MOLECULAR TECHNIQUES FOR THE IDENTIFICATION AND DETECTION OF MICROORGANISMS RELEVANT FOR THE FOOD INDUSTRY

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STELLINGEN

1 Om te kunnen concluderen dat er 16S rRNA genen met verschillende sequenties in één bacteriesoort voorkomen, moet in ieder geval worden aangetoond dat de verschillende 16S rRNA's in één cel aanwezig zijn.

B. Pettersson, F. Lembke, P. Hammer, E. Stackebrandt and F.G. Priest. (1996) Bacillus sporothermodurans, a new species producing highly heat-resistant endospores. Internat. J. System. Bacteriol. 40: (in press); R. Amann, J. Snaidr, M. Wagner, W. Ludwig, and K-H. Schleifer. (1996) In situ visualisation of high genetic diversity in a natural microbial community. J. Bacteriol. 178: 3496-3500.

2 Gezien het veelvuldig voorkomen in microorganismen van insertie-elementen, transposons en plasmiden zal het vast leggen van de biodiversiteit van een microbiële gemeenschap slechts een moment opname zijn.

W. Arber, T. Naas and M. Blot. (1994) Generation of genetic diversity by DNA rearrangements in resting bacteria. FEMS Micobiol. Ecol. 15: 5-14.

3 Een bacterieculture wordt beschouwd als een reinculture zolang het tegendeel niet kan worden aangetoond met de huidig beschikbare analysemethoden.

4 Wanneer de enorme diversiteit aan kaassoorten met hun specifieke smaken en texturen slechts een afspiegeling is van de diversiteit aan gebruikte starterbacteriën, dan is er nog veel werk te doen voor een zuivelmicrobioloog.

5 Producenten van UHT-melk die de steriliteit van het produkt alleen controleren op basis van veranderingen in de pH zullen minder produktuitval hebben dan producenten die steriliteit controleren aan de hand van ATP-gehaltes.

6 Hoewel Metchnikoff in 1908 constateerde dat de feministische beweging nog niet had geleid tot het ontstaan van onvruchtbare werksters, gezien het feit dat vele "geleerde dames" weldegelijk kinderwensen koesterden, blijkt anno 1996 echter dat de emancipatie van de vrouw nog steeds voor een groot deel afhangt van de mate waarin de vrouw haar vruchtbaarheid kan en mag controleren.

E. Metchnikoff. (1908) The prolongation of life. G.P. Putham's Sons, London.

7 Wanneer men de fylogenetische bomen van Haeckel (1866) en Woese (1994) vergelijkt, dan kan men constateren dat ook deze bomen evolutioniare veranderingen ondergaan.

E. Haeckel. (1866) Generelle Morphologie der Organismen. Verlag Georg Reimer, Berlin.
C.R. Woese. (1994) There must be a prokaryote somewhere: Microbiology's search for itself. Microbiol. Rev. 58: 1-9.

8 Om de invloed te bepalen van monsterbehandeling, DNA extractie, PCR primers en de wijze van klonering op de samenstelling van gekloneerde 16S rRNA genen vanuit omgevingsmonsters moet de sequentie worden bepaald van praktisch onhaalbare aantallen gekloneerde 16S rRNA's om statistisch verantwoorde uitspraken te kunnen doen.

P.A. Rochelle, B.A. Cragg, J.C. Fry, R.J. Parkes and A.J. Weightman. (1994) Effect of sample handling on estimation of bacterial diversisty in marine sediments by 16S rRNA gene analysis. FEMS Microbiol. Ecol. 15: 215-226.

9 In de microbiële ecologie is het essentieel om verschillende detectie en identificatie methoden naast elkaar te gebruiken om door complementatie van deze methoden met ieder hun eigen limitering een zo compleet mogelijke weergave van de werkelijkheid te verkrijgen.

R.I. Amann, W. Ludwig and K.H. Schleifer. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59: 143-169; R. Amann, J. Snaidr, M. Wagner, W. Ludwig, and K-H. Schleifer. (1996) In situ visualisation of high genetic diversity in a natural microbial community. J. Bacteriol. 178: 3496-3500.

10 Indien alle getrouwde vrouwen besluiten hetzij hun geboorte naam of de naam van hun echtgenoot te gebruiken, dan zal het aantal namen in literatuur referenties aanzienlijk afnemen.

Stellingen behorende bij het proefschrift:

Molecular techniques for the identification and detection of microorganisms relevant for the food industry

Nicolette Klijn, Wageningen 20 September 1996

Contents

Chapter 1

General introduction

General introduction

In the very early days man learned which of nature's products to eat and which not to eat. As a farmer he learned to grow, harvest, process and store products so as to provide food from one season to the next. He discovered the value of drying, freezing, smoking, curing, fermenting and acidifying as means of preserving his food supply. He also found out that those preserving methods could add specific organoleptic properties to the foods, providing welcome variety in his diet. During the past centuries the means of food preservation were constantly improved and varied, which led to the current diversity of food, in particular fermented products, like beer, wine, bread and cheese.

The introduction of commercially processed foods during the last century, notably that of sterilized canned food, allowed perishable foods to be distributed and stored safely for long periods. At that time also evidence was obtained that such mass-produced foods could cause illness and even death. This led to the recognition of the need to establish a means of assuring safety of food products. In particular, the incidence of botulism acquired from improperly canned foods led to extensive research, mainly focused on the microbiological aspects of the production process. Pioneering work by Espy and Meter (1922) led to the development of a microbiologically safe process based on the recognition of the heat resistance of *Clostridium botulinum* spores (39). Ever since, the food industry has increasingly recognized the need to base microbiological safety and quality of foods on the fundamental knowledge of the microbiology of the raw materials, the production process and the end product, resulting in the recently developed concept of Hazard Analysis of Critical Control Points (7).

Although microbiological knowledge was in the first place needed to prevent food products from spoilage and to avoid contamination with pathogens, it also appeared essential for understanding the behaviour of microorganisms that were used as inoculants in food production in order to obtain a desired fermentation process.

Although centuries of traditional cheesemaking had already led to a wide diversity of successfully produced cheeses, the first evidence for the role of bacteria in the fermentation of milk was provided by Joseph Lister (1873), in his attempts to prove Pasteur's germ theory of fermentation. His experiments resulted in the isolation of the first pure culture of a lactic acid bacterium, *Bacterium lactis* (90), now known as *Lactococcus lactis*. This illustrates not only the importance of microbiology for food production but also the significant role of fermented food products in the early development of microbiology itself. Later on, research into microbial fermentation of foods was continued, leading to the development of industrial starter cultures, which allowed for the large-scale production of fermented foods.

For the production of safe and high-quality foods the specific detection and identification of microorganisms play an important role. Conventional methods for the detection and enumeration of specific bacteria are based on their growth on a selective medium containing defined nitrogen and carbon or other energy sources. Up to the last decade, identification of micro-organisms was largely based on phenotypic and biochemical characterization of pure cultures (13). Although these methods were in general sufficiently precise to distinguish certain groups of bacteria, they did not allow the discrimination of species or strains and the determination of the phylogenetic relationship among certain groups of bacteria. Furthermore, the results obtained by these methods were not always reproducible, due to the variability of phenotypic properties in relation to culture conditions. Therefore, as genotypic properties are very stable,

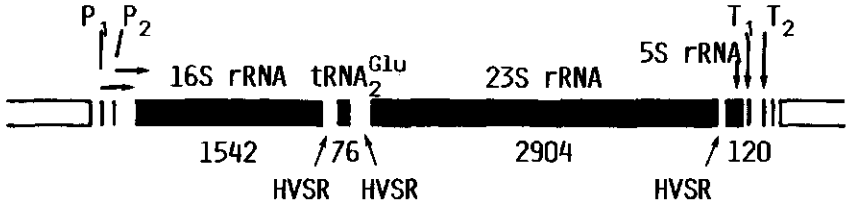

Figure 1

Schematic representation of the ribosomal RNA operon of *E. coli* as described by Brosius *et al.*, 1981. P_1 and P_2 are the rRNA promotors. Numbers under the bars indicate the lengths (in bp) of the indicated regions, genes and spacers. T_1 and T_2 are terminators.

nucleic-acid based methods have been applied in the identification of bacteria and discrimination between species since the scientific and technical tools became available.

The first application of nucleic acids for the determination of the relatedness of microorganisms was in DNA-DNA hybridizations. Due to their unique physical properties complementary DNA strands have the ability to reassociate after being separated by denaturation. When recombining nucleic acids from different organisms, the reassociation values provide an average measurement of nucleotide sequence similarity (67). Particularly in the case of problematic phenotypic differentiation, DNA-DNA hybridizations proved to be essential in obtaining insight into inter- and intra-species relationships. For instance, based on DNA-DNA hybridizations it was established by Johnson *et al.*, 1980, that all lactobacilli belonging to the "species" *Lactobacillus acidophilus* could actually be differentiated into six different groups (68). Each group represented a separate species and those were later renamed as *L. acidophilus, L. amylovorus, L. gasseri, L. johnsonii, L. gallinarum* and *L. crispatus* (45, 46).

Although DNA-DNA hybridization can give very useful information on relatively closely related strains, this technique cannot provide information on the relatedness of certain groups of species or genera. This is only possible by using structural differences between macromolecules such as nucleic acids and proteins as a parameter for evolutionary distance. Already in 1965 Zuckerkandl and Pauling postulated that the comparison of macromolecular sequences could be used to determine the full range of phylogenetic relationships, including bacteria (163). In order to be used as a evolutionary chronometer, a macromolecule had to be universally distributed among bacteria and to fulfil an identical function. Furthermore, the sequence of the molecule chosen should change at a rate corresponding to the evolutionary distance measured. This means that the rate of mutation must not be too high, since this would lead to a complete randomization of the sequence, making it impossible to align sequences of related molecules.

Since the development of efficient methods to determine the sequence of nucleic acids (Maxam and Gilbert, 1977 (98), and Sanger, 1977 (124)) much information has been obtained which drastically altered the current concepts of evolution, in particular for microbiology. Woese determined in 1983 the higher-order structure of 16S-like ribosomal ribonucleic acids by comparing their primary and secondary structure for a number of organisms, including plants, chloroplasts, mitochondria and bacteria (157). This provided the first basis for molecular taxonomy, giving new insights into proposed

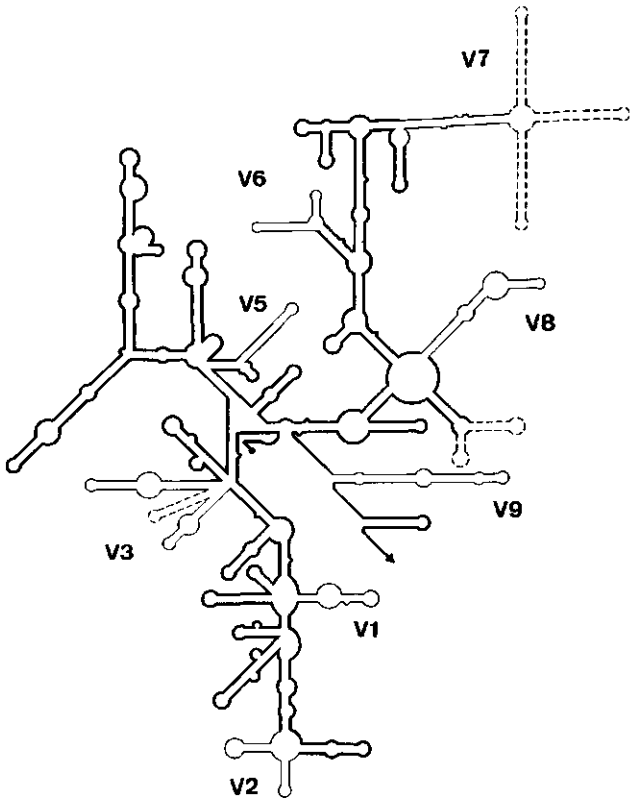

Figure 2

Representation of the secondary structure of the 16S rRNA. The bold lines indicate the conserved regions and the thin lines indicate the variable regions. Adapted from Neefs *et al.*, 1990 (102).

phylogenetic associations (158). Ribosomal sequences appeared to be good evolutionary chronometers, especially because of their universal distribution and essential function in all living organisms.

Ribosomal RNA sequences

The genotype is expressed into the phenotype by the processes transcription and translation. High accuracy of translation is a prerequisite for the functionality of enzymes because of their complexity and specificity. In a ribosome, a complex framework of ribonucleic acids and proteins, messenger-RNA is translated into protein. The two subunits of prokaryotic ribosomes, 30S and 50S, contain, besides approximately 50 proteins, three ribonucleic acids: the 5S rRNA (120 nucleotides), the 16S rRNA (1540 nucleotides) and the 23S rRNA (2900 nucleotides). These rRNAs are encoded by an operon, which was first characterized in *Escherichia coli* and appeared to contain a

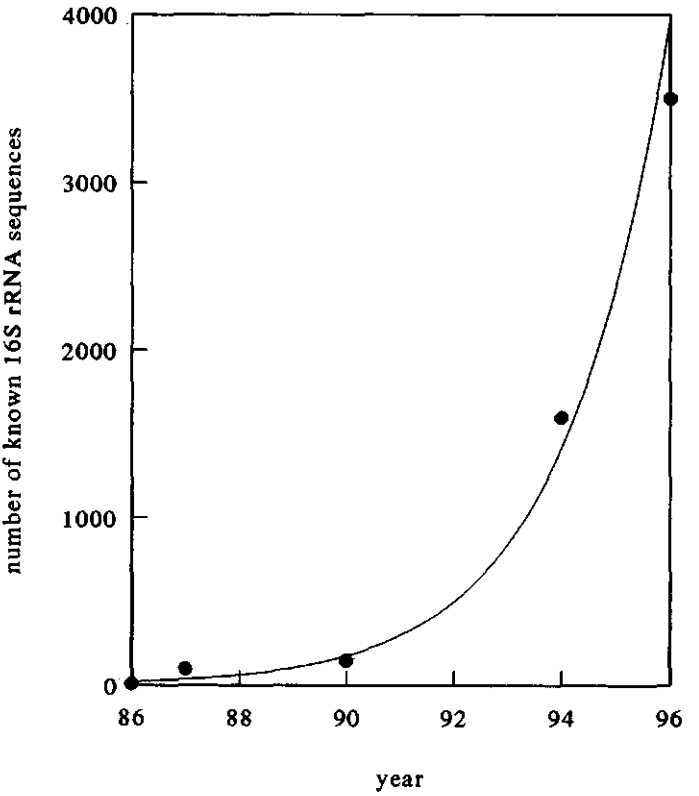

Figure 3

Increase of the number of 16S rRNA genes (only from prokaryotes) sequenced since 1987. Data obtained from references 44, 102, 52 and the RPD database from Woese.

$tRNA^{glu}$ gene in between the 16S and 23S rRNA genes (Brosius *et al.*, 1981 (20)) (Fig. 1). Later it was demonstrated (Wagner, 1994) that *E. coli* contains seven copies of the operon located at different chromosomal positions (143). This operon organization is generally found in all prokaryotes although there are variations in number and identity of the tRNA and also the sequential order of the rRNAs. Most organisms carry more than one copy of the operon in their chromosome, but they usually all contain the same ribosomal sequences. However, in some organisms the presence of two different 16S rRNA sequences has been demonstrated. These differences vary from only a few bases as for *Mycobacterium gordonae* (71), to up to 74 base differences for *Haloarcula marismortui* (101). Occasionally intervening sequences are found within the rRNA sequences (89).

In the first proposed secondary structure of 16S-like rRNAs (157), a number of helical elements (± 50) and the main functional domains were described, but it was not until 1991 that Hubbard and Hearst, with the aid of sophisticated computer software and the first X-ray crystal structures of the 30S ribosomal subunit, presented models of the three-dimensional structure of the 16S rRNA (63). Nevertheless, already in 1987, Woese was able to identify the functionally most important and thereby most conserved regions in the 16S rRNA structure based on comparative sequence analysis of various 16S and

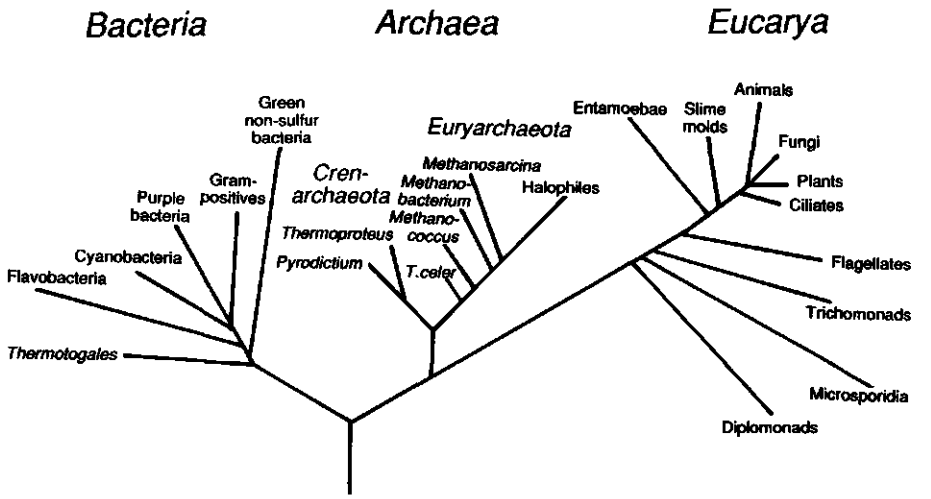

Figure 4

Universal phylogenetic tree in rooted form, showing the three domains, *Archaea, Bacteria* and *Eucarya*. Taken from Woese, 1994 (159).

