic acid (via part of the 6-phosphogluconate/ phosphoketolase pathway) (Kandler, 1983; Axelsson, 1998). The amount of acetic acid is typically 1/10 of the amount of lactic acid (Deketelaere et al., 1974). As indicated in commercial catalogues LAB strains currently most employed in meat starter cultures are *L. casei, L. curvatus, L. pentosus, L. plantarum, L. sakei, P. acidilactici* and *P. pentosaceus*.

8.3 Safety and shelf-life of dry sausage

8.3.1 Hurdle concept

The microbial stability of dry sausages is determined by the combination and timing of different factors referred to as the hurdle-concept (Leistner, 1995). The safety of dry sausage material, which is actually raw meat kept at room temperature for several days, is based on the migration of salt into meat

before the temperature of the meat rises above 10 °C and the addition of nitrite. Salt decreases the initial water activity inhibiting or at least delaying the growth of many bacteria while favouring the growth of starter LAB and starter staphylococci. Nitrite as in the form of undissociated nitrous acid (HNO_2) is able to pass the ion barrier of bacterial cell wall and disturb the function of bacterial enzymes and therefore bacterial growth (Cook and Pierson, 1983; Pierson and Smooth, 1987).

During the first day of fermentation the growth of microbes in sausage material uses up all the oxygen mixed in the sausage matrix during the chopping. This reduces the redox potential (Eh = 100–200 mV) making the nitrite more effective and restricts the growth of aerobic spoilage bacteria (pseudomonads) derived from the raw meat (Lucke and Hechelmann, 1987; Krockel, 1995).

After a few days of fermentation, the high amounts of LAB have produced high amounts of lactic acid resulting in a low pH value (5.0) of dry sausages (Lucke and Hechelmann, 1987). The lower external pH disturbs the homeostasis of different pathogens (e.g. *Salmonella* spp., *Clostridium* spp.) as well as spoilage bacteria, e.g. pseudomonads and enterococci (Leistner, 2000). In a solution, weak acids exist in pH dependent equilibrium between undissociated and dissociated state (lactic acid pKa = 3.86). The low pH favours the uncharged undissociated state of the molecule, which is able to penetrate the target cell membrane. Therefore, the lower the pH the stronger the inhibitory effect (Davidson, 1997; Leistner, 2000). However, at the pH values typical for dry sausage (4.8) only 10% of lactic acid is undissociated, resulting in a fairly moderate inhibitory effect (Lueck, 1980; Cherrington et al., 1991).

The final and most important hurdle for the growth of other bacteria than LAB and staphylococci is the low water activity of the dry sausage (Krockel, 1995). The low pH value decreases the water holding capacity of meat increasing the rate of the drying process. The drying of sausages in ripening chamber leads to the final water activity < 0.90 of the product (Stiebing and Rodel, 1987; Lucke, 1985).

8.3.2 Inhibition of pathogens by hurdle concept

The growth of several bacterial species is inhibited by nitrite, low oxygen level, pH and water activity (Leistner, 1995). The germination of *Bacillus*

and *Clostridium* spores, which may be derived from spices (and to lesser extent from meat), is controlled by low pH and water activity (Nordal and Gudding, 1975). *S. aureus* is tolerant to dry sausage environmental factors, but it is a fairly poor competitor to starter LAB and starter staphylococci at fermentation temperatures 20–25 °C commonly used in North Europe (Hurst and Collins-Thompson, 1979; Metaxopoulos et al., 1981a,b). However, the hurdles present in dry sausage are not sufficient to prevent the survival of *L. monocytogenes* (Farber and Peterkin, 1991; Varabioff, 1992) or *E. coli* O157:H7 (Reed, 1995).

L. monocytogenes is a Gram-positive non-sporulating food pathogen. It is especially dangerous for very young (children), old, pregnant and immunocompromized persons (Farber and Peterkin, 1991). Despite the various hurdles in the dry sausage manufacturing process it is able to survive the commercial dry sausage manufacturing process (Varabioff, 1992; Gahan et al., 1996). An additional hurdle to reduce the risk of *L. monocytogenes* in dry sausage is to utilise specific bacteriocin producing starter cultures in dry sausage manufacturing (Berry et al., 1990; Foegeding et al., 1992; Campanini et al., 1993; Elsser, 1999; Tyopponen et al., in press).

E. coli O157:H7 is highly adapted to acidic conditions and due to its very low infectious dose (~6 CFU) level it poses a serious risk for the consumers (Doyle, 1991; Bolton et al., 1996). Its acid tolerance is inducible and involves the synthesis of acid shock proteins, activation of metabolic enzymes to maintain homeostasis, and the increased in corporation of cyclopropane fatty acids in the cytoplasmic membrane (Leyer et al., 1995; Bearson et al., 1997; Brown et al., 1997). In 1994, in Washington and California 18 people were infected by *E. coli* O157:H7 derived from dry sausage. Therefore, United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) required the processors of dry and semidry sausage to validate at least a 5-log-unit reduction in numbers of *E. coli* O157:H7 cells in dry sausages (Reed, 1995). Several studies concerning the survival of *E. coli* O157:H7 in non-heated dry sausage manufacturing process with starter LAB have shown that the number of *E. coli* O157:H7 can be reduced by 1–3 log units (Hinkens et al., 1996; Clavero and Beuchat, 1996; Calicioglu et al., 1997; Faith et al., 1997, 1998; Yu and Chou, 1998; Erkkila¨ et al., 2000).

8.3.3 Protective culture concept

In recent years, there has been a considerable increase in studies of the natural antimicrobial compounds on and in food produced by LAB, referred as bioprotective cultures. Bioprotective cultures may act as starter cultures in the food fermentation process, such as dry sausage manufacturing process, or they may protect foods without any detrimental organoleptic changes.

Bacteriocins are peptides or proteins, which due to their proteinaceous nature are readily degraded after human digestion to common nutrients (Sanders, 1993; Vandenbergh, 1993). The well-known bacteriocin nisin (Group I) (E234) dissipates the proton motive force of the target cell by forming a pore through the cytoplasmic membrane which causes the flux of essential energy (ATP) and different ions from the cell (Moll et al., 1996; Brotz et al., 1998). The bacteriocins of group II (Klaenhammer, 1993; Nes et al., 1996; Ennahar et al., 2000) are the most interesting for the meat industry. For example curvacin A (Tichaczek et al., 1992) and sakacin A, P and K (Holck et al., 1992; Tichaczek et al., 1992; Hugas et al., 1995) produced by *L. curvatus* and *L. sakei* strains, respectively, are isolated from meat and they are mainly active against other LAB and *L. monocytogenes* while pediocin PA-1/AcH produced by *P. acidilactici*, *P. parvulus* and *L. plantarum* inhibit the growth of *S. aureus*, *L. monocytogenes* and *Clostridium perfingens* (Bhunia et al., 1988, 1991; Christensen and Hutkins, 1992; Ennahar et al., 1996; Luchansky et al., 1992; Klaenhammer, 1993; Eijsink et al., 1998). In dry sausages the utilization of bacteriocin producing *P. acidilactici* JD1–23, *P. acidilactici* PAC 1.0 and *L. plantarum* MSC as fermenting agents resulted 1–2 log units lower number of *L. monocytogenes* per gram dry sausage than in the control sausages (Berry et al., 1990; Foegeding et al., 1992; Campanini et al., 1993).

In contrast to Gram-positive bacteria, Gram-negative bacteria, such as *E. coli* O157:H7, possess in addition to an inner membrane, an outer membrane through which the hydrophobic bacteriocins are not able to penetrate (Nikaido and Vaara, 1985; Helander et al., 1997). As suggested by Helander et al. (1997), food grade permeabilizers, such as lactic acid or citric acid (Cutter and Siragusa, 1995b; Alakomi et al., 2000; Helander and Mattila-Sandholm, 2000), in

combination with bacteriocins would be ideal as part of the hurdle concept in inhibiting Gram-negative bacteria in foods.

Non-proteinaceous low-molecular-mass (LMM) compounds are known to possess a wide antimicrobial spectrum concerning both Gram-positive and Gram-negative bacteria. Currently LMM compounds are poorly characterized due to difficulties in purification as described by Niku-Paavola et al. (1999). The typical LMM compounds are small hydrophobic heterocyclic or aromatic structured compounds similar to benzoic acid (pKa = 4.19), active at low pH and stable in heat processing (Brul and Coote, 1999). However, these compounds most probably do not contribute to the safety and shelf-life of dry sausage due to their extremely low concentrations produced during dry sausage manufacturing process. Furthermore, e.g. diacetyl (Jay, 1982) and reuterin (Chung et al., 1989) are produced by heterofermentative LAB, which also produce carbon dioxide and high amounts of acetic acid (Axelsson, 1998) not desired in dry sausage due to quality defects.

In order to fully utilise the antimicrobial metabolites of LAB a few challenges have to be overcome in the future. Bacteriocins may bind to the food fat or protein particles or the food additives, natural proteases or other inhibitors may inactivate them (Jung et al., 1992; Degnan and Luchansky, 1992; Leroy and De Vuyst, 1999). In addition, the effect may be seen only in a narrow pH range, which excludes their utilisation in many food products (Yang and Ray, 1994; Cutter and Siragusa, 1995a, Mortvedt-Abildgaard et al., 1995; Ga¨nzle et al., 1999b). In case of dry sausage the concentrations produced by LAB in situ may be affected by the low carbohydrate content and low ripening temperature (Vogel et al., 1993; Hugas et al., 1995). Different weight losses and consequently in different salt contents may also have an effect on antimicrobial activity. Especially high salt content may decrease the activity of the bacteriocins, and furthermore, salt may prevent the growth and consequently the production of bacteriocins (De Vuyst et al., 1996; Ga¨nzle et al., 1996; Nilsen et al., 1998; Leroy and De Vuyst, 1999; Tyo¨ppo¨nen et al., in press). However, many bioprotective cultures are known to produce several antimicrobial compounds, which act in cooperation (Hanlin et al., 1993; Niku- Paavola et al., 1999).