18S rRNAs sequences (158) (see Fig. 2). Due to the fact that ribonucleic acids consist of alternating conserved and more variable regions, specified by Neefs *et al.* in 1990 (102) for the 16S rRNA and by Hopfl *et al.*, 1989, for the 23S rRNA (61), they are uniquely suited as molecular chronometers. Due to their high degree of functional consistency, the mutation rate of rRNAs corresponds with the evolutionary distance, a prerequisite for good evolutionary chronometers. Furthermore, since ribonucleic acids could be directly sequenced by using the enzyme reverse transcriptase (83), laborious cloning protocols could be omitted. A relatively large number of 16S rRNAs could be sequenced by one laboratory within a reasonable time. This led to the availability of databases that were sufficiently large to perform the first phylogenetic studies. Since the development of the Polymerase Chain Reaction (PCR;121), rRNA sequences can directly be obtained from lysed cell cultures (16), which is reflected in the exponential increase in known 16S rRNA and 23S rRNA sequences in recent years (see Fig. 3).

Comparative analysis of the sequences of 16S and 23S rRNAs by taxonomists has led to the construction of phylogenetic trees, illustrating the evolutionary relationship between organisms (53). This has recently provided new and revolutionary insights in the development of cellular life (108). Phylogenetic trees based on comparative alignments of 16S rRNAs suggest the existence of more than the two previously formulated kingdoms, the eukaryotes and the prokaryotes. Woese *et al.* postulated in 1990 the existence of three domains (160), namely the Eukarya, containing all former eukaryotes; the Archaea, previously known as Archaebacteria and consisting of the Euryarcheota and the Crenarchaeota; and the Bacteria, consisting of all other prokaryotes (see Fig. 4). This phylogenetic tree illustrates the enormous biodiversity within the prokaryotes. Although ribonucleic acid sequence comparison has resulted in completely new insights into microbial evolution, it has also demonstrated the limitations of our

knowledge. All sequence data used in these studies are derived from culturable microorganisms, but the application of direct 16S rRNA amplification, cloning and sequencing from natural habitats has indicated that most micro-organisms present in the environment have not yet been characterized (147).

Ribosomal RNA sequences in relation to the conventional identification and detection of microorganisms

Problems associated with culture-based methods

Up to a few years ago the successfulness of a microbiologist strongly depended on his or her ability to cultivate microorganisms. Viable plate counts and most-probable number techniques were the only means for the quantification of active microbial cells in specific environments. For the identification of single isolates it was necessary to obtain pure cultures, followed by testing of a range of physiological and biochemical traits. Although it was possible to classify microorganisms based on these phenotypic properties (see *Bergey's manual* (13), a large number of organisms did not fit in this taxonomic frame.

Much research has been devoted to the optimization of culture methods, such as adaptation of incubation temperature and time or modification of the medium components (from high concentration of nutrients to very low concentration of nutrients (21)). However, large discrepancies have still been observed between the total plate counts and direct *in situ* counts of bacteria by microscopical techniques (54), varying from only 0.1% culturable microorganisms in seawater to 1-15% in activated sludge (5).

Although a large proportion of the microorganisms in the environment is not currently culturable, there are also other factors which can influence the recovery of viable cells from an environment. Even microorganisms which can be cultured very easily in the laboratory can, under specific environmental conditions, become non-culturable, although they are still viable. This was very elegantly demonstrated by Colwell *et al.*, 1985, for *E. coli, Salmonella typhimurium* and *Vibrio* spp. (30, 112).

Because of the above-mentioned problems with classical culture methods, alternative techniques were necessary to allow the study of microorganisms in their natural environments. The first alternative developed was the specific detection of micro-organisms using fluorescent antibodies (31). Although this was a step forward, especially for the detection and identification of bacteria that are difficult to culture like methanogenic bacteria and nitrifiers in soil and marine habitats (146), several factors have limited the success of this approach. Problems such as cross-reactivity, non-specificity of the antibody, the necessity for expression of the antigen-coding genes and the stability of the antigen under environmental conditions hampered the routine use of these methods. In order to obtain more reproducible results, detection methods were developed based on genotypic properties, such as specific DNA or RNA sequences (9).

Role of ribosomal RNA sequences in the identification of microorganisms

Despite the fact that taxonomists used 16S rRNA sequences for the construction of phylogenetic trees, it took several years before these sequences were developed into targets for specific DNA probes that could be used for the identification and discrimination of microorganisms (48, 1). The unique structure of the rRNAs, with highly

Table 1: Variable regions of the 16S rRNA containing specific sequences for some important food-associated micro-organisms.

Genus	Region containing genus-specific sequences	Region containing species-specific sequences	references
Lactobacillus	n.a.	V1,V2 and V3	72
Leuconostoc	V1	V3	6
Lactococcus	V2	V1	6
Streptococcus	V1-V2	V2	72
Bifidobacterium	V6	V2 and V3	161
Clostridium	V1-V2	V2 and V6	74
Bacillus	n.a.	V3	72

n.a: not available

conserved functional regions alternating with more variable regions, makes them extremely suitable for the identification of microbial species and ideal targets for specific DNA probes. By comparative sequence analysis of 16S rRNAs of all species within a genus, variable regions can be identified which contain species-specific information (see Table 1). By following the same strategy, genus-specific sequences can also be identified. Both primary and secondary structures contain genus- or group-specific signatures (157). Based on these specific sequences many DNA probes with different levels of specificity have been designed (5). In Table 2 a collection is shown of DNA probes based on 16S rRNA sequences for microorganisms important for food production, including those that are used in research on the optimization of food safety and quality.

A number of researchers have devoted their work to sequencing the 16S rRNA of the type strains of species belonging to relevant genera like *Bacillus* (6, 118), *Lactococcus* (26), *Lactobacillus* (29), *Leuconostoc* (27, 162), and *Clostridium* (28). The data obtained often led to the description of new species, or even new genera. For instance the analysis of the 16S rRNA sequences of all isolates belonging to the genus *Lactococcus* (26), including some motile isolates (125), revealed the existence of a closely related but definitely different genus, *Vagococcus*, containing motile streptococcal isolates from fresh water and diseased fish. During this study also a new species was added to the genus *Lactococcus*, namely *L. piscium* (153).

Already in 1989, during the first phylogenetic studies based on 16S rRNA sequences, it was demonstrated that the species in the genus *Leuconostoc* belonged to at least three distinct branches (162). One branch, including *Leuconostoc paramesenteroides* and some species formerly called *Lactobacillus confusus, L. halotolerans, L. kandleri, L. minor* and *L. viridescens*, has been reassigned to a new genus called *Weissella* by Collins *et al.* (1993). This genus also includes new isolates obtained from fermented sausages (27). Even more recently, Dicks *et al.*, 1995, have proposed reclassifying *Leuconostoc oenos* as *Oenococcus oeni*, since this species forms a very separate and distinct lineage within the *Leuconostoc* and *Weissella* branches (see Figure 5) (35).

Table 2. Published 16S rRNA-based DNA probes for species important to food microbiology.

Specificity	Target position	Appl	Sequence (5'-3') probe	ref.
Genus-specific:				
Legionella spp.	16S, 705-722	IS	CTGGTGTTCCTTCCGATC	94
Lactococcus	16S, 233-212	H	CTTTGAGTGATGCAATTGCATC	122
Bifidobacterium	16S, 164-181	IS	CATCCGGCATTACCACCC	84
	16S, 662-679	IS	CCACCGTTACACCGGGAA	84
Acinetobacter	16S, 652-669	H,IS	ATCCTCTCCCATACTCTA	142
Salmonella	16S, 455-478	H	AACGACGCCAATAATTGGTGTTGT	88
	23S, 341-360	H	ACAGCACATGCGCTTTTGTG	116
Listeria	16S, 454-472	PCR	CAAGGATAAGAGTAACTGC	58
	16S, 1433-1451	PCR	AGGTT/GACCCTACCGACTTC	58
Brevibacterium	23S, 12-30[1]	IS	GGTTAGCATCACTGATTCA	113
Leuconostoc	16S, 73-94	H	CACCTTTCGCTGTGGTT	110
Species-specific:				
Bacillus polymyxa	16S, 172-193	IS	TCCCATGCAGGAAAAAGGATGTATCGGGTAT	69
Bacillus macerans	16S, 1235-1269	IS	CTCCATATCACTACTTCGCTTCCCGTTGTA	69
Campylobacter fetus	16S, 1017-1044	H	CTCAACTTTCTAGCAAGCTAGCACTCTCT	150
Campylobacter hyointesinalis	16S, 1017-1044	H	CACTAATTTCTTGTAAACAAGCACTATCT	150
Campylobacter jejuni subsp. *jejuni*	23S, ± 1700	PCR	TCGAAACATAATCCTAAATG	40
Campylobacter lari	23S, ± 1700	PCR	CTCTTAACGACTACGGCA	40
Campylobacter upsaliensis	23S, ± 1700	PCR	AAAGTAAGTACCGAAGCTT	40
Carnobacterium divergens	16S, n.a.	H	GCGACCATGCGGTCACTTGAAA	19
Carnobacterium mobile	16S, n.a.	H	TCCACCAGGAGGTGGTGGAGT	19
Carnobacterium piscicola/gallinarum	16S, n.a.	H	ATGCATTCCTCAAACTA	19
Corynebacterium glutamicum	23S, 23-40[1]	IS	TTATGGGTGGTTAGTATC	113
Clostridium perfringens	16S, 184-106	PCR	AAAGATGGCATCATCATTCAAC	144
	16S, 440-462	PCR	TACCGTCATTATCTTCCCCAAA	144
Clostridium botulinum type A	23S, 141-160	H	TCAGTGCATGATACATGGGG	115
Clostridium botulinum type D	23S, 139-158	H	CGGTATACAGTATCTAGCAT	115
Clostridium butyricum	16S, ± 1000-1050	H	CTCCATTACAGATAATTCAGGAG	74
	16S, ± 1000-1050	PCR	GTGGCTTGCTCCATTACAGAGTAA	78
Clostridium beijerinckii	16S, ± 1000-1050	H	CCCCATTAAGGGTATTCAGGAG	74
	16S, ± 1000-1050	PCR	CTTCCCCGATTAAGGGTAATTCAG	78
Clostridium acetobutylicum	16S, ± 1000-1050	H	GACTTCATCCATTACGGACTAA	74
	16S, ± 1000-1050	PCR	GGACTTCATCCATTACGGACTAAC	78
Clostridium tyrobutyricum	16S, ± 1000-1050	H	CGCCTATCTCTAGGTTATTCAGGG	74
	16S, ± 1000-1050	PCR	CGCCTATCTCTAGGTTATTCAG	78
Clostridium sporogenes	16S, ± 1000-1050	H	ACCTATCTCTAGGCTATGCAAGGG	74
	16S, ± 1000-1050	PCR	CACCTATCTCTAGGCTATGCAA	78

Table 2 continued.

Specificity	Target position	Appl	Sequence (5'-3') probe	ref.
Enterococcus feacalis	23S, 343-360	IS	GGTGTTGTTAGCATTTCG	10
Enterococcus faecium	23S, 142-158	IS	CACACAATCGTAACATCC	10
Lactobacillus curvatus	23S, n.a.	H	ATGATAATACCCGACTAA	60
Lactobacillus pentosus/plantarum	23S, n.a.	H	TTAATGATAATACTCGATT	60
Lactobacillus sake	23S, n.a.	H	ATCTAGTGGTAACAGTTG	60
Lactobacillus acidophilus	23S, 1159-1180	H	TCTTTCGATGCATCCACA	111
Lactobacillus gasseri	23S, 1160-1178	H	TCCTTTGATATGCATCCA	111
Lactobacillus johnsonnii	23S, 1158-1179	H	ATAATATATGCATCCACAG	111
Lactococcus garvieae	16S, ± 70-90	H	CATAAAAATAGCAAGCTATC	110
Lactococcus lactis	23S, 271-289	IS	CTATAATGCTTAAGTCCACG	5
Lactococcus lactis subsp. *lactis*	16S, ± 70-90	H	AGTCGGTACAAGTACCAAC	110
Lactococcus lactis subsp. *cremoris*	16S, ± 70-90	H	TTCAAATTGGTGCAAGCACC	110
Lactococcus plantarum	16S, ± 70-90	H	CTACGGTACAAGTACCAGT	110
Lactococcus raffinolactis	16S, ± 70-90	H	CGGTGAAGCAAGCTTCGGT	110
Leuconostoc lactis	16S, ± 450-470	H	ATGCTAGAATAGGGAATGAT	110
Leuconostoc mesenteroides	16S, ± 450-470	H	CAGCTAGAATAGGAAATCAT	110
Listeria monocytogenes	16S, 1228-1246	PCR	CACGTGCTACAATGGATAG	145
	16S, 1277-1298	PCR	GATTAGGGTATTTTGATAAGA	145
Streptococcus parauberis	16S, 179-201	H	AGTACATGAGTACTTAATTGTCA	11
	23S, 302-325	H	GACGTGGGATCAAATACTATA	55
	23S, 378-398	H	TAGTAAATGACTCTAGCAGT	55
Streptococcus salivarius	23S, 1866-1883	IS	CATACCTTCGCTATTGCT	10
Streptococcus thermophilus	23S, 1866-1883	IS	CATGCCTTCGCTTACGCT	10
Streptococcus uberis	16S, 179-203	H	AGGGTACATGTGTACCCTATTGTCA	11
	23S, 302-325	H	GAAGTGGGACATAAAGTTAATA	55
	23S, 378-398	H	TTGACTTTAGCCCTAGCAGT	55

E. coli numbering (20) [1]: insertion numbering according to Roller *et al.*,1992 (114); n.a.: not available; H: applied in hybridization; IS: applied in *in situ* detection; PCR: applied in specific PCR amplification.

Recent literature shows many examples of renamed (87) and newly classified microorganisms (41). Some of these are relevant for food microbiologists, such as the definition of the new genus *Alicyclobacillus*, comprizing the former *Bacillus* spp. *acidocaldarius, acidoterrestis* and *cycloheptanicus* (154). All strains in these species are thermophilic and can grow at pH 2, which may give spoilage problems in moderately heat-treated acid food products. Such reclassifications illustrate the importance of 16S rRNA sequencing in relation to the identification of microorganisms.

16S rRNA sequencing as a taxonomic parameter

The basic taxonomic group in the systematic of classification of bacteria is the species. According to the species definition as formulated in the latest edition of

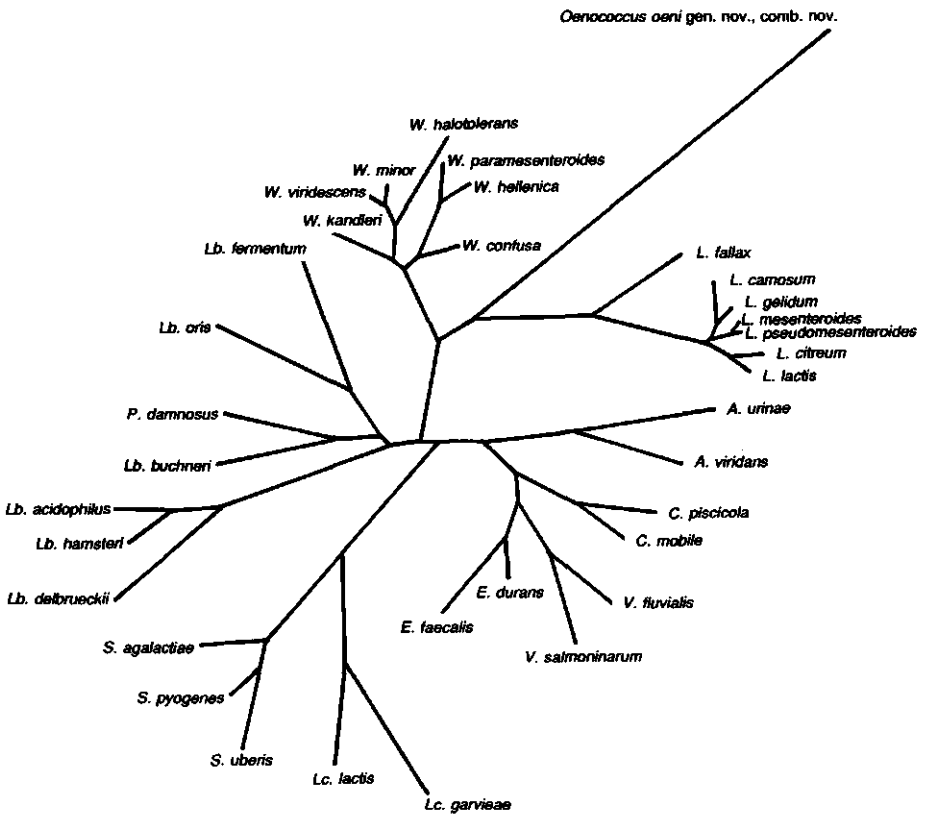

Figure 5

Unrooted phylogenetic tree showing the relationship of O. oeni to *Leuconostoc* spp. and "lactic acid bacteria" based on 16S rRNA sequences.
Abbreviations: *W., Weissella; Lb., Lactobacillus; L., Leuconostoc; P., Pediococcus; A., Aerococcus; C. Carnobacterium; E., Enterococcus; S., Streptococcus; V., Vagococcus; Lc., Lactococcus.* Taken from Dicks *et al.*, 1995 (35).