Table 2.5 Antimicrobial products of lactic acid bacteria (LAB) with broad inhibitory spectrum

Product	Producer	Reference
Lactic acid	All LAB	Axelsson (1998)
Acetic acid	Heterofermentative LAB	
Hydrogen peroxide	All LAB	
Alcohols	Heterofermentative LAB	
Carbon dioxide	Heterofermentative LAB	
Other low-molecular-mass compounds		
Diacetyl	*Lactococcus* spp.	Jay (1982)
Reuterin	*Lactobacillus reuteri*	Chung et al. (1989)
Bacteriocins		
Class I bacteriocins		
Nisin	*Lactococcus lactis*	
Class II bacteriocins		
Sakacin P	*Lactobacillus sake*	Holck et al. (1994)
Sakacin K	*Lactobacillus sake*	Hugas et al. (1995)
Curvacin A/sakacin A	*Lactobacillus curvatus*	Tichaczek et al. (1992, 1993)
Carnobacteriocin A	*Carnobacterium piscicola*	Worobo et al. (1994)
Pediocin AcH/PA-1/SJ-1	*Pediococcus acidilactici*	Gonzalez and Kunka (1987)
	Pediococcus parvulus	Bennik et al. (1997)
	Lactobacillus plantarum	Ennahar et al. (1996)
Leucosin A/B-Talla	*Leuconostoc gelidum*	Hastings and Stiles (1991)
	Leuconostoc carnosum	Felix et al. (1994)
Mesentericin Y105	*Leuconostoc mesenteroides*	Héchard et al. (1992)
Enterocin A	*Enterococcus faecium*	Aymerich et al. (1996)
Enterocin P	*Enterococcus faecium*	Cintas et al. (1997)
Enterocin B	*Enterococcus faecium*	Casaus et al. (1997)

8.4 Probiotics

Probiotics are live microbial food ingredients that are beneficial to health (Salminen et al., 1998a, 1999). Probiotics are lactic acid bacteria or bifidobacteria, currently mainly of Lactobacillus species (Table 2.6). Also other species have been introduced including enterococci, propionibacteria and even clostridia (Sanders and Huis in't Veld, 1999; von Wright and Salminen, 1999).

Table 2.6 Some commercially used probiotic lactobacilli and bifidobacteria and reported clinical effects in humans

Strain	Company	Reported clinical effects in humans
L. rhamnosus GG	Valio, Finland	Adherence to human intestinal cells, lowering faecal enzyme activities, prevention of diarrhoea, immune response modulation, prevention and treatment of food allergies
L. johnsonii La1	Nestle, Switzerland	Modulation of intestinal flora, immune enhancement, adjuvant in *Helicobacter pylori* treatment
L. casei Shirota	Yakult, Japan	Modulation of intestinal flora, lowering faecal enzyme activities, prevention of occurrence of superficial bladder cancer
L. reuteri SD2112	BioGaia, USA	Colonisation of intestinal tract, treatment of diarrhoea
L. plantarum 299V	Probi, Sweden	Adherence to human intestinal cells, modulation of intestinal flora
B. lactis Bb-12	Chr. Hansen, Denmark	Treatment/prevention of diarrhoea, modulation of intestinal flora, improvement of constipation, modulation of immune responces, alleviation of symptoms of food allergy

(Sanders and Huis in't Veld, 1999; von Wright and Salminen, 1999; Mattila-Sandholm and Saarela, 2000).

The ability to produce different antimicrobial compounds, such as bacteriocins and/or low-molecularmass antimicrobial compounds may be one of the critical characteristics for effective competitive exclusion of pathogens and survival in the intestine to express probiotic effect to the host (Ouwehand, 1998; Salminen et al., 1998a). The acidic conditions in the stomach may even enhance the activity of these antimicrobial compounds (Ganzle et al., 1999a,b). Furthermore, the probiotic effect of LAB may partly be based on the production of relevant concentrations of lactic acid in the microenvironment, which in combination with detergent like bile salts inhibit the growth of Gram-negative pathogenic bacteria (Alakomi et al., 2000). In fact, the exact mechanism by which a probiotic strain interacts with other bacteria in the gastrointestinal tract or with the mucus of gastrointestinal tract itself is not known (Havenaar et al., 1992; Fuller, 1992; Playne, 1995; Berg, 1998; von Wright and Salminen, 1999). However, several clinical studies concerning gastrointestinal disorders, food allergies and inflammatory bowel diseases have provided evidence for the claimed health effects (Isolauri et al., 1999; Ouwehand et al., 1999; von Wright and Salminen, 1999). Gastrointestinal disorders mainly cover diarrhoea caused by pathogens (Oksanen et al., 1990; Perdigon et al., 1990; Hilton et al., 1997), antibiotics (Arvola et al., 1999) and rotavirus (Saavedra et al., 1994). Furthermore, probiotics are known to promote the immune function (Perdigon et al., 1995; Schiffrin et al., 1995; Pelto et al., 1998; He et al., 2000). Recently, probiotics have been used for alleviation of symptoms of food allergy in infants and adults as well as prevention of atopic diseases in infants (Isolauri et al., 1999; Kalliomaki et al., 2001).

The utilisation of health aspects of food products in their marketing began in the 1960s and in the 1970s the trend was to remove unhealthy components, such as salt, sugar or fat. The trend continued by removing some of the additives in 1980s and finally in 1990s more healthy components, such as vitamins, antioxidants, fiber and probiotic LAB, were added to the food products. In the 2000s specific functional food products have scientifically proven to benefit the health and well-being of consumers. Proposed functional foods in Europe include 60% of dairy products, 25% of fatbased spreads, 10% of bakery and cereal products, and 5% of drinks (Young, 2000). The concept of probiotic foods being part of the concept of a functional food is due to the successful cooperation of food industry and research in food science and technology

as well as in clinical nutrition. Probiotics have been applied mainly in dairy products, such as yoghurt and other cultured milk products and also in cereal products. For example *L. rhamnosus* GG has been applied to normal and drinking yoghurts, fermented milks, pasteurised milk, dairy-based drinks, fermented whey-based drinks as well as juices (Saxelin, 2000).

The target products in meat processing are the various dry sausages which are processed by fermenting without heating. Arihara et al. (1996, 1998) have shown that the potentially probiotic strain *Lactobacillus gasseri* JCM1131 is applicable for meat fermentation to enhance product safety, and Sameshima et al. (1998) have demonstrated the usefulness of the potential probiotics *L. rhamnosus* FERM P-15120 and *L. paracasei* subsp. *paracasei* FERM P-15121 in meat fermentation. Erkkila et al. (2000, 2001a,b) used probiotic *L. rhamnosus* GG and potentially probiotic *L. rhamnosus* LC-705 and VTT-97800 for dry sausage manufacturing and Andersen (1998) fermented dry sausages succesfully using a mixture of the traditional starter culture Bactoferm T-SPX (Chr. Hansen) and a potentially probiotic culture of *L. casei* LC-01 or a mixture of the same starter and the probiotic *Bifidobacterium lactis* Bb-12.

However, human clinical studies documenting health promoting effects of dry sausage do not exist. At this point, it could be speculated that since the bacteria in general are sensitive to low pH values in the stomach (Conway et al., 1987; Berrada et al., 1990; Hammes et al., 1997; Erkkila and Petaja, 2000), it is more important that probiotics are consumed within a food matrix. Bacteria can survive acidic conditions in vitro when inoculated onto the surface of solid food, whereas the same level of acidity is lethal to the inoculum in an acidified broth environment. Milk has been shown to be an excellent vehicle for probiotic bacteria probably due to its high buffering capacity (Saxelin, 1996). Meat acts also as a buffer in an acidic environment and therefore may also protect bacteria from hostile environment. The protective effect of some solid foods may be due to decreasing the pH of microenvironment of the bacteria on the surface of the food (Waterman and Small, 1998). Furthermore, meat has been found to protect LAB against the lethal action of bile (Ganzle et al., 1999a). In fermented sausages LAB grow in nests (Katsaras and Leistner, 1991) and they are therefore "encapsulated" by the sausage matrix consisting of meat and fat. Thus they may survive better the critical passage through the stomach and the small

intestine compared to their unprotected exposure to low pH and bile salts. Furthermore, it has been argued by Tannock (1999) that food components (meat and fat particles) that have escaped digestion may act as an energy source for the bacteria in the intestine.

The minimum dose of daily ingestion of probiotic bacteria is not known, but it is estimated to be 10^{9-10} viable microbes in order to show a health effect and temporary colonisation as measured by levels of 10^{6-8} viable microbes/g faeces. For a dry sausage containing 10^8 viable microbes/g the minimum dose therefore could be 10–100 g sausage per day. However, the minimal dose is dependent on several factors, such as individual person, strain and food product. Since the exact mechanism by which a probiotic strain interact with other bacteria in the gastrointestinal tract or with the mucus of gastrointestinal tract in itself is not known (Havenaar et al., 1992; Fuller, 1992; Playne, 1995; Berg, 1998), no direct extrapolations of how a strain would affect a host can be made (Mattila-Sandholm et al., 1999). It can also be speculated that a strain particularly well-suited to survive through the gastrointestinal tract could be provided in lower numbers than a poorly adapted strain (Conway et al., 1987; Berrada et al., 1990). Finally, the food product itself is highly important. The number of viable probiotics in the product is affected by several factors, such as temperature, moisture, fat content and levels of different chemicals (Sanders and Huis in't Veld, 1999; Hammes and Hertel, 1998).

9. Lactic Acid Bacteria as Functional Starter Cultures

Nowadays, the consumer pays a lot of attention to the relation between food and health. As a consequence, the market for foods with health-promoting properties, socalled functional foods, has shown a remarkable growth over the last few years (Nutrition Business Journal, 2002). Also, the use of food additives is regarded as unnatural and unsafe (Ray, 1992). Yet, additives are needed to preserve food products from spoilage and to improve the organoleptic properties. The demand for a reduced use of additives and processing seems contradictory with the market preference for products that are fresh, safe, tasty, low in sugar, fat, and salt, and easy to prepare. In cheese-making, for instance, the use of raw milk permits the manufacture of high-value traditional artisan varieties but brings about safety risks, e.g. the development of

Listeria monocytogenes. On the other hand, pasteurisation of the milk results in loss of flavour and gives end products that are perceived by the consumer as ''boring'' (Law, 2001). These market trends put the food industry under pressure to look for alternatives. In food fermentation, one of the key points for intervention seems to be on the level of the starter culture. Unfortunately, industrial starter cultures lack the necessary characteristics for product diversification, and the commercial availability of new interesting starter cultures is limited. The increased understanding of the genomics and metabolomics of food microbes opens perspectives for starter improvement. Through molecular biology it is now possible to express desirable and suppress undesirable properties of starter cultures (Delcour et al., 1999; Law, 2001; Mogensen, 1993).