Bergey's Manual (13), a bacterial species may be regarded as a collection of strains that share many features and differ considerably from other strains. However, in 1987 the ad hoc Committee on Reconciliation of Approaches to Bacterial Systematics decided to define a species as a group of strains that show 70% or greater DNA-DNA reassociation (148). Therefore, in recent years DNA-DNA hybridizations have played an important role in the description of new species. In 1993 more than 75% of the newly proposed species in the *International Journal of Systematic Bacteriology* were defined on the basis of DNA reassociation studies (131).

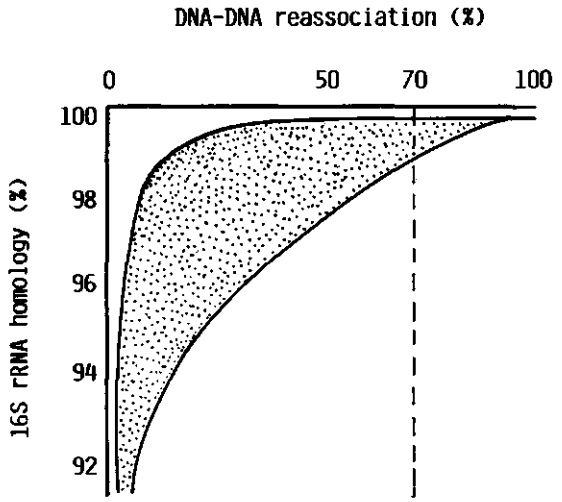

Figure 6
Comparison of 16S rRNA homology and DNA-DNA reassociation values. Adapted from Stackebrandt and Goebel, 1994 (131).

Because of the development of extremely rapid 16S rRNA sequencing protocols and the large databases, like the Ribosomal Data-base Project (617), rRNA sequences have also come to play a significant role in the description of species. Although for some groups of bacteria there is a good correlation between the percentage of DNA relatedness and the percentage of sequence homology between the 16S rRNA genes (147), there are also reports of lack of congruence between these two taxonomical parameters. In these exceptions it is always shown that strains with a low percentage of DNA relatedness share an almost identical 16S rRNA sequence. This was for instance demonstrated for *Rhizobium*, *Bradyrhizobium* and *Aeromonas* species (109, 97). However, when DNA-DNA hybridization percentages are plotted against percentages of 16S rRNA homology, it appears that there is no linear correlation between these two taxonomical parameters (see Fig. 6) (131). This indicates that a high homology of 16S rRNA sequence does not give complete certainty about DNA relatedness, implying that for establishing interspecies relationships DNA-DNA hybridization is still the only correct method. However, it has also been shown that in cases where the 16S rRNA homology was lower than 96%, the DNA reassociation percentage was always lower than 60% (Fig. 6). This indicates that isolates with similarity values below 96% of total 16S rRNA sequences should at least be regarded as distinct species.

Determination of type strains

Molecular identification techniques, like 16S rRNA sequencing and DNA-DNA hybridizations, have had a large impact on the identification of many species, in particular of those groups of bacteria that phenotypically could hardly be discriminated. Type strains are intended to be typical strains which can be compared with other strains

when classification or identification is undertaken. However, since type strains were historically chosen on the basis of phenotypic properties, some serious problems have occurred. For some species the type strain appeared rather exceptional. Collins *et al.* demonstrated that based on DNA-DNA hybridizations *Lactobacillus casei* isolates could be divided into three separate species sharing less than 20% DNA relatedness, *L. casei*, *L. paracasei* and *L. rhamnosus*, instead of the two known subspecies *L. casei* subsp. *casei* and *rhamnosus*. However, it appeared that the type strain *L. casei* subsp. *casei* ATCC 393 represented only a few of all isolates formerly known as *L. casei* subsp. *casei*. Therefore, later on Dellaglio *et al.* proposed designating *L. casei* subsp. *casei* ATCC 334 (named *L. paracasei* by Collins *et al.*, 1989) as the new type strain (34). Although 16S rRNA sequencing confirms the existence of three different groups (see RPD data base 91) the matter of the correct nomenclature is still under debate. This indicates that although molecular identification yields reliable data, it sometimes remains difficult to interpret the results and provide a correct identification.

Complex problems in relation to a correct identification also occur when questions arise with respect to the correct identity of the type strains used. Phylogenetic studies, in general, only involve the rRNA sequence of the type strain of a particular species. Unfortunately, it has been shown that in some culture collections type strains have been mixed up, especially those belonging to phenotypically closely related species. For example Wisotzkey *et al.* (154) describe a 16S rRNA sequence for *B. acidoterrestis* that differs in more than 250 positions from the sequence earlier published by Ash *et al.* (6) for the same strain, allegedly caused by a mislabelling of strains. A similar discrepancy was observed during the development of species-specific DNA probes for some *Clostridium* spp. (74). The published 16S rRNA sequence of *Clostridium acetobutylicum* NCIMB 8052 as determined by Collins *et al.* differed from the sequence found for a range of *C. acetobutylicum* strains, including the type strain *C. acetobutylicum* ATCC 824. The latter strain should be identical to NCIMB 8052 according to the culture collection data. These findings were later confirmed in a phylogenetic analysis of *C. acetobutylicum*, showing that 21 isolates could be separated into three distinct phylogenetic groups, one of which is identical to *C. beijerinckii* (70). This illustates that sequencing of the 16S rRNA of only one reference strain (usually the type strain) is not sufficient to obtain a reliable, representative sequence for a species, especially if this species is phenotypically difficult to discriminate from other species. So both the identity and the representativeness of a type strain should be thoroughly checked before its 16S rRNA sequence is used in phylogenetic studies or for the development of specific DNA probes.

Rapid and reliable identification of isolates

The determination of 16S rRNA sequences is a very useful tool for routine identification of microorganisms. The identification of specific isolates, such as spoilage organisms or isolates from environmental samples, by direct sequencing of partially amplified DNA proceeds very rapidly and is highly reliable. Efficient extraction techniques, requiring as little material as one colony on an agar plate, provide many advantages to this approach, such as a low risk of contamination and speed. However, the colony that is used for the identification should originate from a single cell. In practice problems may occur with regard to the purity of a culture, especially when organisms that form morphologically similar colonies, like *Lactococcus* spp., are studied.

Even three repeated single colony purifications do not guarantee that a pure culture is pure (13a). Colonies deriving from more than one cell could be obtained in the case of strong symbiotic or syntrophic relationships between two microorganisms, but also in case of physical attachments, such as mediated by capsular polysaccharides or flocculation and other aggregation proteins.

Another practical problem associated with the use of 16S rRNA sequences for the identification of microorganisms is the fact that, although more than 3000 ribosomal sequences are now available in computer data-bases (RPD, EMBL), it still frequently happens that the 16S rRNA sequence obtained from an unknown isolate does not correspond with one of the known 16S rRNA sequences. For instance, screening and identification of environmental isolates of *Lactococcus* spp. revealed the existence of a new and undescribed (sub)species based on differences in the 16S rRNA sequence (77).

In addition, during the identification of a *Bacillus* sp. forming ultra-heat-resistant spores and therefore causing spoilage problems in UHT-milk, a 16S rRNA sequence was found that did not correlate with the known 16S rRNA sequences of 52 *Bacillus* species present in the RPD-data base (91). The closest relative appeared to be *B. badius* but significant sequence differences were found in the first part of the 16S rRNA (75). This illustrates that besides the vast range of unknown unculturable microorganisms, also the extent of the biodiversity of all culturable organisms has not yet been established.

Molecular identification and detection methods based on ribosomal RNA sequences

Identification methods based on ribosomal RNA sequences

During recent years many identification and detection methods based on rRNA sequences have been developed. Several of these techniques will be described and discussed, using relevant examples from food or clinical research, or microbial ecology, to demonstrate their specific scope of application.

Ribotyping. Determination of Restriction Fragment Length Polymorphism (RFLP) of genes coding for ribosomal RNA has been used for taxonomic studies of many different bacteria, like *Lactobacillus, Salmonella, Listeria* and *Vibrio* (33, 32, 134, 109, 66). This method, referred to as ribotyping, has proven to be a useful tool to differentiate bacteria at the species or strain level. However, the diversity of the ribotyping patterns among strains within different taxa of the same hierarchical level can vary considerably (49). The method is based on the digestion of total chromosomal DNA by a specific restriction endonuclease, followed by electrophoretic separation of the fragments obtained, which are blotted to a membrane. After hybridization with a DNA probe, directed against an rRNA sequence, a specific band pattern is obtained. Depending on the restriction endonuclease used and the target specificity of the DNA probe the discriminating capacity of this method can vary from species to strain level (49). Especially in epidemiological studies this technique has frequently been applied (152, 134, 38). Using ribotyping Esteban *et al.*, 1993, could demonstrate, for example, contamination routes from one farm to another by comparing *Salmonella* strains found on the different farms (38).

Ribotyping has also been applied in ecological research. Based on ribotyping, Ståhl *et al.* (1994) obtained specific band patterns with *Lactobacillus reuteri* strains, isolated

from the intestine of rats, pigs and man, that correlated with the source of isolation (133). Although this technique has the major advantage that it can potentially discriminate down to the strain level, it requires pure cultures that can be grown to high densities in order to obtain sufficient chromosomal DNA that is suitable for enzymatic digestion (50). The necessary optimization of the procedure (restriction endonuclease, electrophoresis protocol, probe design) to obtain reproducible species- or strain-specific patterns requires a significant research effort. Therefore, the technique is suitable for laboratories characterizing large numbers of the same microorganisms, such as those involved in epidemiological or ecological studies. However, a complete automated system, the Riboprinter™ is now available, developed by Dupont (Wilmington, USA). With this system 32 samples per day can be analysed and if the organisms used are present in the data-base connected to the instrument reliable identifications can be obtained. This instrument may be useful for routine laboratories in the clinical or food area.

Analysis of the spacer regions. The operon coding for rRNAs (see Figure 1) contains, besides the coding regions also spacer regions, which show a large degree of sequence and size variation at the genus and species level. These hypervariable spacer regions not only vary between species, but differences were also found between the spacer regions from the different copies of the operon present in the chromosomal DNA (65). This diversity is partly due to variations in the number and type of tRNA sequences found within the spacers. The specific sequences found within these variable spacer regions can be used as target for specific DNA probes (8, 57). Moreover, amplification of spacer regions, using PCR primers based on conserved flanking sequences of the 16S rRNA and 23 rRNA genes (see Figure 1), results into amplification patterns that are strain-specific for a number of bacteria (65). This technique was used for the identification of clinical isolates of *Staphylococcus aureus* (Dolzani *et al.*, 1994, 37). The patterns obtained demonstrated that strains isolated from the blood of patients were identical to those present in the infected area, even when the samples were taken on different days. This allowed the monitoring of the status of the infection during antibiotic treatment (37).

An advantage of the amplification of variable spacer regions is that no large-scale DNA extractions are necessary, which allows the processing of large numbers of isolates. However, much research effort is still required to optimize the PCR conditions and electrophoresis protocols, since the differences detected are usually only a few nucleotides. Unfortunately, the spacer regions are not equally variable for all species. Strain-specific spacer patterns can be obtained, for instance, for *Leuconostoc* species, but for *Lactococcus lactis* strains no variation is found (127). So in order to use this method large numbers of isolates have to be analysed before the applicability of the method for the specific purpose can be established.

Restriction endonuclease digestion of amplified ribosomal RNA sequences. In order to develop a rapid diagnostic technique, Gurtler *et al.* (1991) described the use of restriction endonuclease digestion of the amplified 16S rRNA gene as a simplified way for the identification of clinical clostridial isolates (51). Analysis of the restriction sites within the sequence of the 16S rRNA for different *Clostridium* spp. allowed the identification of unique restriction sites suitable for the rapid identification of clinical isolates, without the need for laborious sequencing protocols. The same approach was

later used by Carlotti and Funke to discriminate between *Brevibacterium* spp. (23) to demonstrate that a number of clinical isolates belonged to *B. casei*, usually isolated from surface-ripened cheeses, and not to *B. epidermidis*, a normal isolate of human skin. More recently, this method has been applied to estimate the biodiversity in specific environments like hypersaline sediments and hydrothermal vent systems (96, 99). After direct extraction of microbial DNA from environmental samples, the 16S rRNA gene is amplified and digested with specific restriction endonucleases. Based on the characteristic profiles of the digested fragments, estimations can be made of the diversity and of overall similarities between the organisms in different environments.

PCR-single-strand conformation polymorphism of partially amplified 16S rRNA. PCR-single-strand conformation polymorphism (PCR-SSCP) is a rapid technique for the detection of mutations and allelic variants (93). Amplified parts of the 16S rRNA gene are denatured into two single-stranded (ss) DNAs. These strands are analysed on a non-denaturing polyacrylamide gel. Due to the sequence-dependent mobility of ssDNA, differences can be found in the band patterns obtained from amplified 16S rRNA fragments from different bacteria. In this way it was possible to obtain genus- and species-specific patterns for 100 strains from 15 genera and 40 species (Widjojoatmodjo *et al.*, 1994) (156). By combining fluorescence labelling with band pattern analysis on an automated sequencer a reliable and rapid method has been developed for the identification and detection of a broad range of pathogens found in usually sterile body fluids, such as blood and cerebrospinal fluid.

A parallel approach was taken by Muyzer *et al.* by using denaturing gradient gel electrophoresis (DGGE) to analyse the sequence polymorphism in amplified 16S rRNA genes from environmental samples (100). With DGGE, DNA fragments of the same length but with different nucleotide sequences can be separated due to the difference in mobility of partially denatured DNA molecules. Because of the presence of stretches of nucleotides with different melting temperatures within an amplified 16S rRNA gene, an amplified fragment will partially denature when the lowest melting temperature has been reached. This will influence the migration of the molecules depending on their variation in sequence and therefore it is possible to separate them by DGGE. Using DGGE or temperature gradient gel electrophoresis (TGGE) of amplified 16S rRNA genes in combination with specific DNA probes, complex microbial populations can be studied (100). The DDGE technique can also be applied for the specific identification of bacteria. Tsuchiya *et al.*, 1994, used this technique to analyse the sequence polymorphism of the 5S rRNA of some lactic acid bacteria that cause spoilage of beer (138).

Although the DGGE and PCR-SCCP approaches are quick and reproducible, it will take much research effort to build a data-base containing specific band patterns for a large number of micro-organisms. Moreover, when unknown patterns are found for a particular isolate, the only way to correctly identify the organism will be to sequence the 16S rRNA.

Design of specific DNA probes based on ribosomal RNA sequences

rRNA sequences are the ideal target for a direct and rational probe design. Based upon comparative sequence analysis, specific sequences can be identified which can be used for the identification and detection of microorganisms (see Table 2). Depending on

what is desired, probes can be designed that discriminate between groups, genera or species, and in some cases even between subspecies (5). Species-specific probes target the most variable regions of the 16S rRNA, while more general probes (group/genus) are complementary to the more conserved regions of the 16S rRNA. In recent years many reports on the development of specific DNA probes based on 16S rRNA have been published. However, the successful use of rRNA sequences as a basis for the design of specific DNA probes depends on a number of factors.