Table 2.7 Typical examples of functional starter cultures or co-cultures and their advantages for the food industry

Advantage	Functionality	Lactic acid bacteria[a]	Relevant references
Food preservation	Bacteriocin production		
	-Dairy products	*L. lactis* subsp. *lactis*	Maisnier-Patin *et al.* (1992), Roberts *et al.* (1992)
		Enterococcus spp.	Giraffa (1995)
	-Fermented meats	*Lb. curvatus*	Vogel *et al.* (1993)
		Lb. sakei	Hugas *et al.* (1995)
		P. acidilactici	Foegeding *et al.* (1992)
		E. faecium	Callewaert *et al.* (2000)
	-Fermented olives	*L. plantarum*	Ruiz-Barba *et al.* (1994)
	-Fermented vegetables	*L. lactis*	Harris *et al.* (1992)
Organoleptic	Production of exopolysaccharides	Several lactobacilli and streptococci	De Vuyst & Degeest (1999) De Vuyst & Marshall (2001) De Vuyst *et al.* (2001)
	Production of amylase	Several lactobacilli	Mogensen (1993)
	Aroma generation	Several strains	Marshall (1987) Demeyer *et al.* (2000) Kleerebezem *et al.* (2000)
	Enhanced sweetness		
	-Homoalanine-fermenting starters	*L. lactis*[b]	Hols *et al.* (1999)
	-Galactose-positive/ glucose-negative starters	*Lb. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*	under development
	malolactic fermentation	*O. oeni*	Lonvaud-Funel (1999)
Technological	Bacteriophage resistance	Several strains	Forde & Fitzgerald (1999)
	Prevention of overacidification in yoghurt	lactose-negative *Lb. delbrueckii* subsp. *bulgaricus*	Mollet (1996)
	Autolysing starters		
	-Phage-mediated	*L. lactis* subsp. *lactis*	Crow *et al.* (1996)
	-Bacteriocin-induced	*L. lactis*	Morgan *et al.* (1997)
Nutritional	Production of nutraceuticals		
	-Low-calorie sugars (e.g., sorbitol and mannitol)	*Lb. plantarum* *L. lactis*	Wisselink *et al.* (2002)
	-Production of oligosaccharides	*L. lactis*	Ruas-Madiedo *et al.* (2002)
	-Production of B-group vitamins (e.g., folic acid)	*Lb. delbrueckii* subsp. *bulgaricus*, *L. lactis*, *S. thermophilus*	Hugenholtz & Kleerebezem (1999) Wouters *et al.* (2002)
	-Release of bioactive peptides	Several strains	Meisel & Bockelman (1999)
	Reduction of toxic and anti-nutritional compounds		
	-Production of L(+)-lactic acid isomer	L(+)-lactic acid-producing strains	Wouters *et al.* (2002) Holzapfel (2002)
	-Removal of lactose and galactose	*S. thermophilus*	Wouters *et al.* (2002)
	-Removal of raffinose in soy	Several strains	Scalabrini *et al.* (1998)
	-Reduction of phytic acid content, amylase inhibitors, and polyphenolic compounds	*Lb. plantarum* *Lb. acidophilus*	Sharma & Kapoor (1996)
	-Decreased production of biogenic amines	*E. faecalis*	Joosten *et al.* (1995)

[a] *E.* = *Enterococcus*, *L.* = *Lactococcus*, *Lb.* = *Lactobacillus*, *O.* = *Oenococcus*, *P.* = *Pediococcus*, *S.* = *Streptococcus*.
[b] Recombinant strain.

Recently, the use of functional starter cultures in the food fermentation industry is being explored (De Vuyst, 2000). Functional starter cultures are starters that possess at least one inherent functional property. The latter can contribute to food safety and/or offer one or more organoleptic, technological, nutritional, or health advantages. The implementation of carefully selected strains as starter cultures or co-cultures in fermentation processes can help to achieve in situ expression of the desired property, maintaining a perfectly natural and healthy product. Examples are LAB that are able to produce antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, useful enzymes, or nutraceuticals, or LAB with health-promoting properties, so called probiotic strains. This represents a way of replacing chemical additives by natural compounds, at the same time providing the consumer with new, attractive food products. It also leads to a wider application area and higher flexibility of starter cultures. Although probiotic strains may also be classified as functional starter or co-cultures for food fermentations. (Chandan, 1999; Erkkila et al., 2001; Jahreis et al., 2002; Pidcock, Heard, and Henriksson, 2002; Ross et al., 2000).