First of all, it is very important to evaluate the amount of sequence variation between the organisms that have to be discriminated. Furthermore, the regions containing the most variable sequences have to be identified (see Table 2 and 147). The number of nucleotide differences in the most variable region can vary considerably between different genera. While *Clostridium* spp. may have 20 or more nucleotide differences in their most variable regions of the 16S rRNA (V2 and V6), *Bacillus* spp. have only 10 or less in the region containing most sequence variation (V3) (see Table 1). This implies that it will be more difficult to design species-specific DNA probes based on 16S rRNA sequences for *Bacillus* than for *Clostridium*. This can be compensated by using 23S rRNA sequences, which contain more sequence information, as was done by Tatzel *et al.* (1994) for *Bacillus licheniformis*. Unfortunately the number of known and available 23S rRNA sequences is still low (about 350) (5). Therefore, it is not always possible to compare sufficient sequences in order to identify the parts of the 23S rRNA that contain sufficient sequence differences to allow for the design of specific DNA probes.

Another factor which is very important for the design of a specific DNA probe is the technique in which the probe is applied. Specific DNA probes can be used with different detection techniques, like membrane or solution hybridization, PCR amplification, or *in situ* hybridization. Each of these applications demands specific properties of the DNA probe. Probes used in regular solid-phase hybridizations in general have a size of 15-25 nucleotides. In order to obtain a specific hybridization signal a probe should have a size of at least 15 nucleotides (132). The determination of the T_d (temperature corresponding to the mid-point in the dissociation of a short oligonucleotide bound to immobilized DNA) of oligonucleotide probes and its consequences for the washing conditions have been described by Stahl and Amann, 1991 (132). Usually a DNA probe of 20 bases will have a T_d between 50 and 60 °C. The specificity of hybridization probes depends on the position of the specific base pairs. A mismatch near the end of the duplex is generally less destabilizing than an internal mismatch. In addition the nature of a mismatch has its influences on the stability of the duplex formed by the DNA probe and its target sequence. While mismatches involving G-T, G-A and G-G are only moderately destabilizing, strong destabilizing effects are found with A-A, T-T, C-T and C-A mismatches. So, in designing a hybridizing DNA probe, the discriminating nucleotides should be in the internal part of the oligonucleotide, and the optimal specificity will depend on parameters during the hybridization, like the washing temperature and the ionic strength of the wash solution (132).

In case a DNA probe is used as one of the primers in a PCR amplification, its design should follow completely different guidelines. First of all the length and the T_d of both primers used in a PCR should be similar. The optimal primer length is between 18 and 24 nucleotides and the T_d should not be lower than 55 °C (36). The last requirement is important with respect to the elongation temperature of 72 °C (when the *Taq*-polymerase is used). If the annealing temperature is below 50 °C, the efficiency of the PCR will be very low due to the small amount of primer which is still annealed to

the template DNA once 72 °C has been reached. This is even more so since the ramping times have decreased considerably in the newest thermocycling equipment (25 s from 50 to 72 °C for a Thermocycler 480 to 10 s for a Thermocycler 9600 (Perkin Elmer)).

The most important difference in the rational design of PCR primers compared to that of a hybridization probe, is the positioning of the nucleotides determining the specificity of the probe. While for hybridization probes the specific bases should be in the internal part of the probe, for PCR primers the specific nucleotides should be positioned at the 3′-end. Optimal specificity requires a perfect match of the first three nucleotides at the 3′-end, in which in particular the last nucleotide plays a crucial role (22). For elongation with *Taq*-polymerase it has been demonstrated that transitional mispairs at the 3'-end of the primer, like A-C, C-A, G-T and T-G, are 10^{-3} to 10^{-4} times less efficiently extended than their correctly paired counterparts. But the relative elongation efficiencies of *Taq*-polymerase for extending T-C, T-T, A-A, G-A, A-G, G-G and C-C are even less than 10^{-5} (62). This indicates that only a few nucleotide differences can be sufficient when generating specific primers for use in PCR to detect microorganisms. Some other parameters in the PCR amplification can influence the primer specificity, such as the enzyme used and the magnesium concentration (120).

For the design of DNA probes for *in situ* hybridization, again other factors have to be considered. The oligonucleotides used for *in situ* hybridization are usually less than 20 nucleotides (5). They are designed by following the same guidelines as for the regular hybridization probes, but they need to be extensively validated under practical conditions. This is very elegantly shown by Langedijk *et al.*, 1995, who describe the validation of three *Bifidobacterium*-specific probes. One of the probes, targeted at the V8 region of the 16S rRNA, gave very poor hybridization signals, although it had shown good specificity. This could have resulted from the presence of intramolecular reassociation that made the probe target site inaccessible (84, 5). Up to now the *in situ* accessibility of rRNA molecules for oligonucleotides has hardly been examined in a systematic way, although Amman postulated recently that no *in situ* hybridizations could be detected when hybridization sites are shifted towards the 5'-end beyond approximately position 640 (4).

Validation of the specificity of DNA probes

In order to use DNA probes for reliable identification and detection of microorganisms, they should be correctly validated. Usually this is done by testing the probe in hybridizations against blotted chromosomal DNA of a number of strains belonging to the species aimed at and its closest relatives. Problems can arise due to an insufficient or incorrect identification of strains used for the validation, or their impurity. Therefore only correctly validated control strains (verified by 16S rRNA sequencing) should be used for the evaluation of specific DNA probes.

When a DNA probe is used for the specific detection of a microorganism in a certain environment, representatives of the indigenous micro-flora should also be tested. Unfortunately, the whole range of indigenous microorganisms is seldom known completely. When total DNA of a complex population is used, it is therefore sometimes difficult to optimize the hybridization conditions. In such cases it might be preferable to use specific PCR-amplification for the detection of a microorganism, because the correctness of the signal can be checked, either by the size of the product, by hybridization of the amplified product with a specific internal probe or by partial

sequencing of the PCR product. Another advantage of the use of specific PCR is the fact that the range of microorganisms that must be tested for validation is smaller, due to the high degree of specificity of a PCR-amplification and the direct control on the correctness of the signal.

Application of specific DNA probes based on ribosomal RNA sequences

Detection and identification of microorganisms using specific DNA probes in hybridization procedures. DNA probes can be applied for the detection and identification of microorganisms in a variety of methods. The most straightforward approach is the use of specific DNA probes in DNA-DNA or DNA-RNA hybridizations, in which the target molecules are fixed to a membrane. The use of RNA as target during a hybridization protocol has a great advantage. Although usually several chromosomal copies of the rRNA operon are present in microorganisms, this will never reach the abundance of the RNA targets present. All microbial cells contain, depending on their growth stage, up to 10^5 ribosomes. Each ribosome contains one set of rRNAs, leading to a significant increase of sensitivity.

The target DNA or RNA can be immobilized on a membrane by different methods. First of all colonies, grown on an agar plate, can be directly transferred to a membrane after which fixation of the DNA is achieved by cell lysis and covalent binding of the DNA or RNA to the filter. Especially Gram-negative bacteria are easy to detect with these so-called colony-blot techniques, but Gram-positive organisms are difficult to lyse due to the composition of their cell wall. Specific protocols have been developed to prevent cell material resulting from incomplete lysis remaining on the filter, otherwise giving rise to non-specific background signals (14). Such a method has been developed for the specific detection of *Lactococcus lactis* subsp. *cremoris* isolates from environmental samples (123).

Bacterial cells can also be transferred to a membrane by a dot-blot manifold, using vacuum filtration to collect the cells on the membrane. In this way bacterial cells can be concentrated from liquid environmental samples containing low numbers of cells, such as sewage, fresh water or sea water (82).

Many commercially available kits have been developed based upon DNA hybridization, especially for the detection of pathogenic organisms like *Listeria monocytogenes* and *Salmonella*. These kits specifically detect the target organisms after a pre-enrichment in a general medium. During a hybridization step, DNA from the lysed cells is captured on a dip-stick, using a specific capture probe (the Gene-Trak method), followed by a specific detection. This detection may involve a detection probe labelled with an enzyme capable of giving a chemiluminescent or colorimetric signal. With these detection methods it is possible to obtain a quicker and more reliable detection of *L. monocytogenes* or *Salmonella* from the enrichment broths as compared to the classical confirmation methods (103, 139).

The above-mentioned techniques are all based on the initial collection of bacterial cells, that are subsequently lysed, followed by the direct detection of the DNA by a DNA probe. Another frequently used strategy is the application of isolated DNA as target for specific DNA probes. This DNA can either be isolated from pure cultures or environmental samples, or it may be obtained by PCR amplification. Membrane-fixed DNA isolated from pure cultures is often used to validate the specificity of DNA probes (74). This technique enables the testing of several DNA probes to the same collection

of strains. It can also be applied to efficiently identify a large number of isolates at the same time (77). This approach, using a genus-specific DNA probe for *Lactococcus*, was used for the initial identification to the genus level of environmental isolates (77). This technique is also well suited for the specific detection and quantification of a particular microorganism in mixed bacterial populations. The presence of the organism can be monitored by hybridizing specific DNA probes with total bacterial DNA or RNA extracted from an environmental sample fixed on a membrane. Odenyo *et al.*, 1994, demonstrated the suitability of this method in a study monitoring the population dynamics of *Ruminococcus* species in *in vitro* rumen systems (107). After extracting the RNA from the *in vitro*-grown culture, equal amounts of RNA were blotted on a membrane which was subsequently hybridized with ^{32}P-labelled probes for several *Ruminococcus* species. After washing, the membrane was exposed to X-ray film and the amount of signal was quantified using laser densitometry. This allowed monitoring of the influence of different substrate additions to the *in vitro* rumen system on the *Ruminococcus* population and the interaction between different species (106).

The detection limit of the above-mentioned techniques can be lowered by PCR amplification. A more detailed description is given in the work on the specific detection of *Clostridium tyrobutyricum* sequences in DNA extracted from cheese (78). This approach has several advantages. Firstly, relatively clean DNA is obtained which can be easily transferred to a membrane, and secondly the target region for the probe can specifically be amplified, thereby decreasing the chance of non-specific binding. This approach is particularly useful when 16S rRNA probes based on a variable region in the 16S rRNA are used. The initial specific amplification of variable regions yields good-quality template DNA from as little material as one colony on an agar plate. This strategy is extremely suitable for the rapid screening of large numbers of isolates (77).

Use of specific PCR amplification for the detection of microorganisms in food products. PCR amplification can be used to detect microorganisms in food. The two major advantages of this approach are the relatively simple protocols for the preparation of the template DNA and the low detection limit. Using two specific DNA primers based on variable regions in the 16S rRNA for *Campylobacter jejuni* Giesendorf *et al.*, 1992, demonstrated the potential to detect as little as 25 colony-forming units per gram chicken meat (47). This is close to the desired detection levels, demonstrating the potential of PCR amplification for the detection of microorganisms in food. Unfortunately, many food components appear to inhibit the PCR reaction and some food matrices require special extraction methods in order to obtain DNA which can succesfully be used in PCR amplification (78, 86). Rossen *et al.*, demonstrate that components of especially cheese extracts inhibited the PCR reaction, causing increased detection limits because of dilution steps required to eliminate the inhibitors (117). Such inhibitors are not only present in foods but are also found in DNA extracted from environmental samples like soil and faecal material. Generally the exact nature of these inhibiting factors is not known, although humic acids in soil (135) and bile acids in faeces (24) are among the inhibitory components identified. Special DNA extraction methods including extra purification steps have been developed to eliminate these inhibitors from the template DNA. Quia-gen purification can be applied to DNA extracted from human faecal material in order to obtain PCR amplification signals (76). Other methods that eliminate inhibitors include the use of magnetic beads coupled to specific antibodies that specifically extract cells of the target organism from the food matrix (43, 155) or the

inclusion of a pre-enrichment step, during which the inhibiting components are diluted and the number of cells is increased (85). In particular this last approach is applied in the routine detection of pathogens, especially in the PCR-based detection kits now commercially available, like the Bax^{TM} system to detect *Salmonella* (Dupont, USA).

Another problem is posed by the presence of traces of DNA in many commercially available *Taq*-DNA polymerases, especially when general 16S rRNA primers are used for the amplification of a specific part of the 16S rRNA. Due to such contaminations background signals may appear which interfere with the specific detection. The contaminating DNA appeared not to result from *Thermus* or *Escherichia coli* (92) but from Gram-negative organisms, like *Pseudomonas* and *Alcaligenes*, normally present in tap water. Schmidt *et al.* clearly recognized the disposable tubes used for the PCR reactions as the source of the DNA contamination (126). Fortunately, since the levels of contamination are usually extremely low, they do not interfere in the detection assay, because the amount of template DNA usually outcompetes the contaminating DNA.

A two-step PCR using nested primers may be applied to increase the sensitivity of PCR-based detection methods. In this technique, two sets of primers are used, the second of which amplifies an internal part of the fragment that is amplified by the first set of primers. By combining a very efficient DNA extraction method with a two-step PCR-amplification of the listeriolysin O gene, Herman *et al.* (59) were able to detect 10 to 5 CFU of *Listeria* in 25 ml of raw milk. A two-step PCR approach was also applied for the sensitive detection of *C. tyrobutyricum* in cheese (78).

Quantitative PCR amplification. Although PCR is an exponentially proceeding process and thus potentially difficult to control, several methods have been developed in order to obtain quantitative signals (42) enabling direct quantification of micro-organisms or nucleic acids in environmental samples. The most straightforward is the use of limiting dilutions of target DNA. The amount of target DNA is quantified by positive/negative signals obtained from a series of dilutions, similar to classical most-probable-number (MPN) determinations (136). Using the MPN-PCR method Picard *et al.* quantified *Agrobacterium tumefaciens* cells in soil samples with a detection limit as low as 10^4 cells per g soil (110).

Another approach is the use of an internal standard in the amplification of the target DNA (128). Usually the internal standard differs from the target molecule only in size due to an insertion or elimination in the sequence of the internal standard. Since the primers will have the same binding sites in both internal standard and target molecule, the two are quantitatively amplified. Only an excess of one of the target molecules would inhibit the amplification of the other template. The ratio between the signals obtained for both reaction products provides quantitative information on the number of copies of target DNA originally present in the sample. This approach was used to monitor the effects of antibiotic treatment in patients infected with *Mycobacterium tuberculosis*, by quantifying specific *M. tuberculosis* DNA in blood samples (81).

In general, PCR products are quantified by image analysis of ethidium-bromide-stained agarose gels. Although sensitive scanning equipment is available, alternative methods have been developed that allow the direct quantification of PCR products. They are all based on the principle of labelling one of the PCR primers with a reporter molecule that, directly or indirectly, can be quantified by an enzymatic reaction, resulting in a colorimetric or chemiluminescent assay (95). Recently Perkin Elmer developed a semi-automated electrochemiluminescent (ECL)-based post-PCR

quantification system called Q-PCR System 5000. Biotinylated PCR products are captured on streptavidin-coated paramagnetic beads, followed by quantification through measurement of the ECL signal from labelled reporter probes. Using reporter probes for *Escherichia coli* and *Pseudomonas aeruginosa* Blok *et al.* quantified each species by the Q-PCR amplification of the 16S rRNA with DNA isolated from mixed cultures with ratios of 1:100 (15).

Besides PCR amplification many other DNA-amplification techniques have been developed in the recent years, such as ligase chain reaction (151), NASBA (141, 140), self-sustained sequence replication (3SR), and QB replicase. Especially the NASBA, in which DNA sequences are amplified by means of RNA polymerase, has major advantages since only one temperature is necessary for the amplification, allowing it to be performed in regular water baths. Automated quantification, based on the same principles as Q-PCR is now under development at Organon Teknika, who developed the NASBA method, making it more appropriate for routine control of food products (140).

Use of in-situ *hybridization techniques to study the ecology of food micro-organisms.* Although it is possible to quantify a specific microorganism in an environment by quantitative PCR, direct quantification by whole-cell hybridization is still the most accurate method for the quantitative study of microbial populations in natural microhabitats (5). Hybridization of fixed cells with fluorescently labelled DNA probes, followed by fluorescence microscopy, allows the visualization of the arrangement of various microorganisms in a particular habitat. This method also yields information on the spatial distribution of the specific organisms *in situ* (56). An extra dimension of *in situ* detection is the application of several DNA probes, each labelled with a different fluorescent label. This allows for the visualization of more than one type of microorganism in an environmental sample, which permits the study of complex populations. In this way very relevant information can be obtained, as was shown for the distribution of syntrophic propionate-oxidizing bacteria in anaerobic granular sludge (56). Moreover, an important asset of this method is the possibility to detect and to quantify microorganisms which are unculturable or are very difficult to quantify on selective media, such as *Bifidobacterium* (84). Therefore such techniques have frequently been applied to characterize specific microorganisms in the gastro-intestinal tract, one of the most intensively studied ecosystems. Specific microorganisms such as *Fibrobacter intestinalis*, could be directly detected and quantified in a complex natural environment like the caecum of the mouse (2). Still, this technique has its greatest potential in the detection of non-culturable microorganisms. *In situ* hybridization allowed Snel *et al.*, 1993, to verify the identification of segmented filamentous bacteria (SFB) inhabiting the ileum of mice with SBF-specific DNA probes based on 16S rRNA sequences. These sequences were obtained by extracting DNA from mono-associated gnotobiotic mice by using specific PCR primers for the amplification of bacterial 16S rRNAs (129). The organisms, later named *Candidatus* arthromitus (130), appeared to be closely related to the genus *Clostridium*. The correctness of the 16S rRNA sequence obtained was verified by *in situ* hybridization with the specific probes.