9.1 Application of functional starter cultures in food fermentations food preservation and safety

Chemical food additives such as nitrite, sulphite, propionic acid, sorbic acid, and benzoic acid are commonly applied in food preservation technology (Smith, 1993). As an alternative, the antimicrobial activity displayed by LAB strains may help to combat microbial contamination (Holzapfel, Geisen, and Schillinger, 1995; Lucke, 2000). LAB produce several natural antimicrobials, including organic acids (lactic acid, acetic acid, formic acid, phenyllactic acid, caproic acid), carbon dioxide, hydrogen peroxide, diacetyl, ethanol, bacteriocins, reuterin, and reutericyclin. Acetic acid, for instance, contributes to the aroma and prevents mould spoilage in sourdough (Messens and De Vuyst, 2002).

Bacteriocins from LAB are low-molecular-mass peptides or proteins with an antibacterial mode of action restricted to related Gram-positive bacteria. Bacteriocin-producing LAB can be applied for food preservation because of their microbiological, physiological and technological advantages (Cleveland et al., 2001; De Vuyst and Vandamme, 1994; Nettles and Barefoot, 1993; Ray and Daeschel, 1992; Smith, 1993). The in situ production of bacteriocins may increase the

competitiveness of the producer strain in the food matrix and contribute to the prevention of food spoilage (Hugas et al., 1995; Ross et al., 2000; Ruiz-Barba et al., 1994; Vogel et al., 1993). For instance, bacteriocin-producing LAB can be used as an alternative to potassium nitrate to prevent late loss of cheese due to contamination by clostridia (Thomas, Clarkson, and Delves-Broughton, 2000). Another example is the suppression of flavour-disturbing contaminating microbes, e.g., certain strains of *L. lactis* that produce off-flavours in dairy products (Stanley, 1998). In addition, many bacteriocins are active towards foodborne pathogens such as *C. botulinum*, *S. aureus*, and *L. monocytogenes* (Nettles and Barefoot, 1993). Several studies have indicated that LAB starter strains are able to produce their bacteriocins in food matrices and consequently display inhibitory activity towards sensitive food spoilage or pathogenic bacterial strains. The latter has been documented for fermented sausage (Callewaert, Hugas, and De Vuyst, 2000; Foegeding et al., 1992; Hugas et al., 1995; Vogel et al., 1993), fermented vegetables and olives (Harris, 1998; Harris, Fleming, and Klaenhammer, 1992; Ruiz-Barba et al., 1994), and dairy products (Benkerroum et al., 2002; Buyong, Kok and Luchansky, 1998; Foulquie et al., 2003; Giraffa, 1995; Maisnier-Patin et al., 1992; McAuliffe, Hill, and Ross, 1999; Roberts, Zottola, and Mckay, 1992; Rodriguez et al., 1998).

Reuterin (b-hydroxypropionaldehyde) produced by *L. reuteri* is active towards a wide spectrum of bacteria, moulds and yeasts (Talarico and Dobrogosz, 1989), but is not formed in sufficient amounts in the presence of sugars. Reutericyclin, a tetramic acid antibiotic with broad antimicrobial activity produced by *L. reuteri* (Ganzle et al., 2000; Ga nzle and Vogel, 2003), is believed to be responsible for the stability of certain German sourdoughs (Messens and De Vuyst, 2002).

Recently, it has been shown that the production of phenyllactic and 4-hydroxy-phenyllactic acids by *L. plantarum* strains is responsible for a broad antifungal activity in sourdough (Lavermicocca et al., 2000). Besides the production of phenyllactic acid, *L. plantarum* MiLAB 393 displays antifungal activity due to the production of cyclic dipeptides (Strom et al., 2002). Also, caproic acid produced, among other acids, by *L. sanfranciscensis* CB1 plays a key role in inhibiting mould growth (Corsetti et al., 1998).

In addition to using selected natural strains, genetically engineered microorganisms may find applications. The heterologous production of bacteriocins is well known (Rodrı guez et al., 2002). A *Lb. curvatus* strain, harbouring a gene for the expression of the lytic enzyme lysostaphin, was shown to produce it in sufficient quantities to inactivate *S. aureus* during sausage fermentation (Cavadini, Hertel, and Hammes, 1998).

9.2 Functional starters for a more appealing product improvement of texture

To give a desired texture and mouthfeel to yoghurt, skim-milk powder or whey is frequently added to the milk. Although the consumer does not consider this as unnatural, it represents an extra cost for the producer. In some countries, however, gelatin or plant (e.g., starch, pectin, guar gum, and alginate) and microbial polysaccharides (e.g., xanthan and gellan) are added. Polysaccharides increase the viscosity and firmness, improve the texture, reduce susceptibility to syneresis, and contribute to the mouthfeel of low-fat products. Some polysaccharides, e.g., plant carbohydrates, xanthan and gellan, have the additional advantage of being suitable to chemical modification to improve their rheological properties (Harvey and McNeil, 1998). However, the modified molecules are perceived by the consumer as unnatural and the food regulation of several European countries forbids the use of additives in, for instance, yoghurt.