Whole-cell hybridization with fluorescently labelled probes can also be used for the direct quantification of microorganisms by flow cytometry (1). In this way different members of a bacterial population can be quantified simultaneously over a wide range of concentrations (up to 0.8% of the total population). In this and other whole-cell hybridization approaches, the efficiency of the hybridization is very important. The

amount of signal obtained for a cell depends on various parameters, such as the efficiency of the probe entry of the fixed cell and the number of ribosomes present. Especially in Gram-positive bacteria the permeability of the cell-wall limits the entery of the DNA probe. Another limiting factor is the number of rRNA copies available for hybridization with the specific probe. When the physiological activity of the cells is low, the number of ribosomes is also rather low, resulting in a limited amount of hybridization targets (5). This limitation can be overcome by increasing the signal per DNA probe, using enzyme-labelled DNA probes (3). Enzymes, such as horseradish peroxidase, can also be of use when strong background fluorescence interferes with the detection of the fluorescence signal of the probe. Another way to increase the signal per cell is to use more than one probe for a specific organism, for instance one based on the 16S rRNA and the other based on the 23S rRNA (5, 1). In addition the incorporation of more label in a DNA probe can increase the hybridization signals, but usually the gain in signal with this approach is limited (5).

Outline of the thesis

The aim of the research presented in this thesis was to develop and apply sensitive and reliable techniques based on specific 16S rRNA sequences to identify and detect microorganisms relevant for the dairy industry. Such techniques were developed for both desired microorganisms that are used as starter cultures for the fermentation of milk, and unwanted microorganisms causing spoilage of dairy products. Lactic acid bacteria are of great economic importance for the dairy and other sectors of the food industry. They are the most fequently used organisms in starter cultures for the fermentation of various food products, like milk, vegetables and sausages. The techniques developed have been applied to study the microbial ecology of starter during food production, in the food product and in the subsequent waste flows. Also the specific germination of sporeforming spoilage bacteria in cheese was monitored.

In Chapter 1 an overview is given of current developments with respect to the identification and systematics of microorganisms and the role of 16S rRNA sequences. In addition, a summary is presented of available techniques, based on specific 16S rRNA sequences, for the sensitive detection of culturable and unculturable microorganisms. The specific aspects of the application of these techniques in different types of research are discussed.

Since many lactic acid bacteria, including lactococci, have similar physiological characteristics and growth requirements, it is often difficult to identify them by conventional methods. The first part of this thesis deals with the microbial ecology of *Lactococcus* spp. Chapter 2 describes the development of detection and identification methods for mesophilic lactic acid bacteria, such as *Lactococcus* and *Leuconostoc*, based on PCR and specific DNA probing. Large-scale production of fermented milk products relies on the use of starter cultures that have been selected for their performance during the manufacturing of fermented products. In the dairy industry there is a great need for new production strains, which can be used in the development of innovative fermented milk products. This can be achieved either by genetic modification of known production strains (44) or isolation of new strains from natural environments. Both for the application of genetically modified starter strains and to enable an efficient search for strains from natural ecosystems, the ecological niches of lactic acid bacteria should be

known and the ability of lactococci to survive outside the dairy environment has to be determined. Chapter 3 describes an environmental screening in which the natural niches for *Lactococcus* species were identified and the survival of industrial lactococcal strains was monitored.

For future use of lactococcal strains as probiotics (105) or as live vaccines (104, 149) it is essential to determine the survival kinetics in the gastrointestinal tract of these bacteria after human consumption. In Chapter 4 the fate of a genetically marked *Lactococcus lactis* strain after consumption in the human gastrointestinal tract is described.

The second part of this thesis concerns the development of specific identification and detection methods for *Clostridium* species, in particular *C. tyrobutyricum.* The spores of this anaerobic, Gram-positive microorganism survive the heat treatment of cheesemilk and after subsequent outgrowth in cheese are believed to cause the defect called late blowing of cheese (12). To resolve the questions whether all *C. tyrobutyricum* strains can cause butyric acid fermentation in cheese and the defect is always caused by *C. tyrobutyricum,* specific DNA probes were developed for all *Clostridium* species commonly encountered in raw milk. The development of specific DNA probes for *C. tyrobutyricum, C. beijerinckii, C. butyricum, C. acetobutylicum* and *C. sporogenes* is described in Chapter 5. Chapter 6 deals with the identification of *C. tyrobutyricum* as the causative agent of late blowing using species-specific PCR amplification. In Chapter 7 a summary of the thesis is given and the practical relevance is discussed of the use of molecular identification and detection techniques for research and routine analysis in relation to the needs of the dairy industry.

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Chapter 2

Identification of mesophilic lactic acid bacteria by using polymerase chain reaction-amplified variable regions of 16S rRNA and specific DNA probes

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Identification of Mesophilic Lactic Acid Bacteria by Using Polymerase Chain Reaction-Amplified Variable Regions of 16S rRNA and Specific DNA Probes

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Specific DNA probes based on variable regions V1 and V3 of 16S rRNA of lactic acid bacteria were designed. These probes were used in hybridization experiments with variable regions amplified by using the polymerase chain reaction. In this way, a rapid and sensitive method was developed for the identification and classification of *Lactococcus* and *Leuconostoc* species.

Lactic acid bacteria (LAB) are of great economic importance for the dairy and other fermented food industries. For both basic research on LAB and their application in industrial food fermentations, reliable and simple methods for identification of such bacteria are required. Because many LAB have similar nutritional and growth requirements, it is very difficult to identify them by classical methods. Therefore, various approaches that use molecular probes have been described (2, 4, 11). Here we report on a combination of sensitive techniques for identification and detection of LAB that is based on polymerase chain reaction (PCR) (13) and specific DNA probing (9).

In recent years, the use of rRNA sequences for identification and phylogenetic analysis has been generally accepted (1, 5). DNA probes based on highly variable rRNA regions have been applied successfully for the identification and detection of microorganisms in soil, intestinal tract, and clinical samples (6, 12, 16). By comparing the published 16S rRNA sequences of *Lactococcus* spp. (3, 14) and *Leuconostoc* spp. (8, 17), we identified the regions containing the highest variability. For the genus *Lactococcus*, described by Schleifer et al. (15), the V1 region (90 bp) contained sufficient sequence variation to enable the design of DNA probes allowing differentiation between the species *Lactococcus lactis*, *L. garvieae*, *L. plantarum*, and *L. raffinolactis* and *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. The sequences of the V1 region (90 bp) appeared to be identical in all species analyzed in the genus *Leuconostoc*, but those of the V3 region contained sufficient variation to design DNA probes specific for *Leuconostoc* species (Table 1).

To increase the sensitivity of the procedure, we used PCR amplification of the variable regions with primers based on the conserved flanking sequences (Table 1 and Fig. 1). The PCR amplifications were performed by using a BioMed Thermocycler (BioMed, Amstelstad, Holland). The reactions were carried out in sterile Multimax seal tubes with cap locks (1.5 ml), which contained 50 μl of the following buffer: 10 mM Tris HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM NaCl, deoxynucleoside triphosphates at 2.5 mM, and 1 U of Taq polymerase. Template DNA (500 to 100 ng) was added after being heated to 95°C to eliminate all protease activity. The amplification was done in 30 cycles by melting the DNA at 93°C for 1 min, annealing at 54°C for 1.5 min, and elongation at 72°C for 2.5 min.

To allow the identification of small amounts of bacteria, a method was developed to isolate DNA from a single colony grown on an agar plate (7). After the colony (±1.5-mm diameter) was suspended in 50 μl of 10 mM Tris HCl buffer (pH 8.0) containing 400 μg of lysozyme and incubation at 37°C, the cells were lysed by adding 50 μl of 10% sodium dodecyl sulfate and 250 μl of buffer. The DNA was precipitated by adding 60 μl of 3 M sodium acetate and 1 ml of 96% ethanol (stored at −20°C). After centrifugation, the DNA pellet was dissolved in 10 mM Tris HCl buffer (pH 8.0) and precipitated a second time by adding 1 ml of isopropanol. The DNA pellet was washed with 70% ethanol and finally dissolved in 50 μl of TE buffer (10 mM Tris HCl [pH 8.0], 1

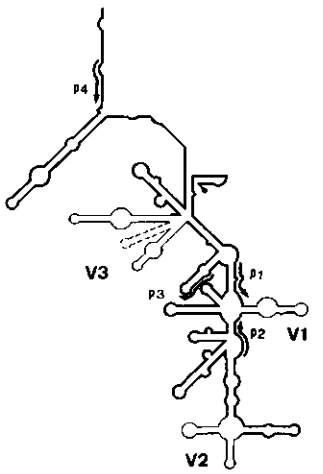

FIG. 1. Representation of the 5′ region of the secondary structure of 16S rRNA (●, 5′ terminus). Conserved areas are drawn in bold lines, and areas that vary in sequence and size are drawn in thin lines (broken lines, structure found only in a few organisms) (10). This part of the 16S rRNA contains the variable regions V1, V2, and V3. The location and direction of the PCR primers used in this study are marked by arrows.

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TABLE 1. List of primers used

Use	Primer or probe[a]	Target DNA (region)	Sequence (5' to 3')
PCR	P1 (S)	41–60 (V1)[b]	GCGGCGTGCCTAATACATGC
	P2 (A)	111–130 (V1)[b]	TTCCCCACGCGTTACTCACC
	P3 (S)	361–380 (V3)[b]	GGAATCTTCCACAATGGGCG
	P4 (A)	685–705 (V3)[b]	ATCTACGCATTTCACCGCTAC
DNA probe	PLl_1 (A)	*L. lactis* subsp. *lactis* V1	AGTCGGTACAAGTACCAAC
	PLl_2 (S)	*L. lactis* subsp. *lactis* and *L. lactis* subsp. *hordniae* V1	GCTGAAGGTTGGTACTTGTA
	PLc (A)	*L. lactis* subsp. *cremoris* V1	TTCAAATTGGTGCAAGCACC
	PLp (A)	*L. plantarum* V1	CTACGGTACAAGTACCAGT
	PLg (A)	*L. garvieae* V1	CATAAAAATAGCAAGCTATC
	PLr (A)	*L. raffinolactis* V1	CGGTGAAGCAAGCTTCGGT
	PLC (A)	*Leuconostoc* spp. V1	CACCTTTCGCTGTGGTT
	PLCl (S)	*Leuconostoc lactis* V3	ATGCTAGAATAGGGAATGAT
	PLCm (S)	*Leuconostoc mesenteroides* V3	CAGCTAGAATAGGAAATCAT

[a] S, sense sequence; A, antisense sequence.
[b] *Escherichia coli* numbering is used (10).

mM EDTA). Five microliters of this solution was used for PCR amplification.

After agarose gel electrophoresis, the PCR-amplified fragments were transferred to GeneScreen Plus (Dupont, Boston, Mass.) with a vacuum blotter (Pharmacia, Woerden, Holland). Prehybridization and hybridization were performed in 0.5 M sodium phosphate buffer (pH 7.2) containing 3% sodium dodecyl sulfate and 1% bovine serum albumin. After 30 min of prehybridization at 40°C, the probe, which had been 5'-end labeled with [γ-^{32}P]ATP (Radiochemical Centre, Amersham, England), was added and the incubation was continued for 4 h. The blots were washed with 0.3 M NaCl–0.03 M sodium citrate at 37°C until a clear signal was found and then were exposed to Kodak X-ray films.

Figure 2 shows that it is possible to identify and discriminate various *Lactococcus* strains with DNA probes that are based on the highly variable V1 region. The specificity of these probes was tested on some closely related LAB (Table 2). The PLl_1 probe did not give a signal with *L. lactis* subsp. *hordniae* containing a sequence in the V1 region that differs in only one nucleotide from that of *L. lactis* subsp. *lactis* and its variant *diacetylactis* (data not shown). Exactly the same substitution is found in the V1 region of some *L. lactis* subsp. *lactis* strains (14). By using the PLl_2 probe, these variants could also be detected (Table 2). The sequences of the V1 regions of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* show too many differences to allow the design of a species-specific *L. lactis* probe.

The identification of *Leuconostoc* spp. is shown in Fig. 3. The larger size of its amplified V1 region (Fig. 3A) confirms that *Leuconostoc paramesenteroides* is related to the genus *Lactobacillus* (V1 = 110 bp), as was proposed recently on the basis of 16S rRNA comparison (18). In spite of the fact that the V3 regions of *Leuconostoc lactis* and *Leuconostoc mesenteroides* contained only three nucleotide differences, a good discrimination between these species was found with the *Leuconostoc* probes (Fig. 3). The specificity of these probes was also confirmed (Table 3).

TABLE 2. Strains tested with DNA probes based on the V1 region[a]

Strain	Specificity of probe:						
	PLl_1	PLl_2	PLc	PLp	PLg	PLr	PLC
Lactococcus lactis subsp. *lactis* NCFB 2597	+	+	–	–	–	–	–
L. lactis subsp. *lactis* NCFB 764	+	+	–	–	–	–	–
L. lactis subsp. *lactis* NIZO R5	+	+	–	–	–	–	–
L. lactis subsp. *cremoris* NCFB 1200	–	–	+	–	–	–	–
L. lactis subsp. *cremoris* NCFB 504	–	–	+	–	–	–	–
L. lactis subsp. *cremoris* NIZO HP	–	–	+	–	–	–	–
L. lactis subsp. *lactis* variant *diacetylactis* NCFB 176	+	+	–	–	–	–	–
L. lactis subsp. *hordniae* NCFB 2181	–	+	–	–	–	–	–
L. plantarum NCFB 1869	–	–	–	+	–	–	–
L. garvieae NCFB 2155	–	–	–	–	+	–	–
L. raffinolactis NCFB 617	–	–	–	–	–	+	–
Leuconostoc mesenteroides NCFB 523	–	–	–	–	–	–	+
Leuconostoc lactis NCFB 533	–	–	–	–	–	–	+
Vagococcus fluvialis NCFB 2497	–	–	–	–	–	–	–
Streptococcus mutans ATCC 10449	–	–	–	–	–	–	–
Streptococcus sanguis ATCC 10556	–	–	–	–	–	–	–
Streptococcus thermophilus NIZO St1	–	–	–	–	–	–	–
Enterococcus faecalis LMG 7937	–	–	–	–	–	–	–
Staphylococcus aureus ATCC 14459	–	–	–	–	–	–	–

[a] Designation of probes is according to Table 1.

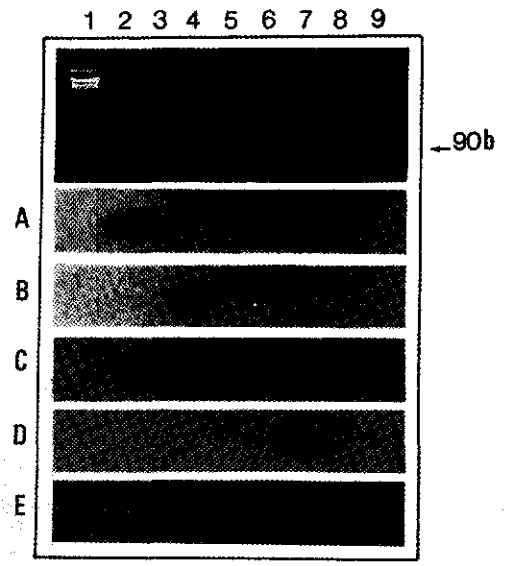

FIG. 2. Identification of *Lactococcus* species and subspecies. The top panel shows an ethidium bromide-stained 1% agarose gel used for separation of the PCR-amplified V1 region of 16S rRNA genes, using primers P1 and P2. Lane 1, lambda DNA digested with *Hin*dIII, used as a negative control for background hybridization; lane 2, *L. lactis* subsp. *lactis* NCBF 2597; lane 3, *L. lactis* subsp. *lactis* variant *diacetylactis* NCBF 176; lane 4, *L. lactis* subsp. *cremoris* NCBF 1200; lane 5, *L. lactis* subsp. *hordniae* NCBF 2181; lane 6, *L. plantarum* NCBF 1869; lane 7, *L. garvieae* NCBF 2155; lane 8, *L. raffinolactis* NCBF 617; lane 9, *Vagococcus fluvialis* NCBF 2497. Gels run in parallel, which contained identical samples, were blotted and hybridized with the PLl_1 (A), the PLc (B), the PLp (C), the PLg (D), or the PLr (E) probe.

The specific DNA probes designed and evaluated in this study allow the identification of small amounts of LAB. The described methods have two major advantages compared with classical identification techniques. First, it is possible to obtain a reliable identification within 1 or 2 days. Second, it is possible to perform a simultaneous identification of a large number of strains with only a small amount of cells, one colony on an agar plate being sufficient. Because of these advantages, the methods are well suited to characterize isolates from starter cultures and environmental samples.

TABLE 3. Strains tested with DNA probes based on the V3 region[a]

Strain	Specificity of probe:	
	PLCl	PLCm
Leuconostoc mesenteroides NCFB 523	−	+
Leuconostoc mesenteroides NIZO 3406	−	+
Leuconostoc mesenteroides NIZO 3411	−	+
Leuconostoc lactis NCFB 533	+	−
Leuconostoc lactis NIZO 6009	+	−
Leuconostoc lactis NIZO 6070	+	−
Leuconostoc paramesenteroides NCFB 803	−	−
Lactobacillus casei ATCC 7469	−	−
Lactobacillus helveticus ATCC 10797	−	−
Lactobacillus bulgaricus ATCC 1489	−	−
Lactobacillus acidophilus ATCC 11842	−	−
Lactobacillus plantarum ATCC 8014	−	−
Lactobacillus fermentum ATCC 9338	−	−

[a] Designation of probes is according to Table 1.

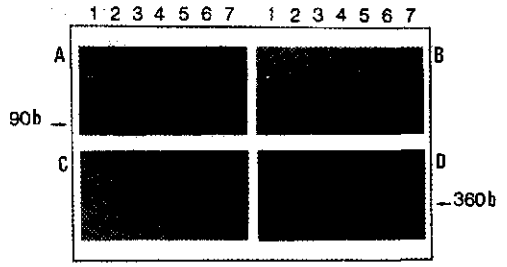

FIG. 3. Identification of *Leuconostoc* species. PCR-amplified DNAs of regions V1 (lane 2 to 4) and V3 (lane 5 to 7) of 16S rRNA genes obtained by using primers P1 + P2 and P3 + P4, respectively, were separated on a 2% agarose gel (A) and stained with ethidium bromide. Lane 1, pUC18 digested with *Hpa*II (19); lanes 2 and 5, *L. mesenteroides* NCFB 523; lanes 3 and 6, *L. lactis* NCFB 533; lanes 4 and 7, *L. paramesenteroides* NCFB 503. Gels run in parallel, which contained identical samples, were blotted and hybridized with the PLC (B), the PLCl (C), or the PLCm (D) probe.

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Chapter 3

Detection and characterization of lactose-utilizing *Lactococcus* spp. in natural ecosystems

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Detection and Characterization of Lactose-Utilizing *Lactococcus* spp. in Natural Ecosystems

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The presence of lactose-utilizing *Lactococcus* species in nondairy environments was studied by using identification methods based on PCR amplification and (sub)species-specific probes derived from 16S rRNA sequences. Environmental isolates from samples taken on cattle farms and in the waste flow of a cheese production plant were first identified to the genus level, using a *Lactococcus* genus-specific probe. Isolates which showed a positive signal with this probe were further identified to the (sub)species level. *Lactococcus lactis* isolates were also characterized at the phenotypic level for the ability to hydrolyze arginine, to ferment citrate, and to produce proteases and bacteriocins. With specific PCR amplifications, the presence of sequences related to *citP*, coding for citrate permease; *prtP*, coding for protease; and *nisA* or *nisZ*, the structural genes for production of nisin A or nisin Z, respectively, was verified. By these methods, it was possible to isolate lactococci from various environmental sources, such as soil, effluent water, and the skin of cattle. The strains of *L. lactis* isolated differed in a number of properties, such as the ability to hydrolyze arginine or the absence of *citP*-related sequences, from those found in industrial starter cultures. The results indicate that the majority of the industrially produced lactococci do not survive outside the dairy environment, although natural niches are available. However, from those niches strains with the potential to be developed into novel starter cultures may be isolated.

In the dairy industry, large amounts of lactic acid bacteria are involved in the daily manufacturing of fermented milk products such as cheese, butter, and quark. Strains belonging to the species *Lactococcus lactis* are the most important organisms in the manufacture of these products at moderate temperatures. Large-scale industrial processes rely on the use of starter cultures that have been selected for their performance during milk fermentation and product formation (15). As a result, the variability among strains used in industrial dairy fermentations is low (19). However, some traditional dairy products still rely on spontaneous milk fermentations, which result in a large variety of products with different flavors, consistencies, and microbiological quality. There is a great need in the dairy industry for new production strains which result in different product properties. This can be achieved either by genetic modification of known production strains (8) or by isolation of new strains from natural ecological niches. Both for the application of genetically modified starter strains and to allow for an efficient search for strains from natural ecosystems, it is important to know if and where lactococcal strains survive outside the dairy environment. Previous studies have already shown that it is possible to isolate *Lactococcus* spp. from environments other than raw milk. Early investigations identified the cow and the milking equipment as the source of *L. lactis* in raw milk (6; see reference 21 for a review), although other studies could not confirm these results (12, 24). Most of the investigations at that time were rather controversial due to unreliable identification methods. In more recent studies, the isolation of *Lactococcus* spp. from sources other than raw milk has been reported. *L. lactis* subsp. *hordniae* was isolated from the hindguts of wood-eating termites (22, 23), *L. plantarum* was isolated from frozen peas (2), and *L. piscium* was isolated from diseased fish (28). In addition, the detection of *L. lactis* and *L. garvieae* in clinical samples has been reported (5), in-

TABLE 1. PCR primers used

Primer[a]	Target DNA	Base positions (reference)	Sequence	Size of PCR product (bp)
P1 (S)	16S rRNA	41–60 (14)[b]	GCGGCGTGCCTAATACATGC	
P2 (A)	16S rRNA	338–358 (14)[b]	CTGCTGCCTCCCGTAGGAGT	319 (P1 + P2)
P3 (A)	16S rRNA	686–705 (14)[b]	ATCTACGCATTTCACCGCTA	610 (P1 + P3)
P4 (S)	Citrate permease	795–814 (3)	GGAGTTGGTGCTGGTATTGTG	497 (P4 + P5)
P5 (A)	Citrate permease	1273–1292 (3)	CCAACCCTGCTGTAATAGCAG	
P6 (S)	Protease	1198–1217 (7, 27)	CAACACGGCATGCATGTTGC	393 (P6 + P7)
P7 (A)	Protease	1573–1591 (7, 27)	CTGGCGTTCCCACCATTCA	
P8 (A)	Nisin	−99–−78 (16)	CGCGAGCATAATAAACGGCT	319 (P8 + P9)
P9 (S)	Nisin	201–220 (16)	GGATAGTATCCATGTCTGAAC	

[a] S, sense sequence; A, antisense sequence.
[b] *Escherichia coli* numbering is used.

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dicating that these organisms are probably widespread in the environment and not strictly dairy related. In all of these recent studies, the identification of the strains was based on modern methods, including cell wall component determination, DNA-DNA hybridization, and 16S rRNA analysis.

Recently, we have developed reliable methods for the identification and detection of *Lactococcus* spp. based on specific sequences in the variable regions of 16S rRNA (14). We have applied these methods in combination with a genus-specific probe for the reliable and rapid screening of large numbers of isolates from various environmental samples. To assess the dissemination of starter lactococci used in industrial fermentations, we sampled the waste flow of a cheese production plant. Since the effluent of this particular plant is finally discarded by spraying onto a meadow, samples of the soil and grass were taken also. In order to locate natural niches of *Lactococcus* spp., the presence of these bacteria on the udder and skin and in the surroundings of cows was determined since these animals have been implicated as carriers of these organisms (21). To assess a possible relation with the presence of milk, similar sampling was also done on a farm where only bulls were present. Samples from effluent water from the cheese pilot plant as well as those from whey, milk, feed, soil, and grass were collected in sterile containers. Samples from the cow's udder, the skin and saliva from both cows and bulls, and the milking equipment were taken with sterile swabs premoistened with a sterile physiological salt solution. Dilutions of the samples were directly plated on M17 agar (Oxoid, Hampshire, England) containing 0.5% lactose (LM17). For enrichment cultures, 1 ml (or 1 g for the feed, soil, and grass samples) was added to LM17, and the cultures were incubated at 30°C for 24 h. Dilutions of the enrichments were plated on LM17 agar. Single colonies from the plates were grown overnight in a microtiter plate in 250 μl of LM17. The isolates were first identified to the genus level. The DNA of the cultures grown in the microtiter plates was fixed to a nylon membrane filter (Gene Screen Plus; Dupont, Boston, Mass.) by transferring 100 μl of a lysed cell suspension with a dot blot manifold (Schleicher & Schuell, Inc., Keene, N.H.). The filters contained DNA of cell cultures from colonies obtained from the enrichment cultures and the direct platings. From each environmental sample, 48 colonies from the direct plating and 48 colonies from the enrichment culture were selected.

These filters were subsequently hybridized with a genus-specific *Lactococcus* probe, labeled by nick translation (19), consisting of the amplified V1 and V2 regions of lactococcal 16S rRNA. The probe was obtained in a PCR of a mixture of equal amounts of total DNA from *L. lactis*, *L. garvieae*, *L. raffinolactis*, and *L. plantarum*, using primers P1 and P2 (Table 1), and validated with a collection of strains belonging to the genera *Lactococcus* (20 strains), *Lactobacillus* (6 strains), *Streptococcus* (8 strains), and *Leuconostoc* (7 strains). Under stringent washing conditions, no signal was found with the genus-specific *Lactococcus* probe and DNA of strains from the other genera (data not shown). However, when cell lysates of four *Lactococcus* species were transferred to a filter and hybridized with a general 16S rRNA probe and the genus-specific *Lactococcus* probe, significant hybridization signals were obtained in both cases (Fig. 1A and B). *L. plantarum* was not tested in this way since this species cannot use lactose, which was included as the sole sugar to allow for specific enrichment of lactococci. Results of hybridizations with a general 16S rRNA probe and the genus-specific *Lactococcus* probe are shown in Fig. 1C and D. The results of the hybridization with the general 16S rRNA probe reflect the growth and efficiency of the cell lysis procedure (Fig. 1C).

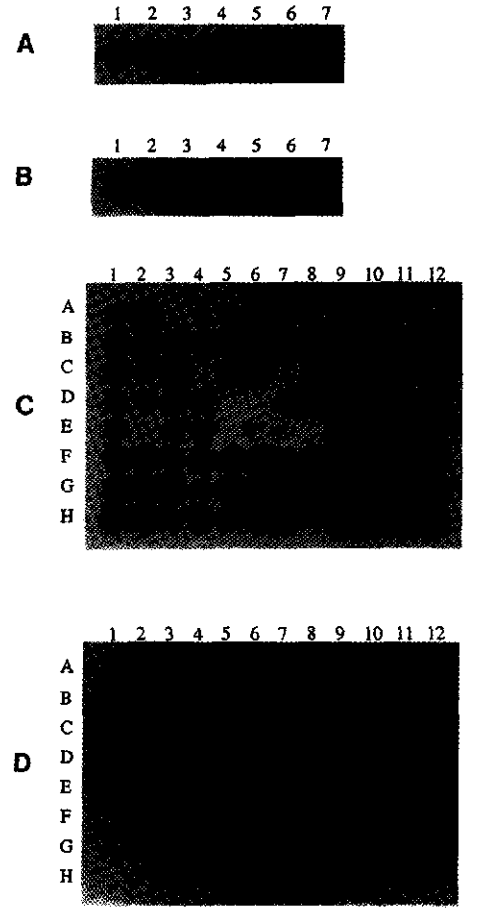

FIG. 1. Validation and use of a *Lactococcus* genus-specific probe based on 16S rRNA. (A and B) Control hybridizations for the four *Lactococcus* strains, *L. lactis* subsp. *lactis* (lane 1), *L. lactis* subsp. *cremoris* (lane 2), *L. raffinolactis* (lane 3), and *L. garvieae* (lane 4), together with *L. casei* ATCC 393 (lane 5) and *L. plantarum* ATCC 245 (lane 6). After lysis of the cultures in a microtiter plate, the DNA was fixed to a nylon membrane. In addition, a mixture of chromosomal DNA, used for generation of the probe, was included as a positive control for the hybridization (lane 7). This membrane was first hybridized with a general probe for 16S rRNA (P1) to check cell lysis and DNA fixation (panel A). Subsequently, the probe was removed and the membrane was hybridized with the *Lactococcus* genus-specific probe (16S rRNA fragment amplified with P1 and P2) (panel B). (C and D) Identification of isolates from a soil sample on the genus level. Lanes A1 and A2 contain DNA from *L. lactis* strains that were included as positive controls. First a hybridization was performed with the general 16S rRNA probe (panel C), and subsequently the blot was hybridized with the *Lactococcus* genus-specific probe (panel D).

Lactococcal isolates were identified with the genus-specific probe (Fig. 1D) and further characterized at the (sub)species level with the (sub)species-specific probes (14). In this way, approximately 80% of the presumed *Lactococcus* isolates obtained from environmental sources could be identified. Isolates that initially showed a signal with two probes, due to impurity

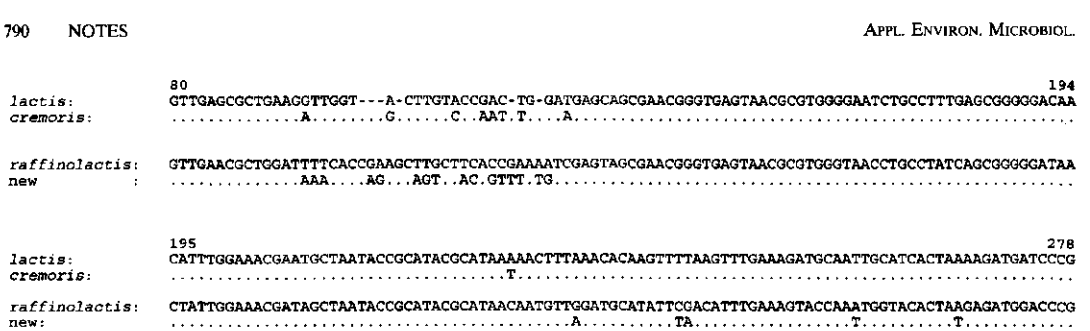

FIG. 2. Alignment of the first part of the 16S rRNA sequence of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. raffinolactis*, and an unidentified *Lactococcus* isolate (new). Only nucleotides which differed with respect to the sequences of *L. lactis* subsp. *lactis* and *L. raffinolactis* are presented.

of the colony, were purified, which always resulted in an unequivocal hybridization signal. The remaining 20% of the isolates were identified by direct sequencing of the first part of the 16S rRNA, using primers P1 and P3 (1). On the basis of this analysis, most of these isolates could be identified as members of the genus *Streptococcus*, in particular, *S. uberis* and *S. parauberis*, which grew to much higher densities than the lactococcal isolates and therefore generated a detectable hybridization signal in spite of the low complementarity of their rRNA to the *Lactococcus* genus-specific probe. Sequence analyses also revealed strains with a 16S rRNA sequence that was very similar to that of *L. raffinolactis* but differed in parts of the V1 region (Fig. 2). These strains probably belong to a new *Lactococcus* (sub)species, and further research is in progress to determine their taxonomic position (13).

In all samples, both the distribution of *Lactococcus* spp. and the estimated total number of lactococci were determined (Table 2). Lactococcal isolates were obtained from all samples, although in many cases only after an enrichment culture, since their initial numbers were low (Table 2), in particular, in the soil and grass samples. In the sampled environments, which are not directly associated with cheese and whey, the *Lactococcus* species *L. lactis*, *L. garvieae*, and *L. raffinolactis* were found to be present (Table 2). There was no difference found in lactococcal content between the samples taken from the cows and those from bulls, indicating that milk is not a prerequisite for the presence of lactococci. Strains belonging to the new (sub) species were isolated also from the wastewater tank and the raw milk, and strains of *S. uberis* and *S. parauberis* were isolated from various samples. The detection of *S. uberis* and *S. parauberis* in most samples derived from the farms is in agreement with the observation that these organisms are frequently associated with cattle and their environment (29).