Recently, the in situ production of natural texture improving sugar polymers (exopolysaccharides) produced by LAB for the manufacturing of yoghurts (De Vuyst and Degeest, 1999, De Vuyst et al., 2001), of sour cream and whipped toppings (Duboc and Mollet, 2001), of ice cream (Christiansen, Madeira, and Edelsten, 1999), and of low-fat Mozzarella (Broadbent et al., 2001; Low et al., 1998) is being explored. Functional, exopolysaccharide-producing starters of *L. delbrueckii* subsp. *bulgaricus* or *S. thermophilus* are promising. It is not likely that these exopolysaccharides will be utilized in other areas than the dairy industry since they would have to compete with established gums (Harvey and McNeil, 1998). However, they can be produced directly in the food matrix through the in situ use of functional starter cultures (De Vuyst et al., 2001; De Vuyst and Marshall, 2001). Another application can be found in the bakery industry for a beneficial effect on bread

volume and staling (Tieking et al., 2003). Current research is investigating the biodiversity of exopolysaccharides produced by LAB from artisan yoghurts, fermented milks, vegetables, and cereals, the conditions for optimal production, and their technological implementation in the industrial production of fermented foods (De Vuyst and Degeest, 1999; De Vuyst et al., 2001; Marshall et al., 2001).

Another example of texture improvement of foods through functional starter cultures is the use of amylase producing LAB. LAB producing thermostable amylases have potential in cereal fermentations, in particular in sourdough technology for the natural inhibition of staling in bread (Mogensen, 1993).

9.3 Production of aroma and flavour

LAB contribute to the aroma and flavour of fermented products. They acidify the food, resulting in a tangy lactic acid taste, frequently exert proteolytic and lipolytic activities, and produce aromatic compounds from, for instance, amino acids upon further bioconversion (van Kranenburg et al., 2002; Williams, Noble, and Banks, 2001; Yvon and Rijnen, 2001). Control over the activities of peptidases from LAB is a key target of cheese ripening technology (Law, 2001). As an example, overexpression of certain peptidases of *L. lactis* subsp. *cremoris* improved the sensory quality of cheese (Guldfeldt et al., 2001).

Wild strain starter cultures and NSLAB play an important role in flavour formation because they have a high biosynthetic capacity and produce interesting aromatic compounds (Ayad et al., 1999; Bouton, Guyot, and Grappin, 1998; Weerkamp et al., 1996). For instance, Italian ewe milk cheeses are characterised by a very heterogeneous NSLAB flora which is influenced by geographical and technological factors, and which could be responsible for cheese diversity (De Angelis et al., 2001). Because such strains offer an important base for product innovation, research is going on to study their application in the food fermentation industry. The addition of NSLAB as adjunct cultures for cheese manufacturing increases the level of free amino acids, peptides, and free fatty acids, leading to flavour intensity and accelerated cheese ripening (Crow, Curry, and Hayes, 2001). Furthermore, they help to reproduce the flavour of raw milk cheeses when pasteurised milk is used (De Angelis et al., 2001).

Homofermentative LAB convert the available energy source (sugar) almost completely into lactic acid via pyruvate to produce energy and to equilibrate the redox balance. However, pyruvate can lead to the generation of many other metabolites such as acetate, ethanol, diacetyl, and acetaldehyde. In this way, LAB produce volatile substances that contribute to the typical flavour of certain fermented products, such as sourdough (determined by the lactate/acetate ratio), kefir and koumiss (ethanol), butter and buttermilk (diacetyl), and yoghurt (acetaldehyde). Optimal fermentation control leads to improved production of some of these volatiles whereas metabolic engineering focuses on the steering of the metabolic flux in a well-defined direction. Strategies aiming at a direct modification of the redox balance have led to overproduction of the desired metabolites mentioned above (Kleerebezem, Hols, and Hugenholtz, 2000). Examples include the enhancement of diacetyl production by *L. lactis* subsp. *lactis* biovar. *diacetylactis* in buttermilk by redirection of pyruvate catabolism (Henriksen et al., 1999; Hugenholtz et al., 2000), and metabolic engineering of acetaldehyde production by *S. thermophilus* in fermented dairy products (Chaves et al., 2002).

Alternatively, the introduction of novel enzymatic activities into LAB may lead to cells that produce interesting metabolites from the supplemented sugar. Overproduction of alanine dehydrogenase in suitable *L. lactis* cells has lead to a homofermentative, stereospecific production of L-alanine from pyruvate (Hols et al., 1999). L-alanine is used as a sweetener in the food industry and its in situ production can lead to dairy products with an intrinsic sweetness.

9.4 Functional starters with a health advantage

Production of nutraceuticals

Nutraceuticals are food components that, through specific physiological action, contribute to the health of the consumer (Andlauer and Furst, 2002). Several nutraceuticals from bacterial origin have been added to food products (Hugenholtz et al., 2002). Through strain selection and process optimisation, the activity of LAB can be modified to increase the content of nutraceuticals in fermented foods such as fermented dairy products. As an example, fermented milks can be produced with LAB starter strains that produce high amounts of low-calorie polyols so as to reduce the sugar content (Wisselink et al., 2002).