The cheese milk inoculated with a common mesophilic mixed-strain starter and the whey produced from it contained only arginine-hydrolyzing *L. lactis* subsp. *lactis* and *L. lactis*

TABLE 2. *Lactococcus* species isolated during environmental screening[a]

Sample	No. of isolates[b]	No. of isolates identified as:							Avg concn[c]
		L. lactis subsp. *lactis*	*L. lactis* subsp. *cremoris*[d]		*L. garvieae*	*L. raffinolactis*	New *Lactococcus* sp.[e]	*S. uberis* or *S. parauberis*	
			Arg$^+$	Arg$^-$					
Cheese plant samples									
Cheese milk	30[f]	11		24					10^8/ml
Cheese whey	30[f]	30		19					10^6/ml
Waste whey	10 (7)[f]	7 (5)	8 (1)		(1)				10^5/ml
Wastewater tank	24	6	7		4	3	4		10^3/ml
Wastewater disposal site soil	(14)	(5)	(2)			(2)		(5)	$<10^3$/g
Grass	(10)	(4)	(5)		(1)	(1)			
Farm samples									
Raw milk	22	8	2		2	1	7	2	10^4/ml
Milk machine	8 (8)	6 (8)	2						10^4/cm^2
Udder	12	4	6					2	10^3/cm^2
Saliva, cow	(10)	(9)	(1)						
Saliva, bull	(5)	(5)							
Skin, cow	(8)	(4)			(1)			(3)	$<10^2$/cm^2
Skin, bull	(7)	(4)			(3)				
Grass	(14)		(7)			(5)		(2)	
Soil	(30)	(21)			(2)	(1)		(6)	$<10^3$/g
Silage	(10)	(10)							

[a] Values given in parentheses are results obtained from enrichment cultures; other values are from direct plating.
[b] Isolates that gave a positive signal with the *Lactococcus*-specific probe and could be identified with the species-specific probes.
[c] Only results from direct platings are given.
[d] All *L. lactis* subsp. *cremoris* isolates were tested for the ability to hydrolyze arginine.
[e] See Fig. 2.
[f] Total number of isolates is lower than the number of species found because of the presence of double signals.

TABLE 3. Phenotypic and genotypic properties of *L. lactis* subsp. *lactis* isolated from environmental sources

Source	No. of isolates tested	No. with given property[a]					
		Cit$^+$	*citP*	Prt$^+$	*prtP*	Bac$^+$	*nisA* or *nisZ*
Waste disposal land[b]	5	2	0	3	3	0	ND[c]
Soil	5	2	0	0	ND	0	ND
Raw milk	10	0	ND	10	8	1	1
Udder and skin	5	0	ND	5	3	2	2

[a] Cit$^+$, capacity to metabolize citrate; Prt$^+$, capacity to produce proteinase; Bac$^+$, capacity to produce antimicrobial activity.
[b] From soil and grass of the area sprayed with wastewater from the cheese plant.
[c] ND, not done.

subsp. *cremoris*, which did not hydrolyze arginine. However, it appeared that all *L. lactis* subsp. *cremoris* strains isolated from the wastewater and outside the plant were able to hydrolyze arginine (Table 2). It has recently been established that there is a discrepancy between the phenotypic and genotypic identifications of the two *L. lactis* subspecies (10, 18, 19). Strains with an *L. lactis* subsp. *cremoris* genotype can be grouped into those conforming to the known *L. lactis* subsp. *cremoris* phenotype (i.e., no growth at 37°C, 4% NaCl, and inability to hydrolyze arginine) and those with an *L. lactis* subsp. *lactis* phenotype (i.e., growth at 37°C, 4% NaCl, and able to hydrolyze arginine) (10, 18). Therefore, we tested all strains containing the *L. lactis* subsp. *cremoris* genotype for the ability to hydrolyze arginine (Table 2) as an indicator of the *L. lactis* phenotype. The results indicate that strains of *L. lactis* subsp. *cremoris* present in industrial starters and showing the typical *L. lactis* subsp. *cremoris* phenotype are not retrieved from the waste flow of a cheese production plant and other nondairy environments. Probably, the range of organisms in the starter cultures has narrowed due to the selection for better industrial performance, resulting in special varieties of *L. lactis* subsp. *cremoris* which have lost properties that are important for their survival outside a dairy environment. By using an in vitro effluent system, it was shown that *L. lactis* subsp. *cremoris* isolates from starters were outcompeted by environmental isolates (13). This suggests that strains with the typical *L. lactis* subsp. *cremoris* phenotype used in the production of fermented milk products would not be easily isolated from environmental sources, although Salama et al. (19) reported the isolation of strains from supposedly wild fermentations that showed a phenotype closely resembling that of the starter strains.

Some *L. lactis* strains isolated from environmental sources show industrially important properties such as the capacity to metabolize citrate or to produce proteases and bacteriocins (Table 3). A number of *L. lactis* strains isolated during this study were analyzed at both the phenotypic and genotypic levels. The ability to ferment citrate was measured with WACCA medium according to the method of Galesloot et al. (9). The presence of protease activity was determined by using GMA agar plates (11). The ability to produce bacteriocins was determined as described previously (4, 26), using *Micrococcus flavus* and *L. lactis* subsp. *cremoris* SK11 as indicator organisms.

Using specific primers in PCR reactions on the DNA extracted from the isolates, we determined the presence of the *citP* gene, coding for citrate permease (3); the *prtP* gene, coding for the cell envelope proteinase (7, 27); and the *nisA* or *nisZ* gene, the structural genes for production of nisin A or nisin Z, respectively (4, 16) (Table 1). For all primer combinations, a standard PCR protocol was used: 94°C, 1 min, denaturation; 55°C, 1.5 min, annealing; 72°C, 2.5 min, elongation. The results (Table 3) show that strains with these properties can be found in various locations, including soil. Most of the strains which were positive for protease production on GMA agar contained sequences that could be PCR amplified with the specific primers for the *prtP* gene (Table 1) (7). The fact that some strains which were positive on the GMA agar did not contain sequences related to that of the cell envelope proteinase can be explained by either the presence of unrelated proteases or their ability to satisfy their amino acid requirements in another way. It is noticeable that those originating from the udder and skin from cattle and from raw milk show proteolytic activities. In contrast, strains isolated from soil were mostly nonproteolytic, but some of them were able to ferment citrate. The citrate-fermenting strains, however, did not seem to possess sequences related to that of the *citP* gene coding for citrate permease, which is so far the only carrier found in dairy strains that is involved in the translocation of citrate across the cell membrane (3). More detailed studies elucidated that these strains fermented citrate at a much lower rate than the industrially used *L. lactis* subsp. *lactis* var. *diacetylactis* strain (data not shown). These strains may use a different, possibly aspecific, transport system with a lower affinity for citrate.

Three of 25 strains showed antimicrobial activity against the indicator strains tested. All three of them contained DNA that could be PCR amplified with specific primers for the *nisA* or *nisZ* gene (Table 1). The fact that all three producers of antimicrobial activity appeared to be nisin producers conforms to the expectation, since it is the most common antimicrobial peptide found to be produced by *L. lactis* subsp. *lactis* strains (4). All examined strains from environmental sources were found to be resistant to phages present in whey preparations from industrial cheese plants that used the same starter culture as that analyzed in this study (data not shown) (25).

Using a genus-specific probe in combination with direct platings and enrichment cultures, we were able to isolate *Lactococcus* spp. from various environments, indicating that lactococci can survive outside the dairy environment and that some are able to persist in soil and effluent water, on vegetation, and on cattle. The detection of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* strains in nondairy environments supports the hypothesis that *L. lactis* strains present in starter cultures and in spontaneous milk fermentations originate from cattle and their surroundings (20). However, the isolated strains of *L. lactis* differ from those present in starter cultures in a number of properties, such as the ability to hydrolyze arginine and to grow at 37°C and 4% NaCl for *L. lactis* subsp. *cremoris* isolates or the absence of *citP*-related sequences and the resistance to industrially important phages for the *L. lactis* subsp. *lactis* isolates. In addition, the numbers of lactococci found in the nondairy environments were considerably lower than the amounts which are daily released into the environment due to the industrial production of fermented milk products. This also suggests that most starter organisms are not able to persist in nondairy environments, although natural niches are available. The industrially relevant properties found in *L. lactis* strains isolated from nondairy environments and the resistance of these strains to dairy-related phages may allow their use in the development of new fermented milk products, which is currently being evaluated.

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Chapter 4

Genetic marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract

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Genetic Marking of *Lactococcus lactis* Shows Its Survival in the Human Gastrointestinal Tract

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A human feeding study was performed with *Lactococcus lactis* TC165.5, which is genetically marked by insertion of the sucrose-nisin conjugative transposon Tn*5276* and chromosomal resistance to rifampin and streptomycin. The fate of strain TC165.5 and its nucleic acids was monitored by conventional plating methods and by molecular detection techniques based on specific PCR amplification of the nisin (*nisA*) gene from DNA extracted from human feces. A method was developed for the efficient extraction of microbial DNA from human feces. The results show that a fraction of viable cells of *L. lactis* TC165.5 survived passage through the human gastrointestinal tract. Only cells that passed within 3 days of ingestion could be recovered from the feces of the volunteers, and they accounted for approximately 1% of the total number of cells consumed. The presence of *nisA* in DNA extracted from feces could be detected up to 4 days, when viable cells were no longer present.

The use of lactic acid bacteria in the production of fermented foods has a long history (12). The application of these starter bacteria is aimed mainly at the production of foods with longer shelf life and better organoleptic properties. Much research effort has been focused on optimizing the performance of lactic acid bacteria during product formation or on broadening their applications, such as their use as probiotics (15) or as live vaccines (5, 14, 24).

The most commonly used starter bacteria include strains of *Lactococcus lactis*, which are used in the manufacturing of many cheeses and other fermented dairy products. In recent years, the genetics of mesophilic lactic acid bacteria have been greatly advanced and several tools have been developed for homologous and heterologous gene expression (5). This has resulted in the availability of genetically modified strains with prospects for applications in dairy product manufacturing and as oral vaccines (14, 24). For the application of genetically modified lactococci both as starter cultures and in health improvement, as a probiotic or a live vaccine, it is important to determine whether these bacteria survive in the gastrointestinal tract after consumption by humans.

The presence of *Lactococcus* spp. in the flora of the human gastrointestinal tract has been demonstrated in a few studies, but the discrimination between *Lactococcus* spp. and *Enterococcus* species is not always possible with classical identification methods (4). Stable populations of *L. lactis* can be established in monoassociated gnotobiotic mice, indicating that lactococcal strains can be maintained in an intestinal environment, although their optimum growth temperature is 30°C (8, 21). To obtain more information about the survival and the stability of lactococci in the human gastrointestinal tract, a study was performed with a genetically marked *L. lactis* strain in human feeding trials, during which the survival and persistence of the chromosomal DNA was monitored by conventional plating methods and molecular detection techniques (21).

DNA extraction from human feces. A sensitive detection method for *L. lactis* in human feces, based on DNA extraction and specific PCR amplification, was developed. Several methods have been described for the extraction of DNA from human fecal material (20, 22). We have developed a protocol based on the isolation of the microbial fraction, followed by cell lysis and chloroform-phenol extraction. The freshly collected fecal sample was stored at 4°C (up to 24 h), and 5 g was resuspended in 100 ml of a 300 mM sucrose solution. A 2-ml portion of this suspension was centrifuged at 2,750 × *g* for 10 min. The supernatant was removed, and the pellet was resuspended in 2 ml of sucrose solution. After centrifugation for 1 min at 750 × *g*, the supernatant, containing over 80% of the microbial fraction, was transferred to another tube. The efficiency of isolation of the microbial fraction was estimated by aerobic total plate counts of the suspension of freshly collected fecal sample on Columbia blood agar (Difco) plus 5% (vol/vol) sterile sheep blood and of the supernatant which contains the microbial fraction. The microbial fraction was collected by centrifugation at 2,750 × *g* for 10 min, and the cell pellet was resuspended in 1 ml of THMS (30 mM Tris · HCl [pH 8.0], 3 mM $MgCl_2$, 25% [wt/vol] sucrose) containing 2 mg of lysozyme per ml. Protoplasts were formed at 37°C for 1 h, after which the cells were lysed by adding 1 ml of TES (50 mM Tris · HCl [pH 8.0], 5 mM EDTA, 50 mM NaCl) containing 1% sodium dodecyl sulfate. DNA was obtained by chloroform-phenol extraction and ethanol precipitation (19) and further purified with genomic tips by following the manufacturer's protocol (QIAGEN, Chatsworth, Calif.). This preparation could be used in PCR without interference by inhibitory substances that are reported to be coextracted from feces (1).

Specific detection of the indicator strain by PCR amplification. *L. lactis* TC165.5 (18) was used as the indicator strain for consumption studies. This strain is a transconjugant of *L. lactis* MG1614 harboring a single copy of the sucrose-nisin transposon Tn*5276* (18). Strain MG1614 is a spontaneous mutant of the plasmid-free strain MG1363 (6) and shows resistance against rifampin and streptomycin. The presence of transposon Tn*5276* in strain TC165.5 allowed its specific enumeration on sucrose indicator plates consisting of Eliker agar (3) with 0.05% bromocresol purple and 0.5% sucrose and supplemented with rifampin (50 μg/ml) and streptomycin (100 μg/ml). MG1363 is a derivative of NCDO 712 and contains a 16S

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TABLE 1. Primers and probes used in this study

Primer or probe[a]	Target	Base positions	Sequence	Reference
Primers				
P1(S)	16S rRNA	41 to 60[b]	GCGGCGTGCCTAATACATGC	9
P2(A)	16S rRNA	686 to 705[b]	ATCTACGCATTTCACCGCTA	9
Pnis1(A)	Nisin	−99 to −78	CGCGAGCATAATAAACGGCT	12
Pnis2(S)	Nisin	201 to 220	GGATAGTATCCATGTCTGAAC	12
Probes				
Pgen(A)	16S rRNA	338 to 358[b]	CTGCTGCCTCCCGTAGGAGT	9
Pnis(S)	Nisin	1 to 20	ATGGGTTGTAATATGAAAAC	This study
PLc(A)	16S rRNA	V1 region	TTCAAATTGGTGCAAGCACC	12

[a] S, sense sequence; A, antisense sequence.
[b] *Escherichia coli* numbering is used.

rRNA sequence specific for *L. lactis* subsp. *cremoris* (7). Since the genotypically *L. lactis* subsp. *cremoris* indicator strain contains the structural nisin (*nisA*) gene, it could very well be distinguished from natural *L. lactis* subsp. *lactis* strains carrying the nisin operon (2).

To determine the sensitivity and specificity of the molecular detection method, different concentrations of *L. lactis* TC165.5 were added to fecal suspensions. The extracted DNA was used in a PCR amplification of the *nisA* gene with specific primers Pnis1 and Pnis2 (Table 1) (13). The products were blotted on a nylon membrane (GeneScreen plus; Dupont, Boston, Mass.) and hybridized with an internal specific probe Pnis (Table 1). The results (Fig. 1) show that it is possible to detect 10 cells per ml of resuspended feces (approximately 1,000 cells per g of feces). All extracted DNA was checked for the presence of PCR-inhibiting components by performing control amplifications with primers P1 and P2 based on conservative regions of the 16S rRNA, which, after blotting, were hybridized with the general 16S rRNA probe Pgen (Table 1) (9).

The use of specific PCR amplification in combination with an efficient DNA extraction of DNA from human fecal material is especially suitable for the specific and sensitive detection of bacteria which are present in relatively small numbers in human feces and are difficult to enumerate by conventional plating techniques, e.g., for the study of probiotic bacteria and the microbial ecology of the gastrointestinal tract.

Survival of lactococcal cells in the gastrointestinal tract. A double-blind human feeding trial was performed with six volunteers to establish whether lactococci survive the passage of the gastrointestinal tract (Fig. 2, experiment 1). This investigation was approved by the Medical Ethical Committee of Wageningen Agriculture University. Written informed consent was obtained from each subject. The six volunteers were divided into two groups; one group consumed a nonfermented product (four-fifths sterile milk and one-fifth orange juice [pH 5.2]) containing 10^8 cells of *L. lactis* TC165.5 per ml, and the other group received the same product but without added bacteria. The products were consumed twice a day for 4 days (first period). One week after the final consumption in the first period, a second consumption period started in which the type of product consumed by the two groups was reversed. Samples of feces and saliva were taken, as indicated in Fig. 2. Dilutions of the samples were plated on the sucrose indicator plates, on which *L. lactis* TC165.5 produces yellow colonies. The authenticity of the colonies appearing on the plates was verified by using the *nisA* and *L. lactis* subsp. *cremoris*-specific PCR primers (Table 1) by amplifying from DNA isolated from the colonies. All yellow colonies appeared to be *L. lactis* TC165.5. In some cases, white colonies with a dissimilar morphotype were detected on the plates; on microscopic analysis, these appeared to be yeasts. DNA was also extracted from the fecal samples and analyzed by specific PCR amplification for the presence of the *nisA* gene. Nonstimulated saliva samples, collected in sterile glass tubes 16 h after the fourth consumption of each period, were applied directly to the sucrose indicator plates. The indicator strain could not be recovered, showing that the strain does not persist in the oral cavity.