Also, the use of oligosaccharide-producing LAB that produce sugar polymers with a controlled structure and chain length (and hence molecular mass) may yield fermented products with health applications (Ruas-Madiedo, Hugenholtz, and Zoon, 2002). Health effects of such oligosaccharides are ascribed to their low-calorie character, their fibre-like nature, and their bifidogenic effect (Voragen, 1998). Certain LAB, such as the yoghurt bacteria *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, are able to produce vitamins such as folate. A controlled use of these bacteria may lead to dairy products with increased folate content (Crittenden, Martinez, and Playne, 2002; Hugenholtz and Kleerebezem, 1999; Lin and Young, 2000a,b).

The proteolytic system of LAB can contribute to the liberation of health-enhancing bioactive peptides from milk (Meisel and Bockelmann, 1999; Wouters et al., 2002). The latter may improve absorption in the intestinal tract, stimulate the immune system, exert antihypertensive or antithrombotic effects, display antimicrobial activity, or function as carriers for minerals, especially calcium.

9.5 Reduction of toxic or antinutritive factors

The fermentative action of specific LAB stains may lead to the removal of toxic or antinutritive factors, such as lactose and galactose from fermented milks to prevent lactose intolerance and accumulation of galactose (Wouters et al., 2002). Other examples are the removal of raffinose, stachyose, and verbadose from soy to prevent flatulence and intestinal cramps (Holzapfel, 1997, 2002; Hou, Yu and Chou, 2000; Scalabrini et al., 1998), proteinase inhibitors from legumes and cereals to prevent maldigestion (Holzapfel, 2002), phytic acid and tannins from cereals and legumes to increase mineral bioavailability (Holzapfel, 1997, 2002; Sharma and Kapoor, 1996), and natural toxins such as cyanogenic glucosides from cassava (Holzapfel, 2002; Kimaryo, Massawe, Olasupod, and Holzapfel, 2000) as well as biogenic amines from traditional fermented foods (Holzapfel, 2002).

10. Conclusions and Future Trends

LAB have been used for dry sausage manufacturing process since 1950s in order to ensure the safety and quality of the end product. Furthermore, by selecting bacteriocin and low-molecular-mass antimicrobial compounds producing strains for dry sausage fermenting process the risk for low numbers of *L. monocytogenes* or *E. coli* O157:H7 derived from raw dry sausage material may be further reduced. As discussed by Ganzle et al. (1999a) bioprotective LAB derived from food may also be useful in the small intestine against food pathogens—as long as they are able to survive the environment of gastrointestinal tract. Likewise, probiotic strains with antimicrobial effects on food act similarly and therefore might be more successful than commonly used food fermenting bacteria. It could be concluded that dry sausage is suitable carrier for probiotic and bioprotective bacteria. However, human clinical studies are needed before the final answer concerning the health promoting effects of probiotic dry sausage.

Moreover, a great number of mostly molecular techniques are available for the identification of LAB, for instance in industrial processes and food products. For each specific type of research or analysis, a well-considered choice has to be made of the methodology to be applied, in relation to taxonomic resolution, workload and cost. It is important to realize that every technique cannot be used for any purpose. For instance, RFLP and PFGE are only suitable for typing purposes, but will generally not yield a species identification. In the course of safety assessments, it is crucial to use multiple techniques working on the strain level in order to obtain a detailed fingerprint of individual isolates. However, most situations only demand identification to the species level, for which techniques such as biochemical characterization, 16S rDNA sequencing or DNA–DNA hybridisation are used. If species-specific primers or probes are available, these offer a very fast way to detect the target organism(s). For routine quality control, commercial easy-to-perform phenotypic tests may provide satisfying results if performed under standardized conditions.

Analyses of food fermentations involving pure cultures will benefit from the use of culture-dependent techniques, although most food fermentations in fact involve more complex bacterial communities. At present, DGGE is one of the few techniques

allowing a fast microbial analysis of these communities. Undesirable changes in the bacterial population caused by fluctuation of ingredients or false fermentation conditions can be rapidly detected using PCR-DGGE, facilitating subsequent adjustments by means of technological measures. However, at the moment quantitative data can only be obtained using culture-dependent methods, which are also required for live/dead discrimination at the cellular level. Current research is focussed on the further optimisation of the DGGE technique, including the coupling of DGGE to real-time and Reverse Transcriptase PCR, which may allow the culture independent quantitative analysis of investigated microbial communities.

An additional area of interest for the near future is the evaluation of new techniques for (routine) analyses of LAB (food) samples. The rapid evolution in total genome sequencing may open new possibilities for identification or detection, when implemented in microarray technology (Wang et al., 2002). Also, a number of non-genomic whole-cell analysis techniques with high discriminatory power are in full expansion, such as those based on mass spectra (Reid et al., 2002).

It is clear from the above discussion that a careful consideration of numerous factors has to be made in order to find the most suitable technique for a certain purpose. Some studies will benefit optimally from the use of a fast, inexpensive, though less discriminating method, whereas other applications will require multiple high-resolution techniques. Although in the future high-tech approaches will make some of the currently used methods redundant, it is expected that both culture-dependent and culture-independent approaches will remain necessary in many studies targeting the speciation or fingerprinting of LAB.