It is evident that *L. lactis* TC165.5 survived passage of the gastrointestinal tract in all volunteers (Table 2). A selection of

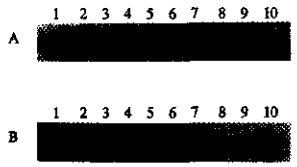

FIG. 1. Determination of the detection limit of *L. lactis* TC165.5 by PCR amplification. DNA was extracted from fecal suspensions (0.05 g [wet wt]/ml of 300 mM sucrose solution) to which different numbers of TC165.5 cells were added. This DNA was used in PCR amplification of the first half of the 16S rRNA with probes P1 and P2 (A) and of the *nisA* gene with probes Pnis1 and Pnis2 (B). Blots of the PCR products were hybridized with a general 16S rRNA DNA probe, Pgen (A), and a *nisA*-specific DNA probe, Pnis (B) (see Table 1 for a list of primers and probes). The following DNA solutions were used in the PCR amplifications: lanes 1 and 2, DNA isolated from fecal material with no TC165.5 added; lanes 3 to 8, 10^6, 10^5, 10^4, 10^3, 10^2, and 10 cells, respectively, of TC165.5 added per 5 g of fecal material; lane 9, purified DNA from TC165.5; lane 10, DNA isolated from fecal material to which purified TC165.5 DNA was added.

Outline experiment 1

day	1	2	3	4	5	12	14	15	16	17	18
group 1	C	C	CF_1	C	SF_2	F_3	C	C	CF_4	C	SF_5
(n=3)	Indicator strain						Placebo				
group 2	C	C	CF_1	C	SF_2	F_3	C	C	CF_4	C	SF_5
(n=3)	Placebo						Indicator strain				

S: saliva sample, F: fecal sample C: consumption of 2 X 100 ml product

Outline experiment 2

day	1	2	3	4	5	6	7	8	10	11
	FC	C	C	CF	F	F	F	F	F	F

F: fecal sample C: consumption of 100 ml product

FIG. 2. Outline of the two human feeding trials (experiments 1 and 2).

TABLE 2. Enumeration of *L. lactis* TC165.5 from the first human feeding experiment

Order of indicator and placebo administration	Person	*L. lactis* TC165.5 counts (log CFU/g of feces)[a]				
		Week 1		Week 2,	Week 3	
		F_1	F_2	F_3	F_4	F_5
Placebo/indicator	1	<2[b]	<2	<2	5.38	5.66
Indicator/placebo	2	3.45	6.53	<2	<2	<2
Indicator/placebo	3	5.08	4.62	<2	<2	<2
Placebo/indicator	4	<2	<2	<2	4.30	3.41
Indicator/placebo	5	3.98	3.83	<2	<2	<2
Placebo/indicator	6	<2	<2	<2	2.25	4.50

[a] F_1 to F_5, fecal samples in experiment 1 (Fig. 2).
[b] <2, below detection limit.

isolates were analyzed by specific PCR; they all contained the *nisA* gene and were identified as *L. lactis* subsp. *cremoris* (data not shown). The number of cells (approximately 10^4/g of feces) was such that on general specific media, they would have been overgrown by *Enterococcus faecalis* and other streptococci, which are generally present at concentrations of 10^8 cells per g of feces (4).

The results of the molecular detection experiment (Fig. 3) show that the *nisA* gene was present only in samples that contained viable cells of the indicator strain, although even samples containing less than 10^3 cells per g of feces (established detection limit, Fig. 1) yielded positive signals.

Quantification of the survival rate. To quantify the survival of *L. lactis* TC165.5 in the gastrointestinal tract, a second human feeding trial was performed (Fig. 2, experiment 2). For 4 days, four volunteers consumed (per day) 100 ml of a product similar to that in the previous experiment, containing 10^9 *L. lactis* cells per ml. In addition to lactococci, *Bacillus stearothermophilus* spores were added to the product as a microbial passage marker (10^5 spores [Merck, Darmstadt, Germany] per ml of product). The spores do not grow at 37°C and do not germinate in the gastrointestinal tract. Spores in the fecal samples were enumerated on plate count agar (Difco, Detroit, Mich.) incubated at 65°C (16). During a period of 9 days, all fecal material was collected and analyzed by selective plate counting and PCR amplification of the *nisA* gene.

In the fecal material collected shortly after consumption, about 0.1 to 2% of *L. lactis* TC165.5 cells survived passage through the intestinal tract (Fig. 4). After 2 days, the number of cells that had survived decreased rapidly, and after 3 days, no viable cells could be detected (detection limit, 10^2 cells per g of feces in the plate counts). Excretion of spores, however remained at a constant level for 4 days and then dropped gradually to below the detection limit on day 8 (Fig. 4). This result is in agreement with the passage kinetics of *B. stearothermophilus* spores observed by other investigators, and comparable passage kinetics have been found for *Bifidobacterium* spp., indicating that they can survive very well in the gastrointestinal environment (16, 17). The lactococcal counts in feces (Fig. 4) suggest that during the 4 days when the passage of spores is at a constant level, the numbers of lactococci decrease according to a first-order kinetics, indicating that the viability of the consumed lactococci declines consistent with the response of a homogeneous population to stress (10).

The decline of living lactococcal cells is also reflected in the gradual disappearance of DNA coding for the *nisA* gene in the fecal material after 3 to 4 days. Similar to the first experiment, the PCR detection method appeared to be more sensitive than the plating method, since some samples in which no viable cells could be detected showed a positive signal in the PCR amplification. This suggests the presence of nonculturable cells of TC165.5 or of naked DNA derived from lysed cells. Such DNA might be protected against nuclease activity by binding to particles, as has been demonstrated in soil and other environments (11).

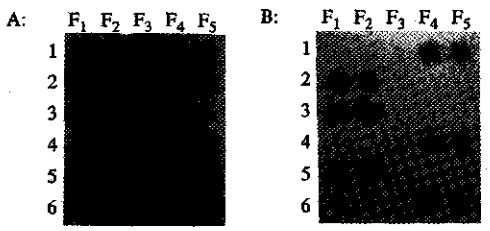

FIG. 3. Detection of the indicator strain *L. lactis* TC165.5 in human feces obtained in experiment 1. (A) Results of the PCR amplification of the first part of the 16S rRNA (with probes P1 and P2) and the hybridization with the general 16S rRNA probe Pgen (control for the absence of PCR-inhibiting components in the DNA isolation). (B) Results of the PCR amplification of the *nisA* gene (with probes Pnis1 and Pnis2) and the hybridization with the *nisA*-specific DNA probe Pnis are shown. Rows 1 to 6, volunteers; columns F_1 to F_5, fecal samples (Table 2).

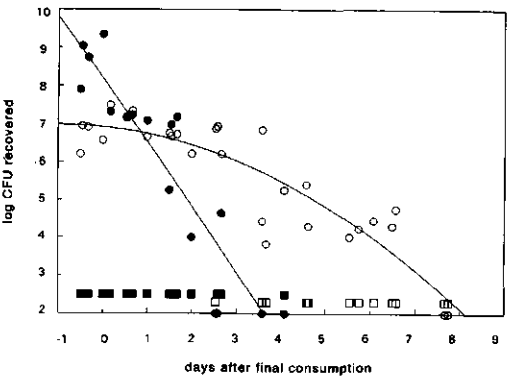

FIG. 4. Recovery of *L. lactis* TC165.5 and *B. stearothermophilus* spores in the feces of four volunteers. Viable counts of *L. lactis* TC165.5 and *B. stearothermophilus* recovered from fecal samples obtained from four volunteers during experiment 2 (outline in Fig. 2) are shown. In addition, the persistence of the *nisA* gene was determined in the same samples by specific PCR. 0 represents the time of final consumption. Symbols: ●, *L. lactis* TC 165.5; ○, *B. stearothermophilus* spores; ■, positive PCR signal for *nisA*; □, negative PCR signal for *nisA*. When general PCR primers were used, all DNA extractions were shown to be free from PCR-inhibiting factors.

This study shows that a substantial proportion of *L. lactis* cells, consumed in a dairy product, survive the passage of the gastrointestinal tract, provided that they do pass within 3 days after consumption. This is in agreement with in vitro work (23) showing that lactococci had a relatively high resistance to conjugated bile salts, comparable to that of intestinal bacteria. Both the fact that only up to 2% of the total amount of consumed bacteria are recovered and the fact that their numbers in the feces decrease more rapidly than those of the microbial passage marker, however, indicate that the viability of the lactococci is negatively influenced by the gastrointestinal environment. The question whether they are metabolically active in the gastrointestinal tract still remains to be answered. The partial survival of lactococci gives a positive prospective for the use of *Lactococcus* strains in the development of oral vaccines (12). On the other hand, the survival of lactococcal cells in the gastrointestinal tract may have consequences for the evaluation of genetically modified strains, notably those containing antibiotic resistance genes, used in the production of foods. It has been shown that gene transfer between lactococcal cells and other microorganisms, especially via conjugation, is possible in the gastrointestinal environment (7). The experimental outline in this study has been shown to be suitable for determining the fate of microorganisms and their nucleic acids during the passage of the gastrointestinal tract and can be used in future research on probiotics and on the risk assessment of the use of genetically modified microorganisms in food.

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Chapter 5

Identification of *Clostridium tyrobutyricum* and related species using sugar fermentation, organic acid formation and DNA probes based on specific 16S rRNA sequences

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Identification of *Clostridium tyrobutyricum* and Related Species using Sugar Fermentation, Organic Acid Formation and DNA Probes Based on Specific 16S rRNA Sequences

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Summary

Spores of *Clostridium tyrobutyricum* are considered as the causative agents for butyric acid fermentation (late blowing) in brine-salted, semi-hard and hard cheeses. In order to enable the early and specific detection of these bacteria, clostridia commonly present in raw milk and farm environments were classified using phenotypic and genotypic properties. In addition specific DNA probes were designed for the identification of clostridia commonly present in these environments.

Phenotypic characterization based on the API 20A system, the formation and conversion of organic acids and the litmus milk reaction, only allowed the differentiation of 71 clostridial strains into three groups, comprising the species *C. tyrobutyricum* (group A), *C. acetobutylicum, C. beijerinckii* and *C. butyricum* (group B) and *C. sporogenes* and *C. bifermentans* (group C) respectively. Strains belonging to the *C. tyrobutyricum* group formed a well-defined and homogenous collection. Using DNA probes based on specific 16S rRNA sequences and DNA-DNA hybridizations a perfect correlation was obtained with the phenotypic classification. The 16S rRNA probes allowed the specific identification of this species.

Using specific 16S rRNA probes, the species in phenotypic group B could be readily differentiated from each other and from other clostridia. A 16S rRNA probe was also developed for *C. sporogenes* belonging to group C. The probe allowed the differentiation of this species from all other clostridia. The identification based on the probes was fully compatible with the results from DNA-DNA hybridization studies.

Key words: rRNA targeted oligonucleotide – *C. tyrobutyricum* – Clostridia phenotypic characterization – DNA-probe – Cheese defect

Introduction

Clostridium tyrobutyricum is most commonly associated with late blowing of cheese (*Bergère and Sivelä*, 1990). For the production of brine salted, semi-hard and hard cheeses, e. g. Gouda, Edam or Emmental cheese, it is very important to limit the number of spores of this bacterium in raw milk (to less than 1 per ml) to prevent this cheese defect. Currently available methods to detect and enumerate these spores are rather inaccurate and non-specific, and take at least several days before the results become available (*Bergère* and *Sivelä*, 1990).

Recently molecular methods have been described for the sensitive and specific identification of micro-organisms (*Keller* et al., 1989). DNA probes, based on variable regions of 16S rRNA have been successfully applied for identification of or discrimination between various micro-organisms (*Barry* et al., 1990; *Wilson* et al., 1988). The use of a DNA probe for the identification of *C. tyrobutyricum* has also been reported (*Colleran* et al., 1990). The application of a specific detection method for *C. tyrobutyricum* in the diary industry will, however, not only depend on its ability to specifically detect all *C. tyrobutyricum* but also on the questions whether all *C. tyrobutyricum* strains can cause butyric acid fermentation in cheese and if butyric acid fermentation in cheese is always caused by *C. tyrobutyricum* only. The clostridial species *C. tyrobutyricum, C. beijerinckii, C. butyricum* and *C. sporogenes* are

commonly present in raw milk and farm surroundings. They have also been frequently isolated from processed cheese and spoiled diary products, which indicates that they are able to grow in cheese and other diary products. The current detection methods (*Bergère* and *Sivelä*, 1990) are not specific for these clostridia in milk, in that all have the ability to form butyric acid and hydrogen in several media. Specific media for the discrimination of these species do not exist and also phenotypic discrimination of *C. acetobutylicum*, *C. beijerinckii* and *C. butyricum* is almost impossible (*Cato* et al., 1988). Therefore it is necessary to develop specific and sensitive identification methods for all relevant clostridial species in order to estimate the relevance of the presence of clostridial spores other than those of *C. tyrobutyricum* in milk in relation to butyric acid fermentation in cheese.

This paper describes the development and the evaluation of the use of DNA probes based on the V6 region of the 16S rRNA for the identification of *C. tyrobutyricum*, *C. acetobutylicum*, *C. beijerinckii*, *C. butyricum* and *C. sporogenes*. To this end we tested a set of strains of these species for their phenotypic properties, performed DNA-DNA hybridization and sequenced part of the 16S rRNA.

Materials and Methods

16S rRNA sequences. The sequences of 16S rRNA were obtained from the EMBL Nucleotide Sequence Database. Those of *C. aminovalericum* (accession number M23929), *C. barkeri* ATCC 25849 (M23927), *C. innocuum* (M23732), *C. mayombei* sp. nov. (M62421), *C. pasteurianum* (M23930), *C. ramosum* (M23731), *C. sticklandii* (M26494) originated from *Weisburg* et al. (1989), except that of *C. mayombei* sp. nov. which originated from *Kane* et al. (1991). *C. R. Woese* supplied the sequences of *C. tyrobutyricum* ATCC 25755 (M59113), *C. sporogenes* ATCC 3584 (M59115), *C. butyricum* ATCC 19398 (M59085) and *C. perfringens* ATCC 13124 (M59103).

Bacterial strains and growth conditions. 88 Clostridia, including reference strains were obtained from ATCC and DSM culture collections, from dairy research institutes in Bern (Switzerland), Kiel (Federal Republic of Germany), Jouy-en-Josas (France), and Wolfpassing (Austria), from IVVO (Lelystad, Netherlands), ADRIA (Quimper, France) and our laboratories. Most of these strains were originally isolated from dairy products or the farm environment. Some strains were received as unidentified and were classified during the investigation. The collection comprised the species *C. tyrobutyricum* (30 strains), *C. acetobutylicum* (4), *C. beijerinckii* (24), *C. butyricum* (5), *C. sporogenes* or sporogenes-like (20) and *C. bifermentans* (5). The strains were maintained in AC broth (Difco, Detroit). Ampoules containing AC broth were evacuated and sealed after inoculation. Following pasteurisation for 15 min at 75 °C they were incubated for a minimum of 4 days at 30 °C and stored at 4 °C after good growth was obtained. For the production of clostridial cells growth was carried out at 30 °C in anaerobic jars equipped with Anaerocult A packs (Merck, Darmstadt) unless otherwise stated.

Identification. The API 20 A system (Bio Mérieux, Marcy-l'Etoile, France) was used essentially as described in the instruction manual. Bacteria were harvested by swabbing RCM agar (Merck) plates which had been incubated anaerobically for a maximum of 3 days at 30 °C, and suspended in sterile water. The optical density of the bacterial suspension was visually adjusted to a McFarland 3 tube. The strips were incubated at 30 °C for two days under anaerobic conditions. Further identification was done by HPLC analyses of organic acids (lactic, formic, acetic, propionic, butyric, iso-butyric, valeric, iso-valeric and caproic acid) present after incubating for 7 days at 30 °C in an anaerobic jar in two liquid media, i.e. AC broth and PAN broth (Composition: trypton, 5 g; L(+)-sodium lactate syrup [60%, wt/wt], 25 g [or 15 g sodium lactate]; yeast extract, 10 g; sodium acetate 3.aq, 8 g; water, 1 l. Sterilize 15 min at 121 °C. Final pH 6.5 ± 0.2). Then 0.4 ml of the grown culture was mixed with 3.6 ml of 0.01 M sulphuric acid, centrifuged (3,000 × g for 15 min) and membrane filtered (0.22 µ; Millex-GS, Millipore). These samples were stored at 4 °C. The HPLC analysis (injection volume 25 µl) was performed at room temperature on an Aminex Ion Exclusion column (HPX-87H, 300 × 7.8 mm; Biorad), using 0.01 N sulphuric acid (flow 0.6ml/min) as eluent and a refractive index detector (Erma-7510). Also the reaction in litmus milk was used for the identification. The reaction was judged after incubation at 30 °C for 7 days under anaerobic conditions and standing open to the air for one hour after incubation. The spore production was microscopically verified in cultures grown for 1 week at 30 °C under anaerobic conditions in three different liquid media, i.e. AC broth, PAN broth and RCM (Merck).

Isolation of DNA. For DNA isolation the strains were grown in AC broth or PAN broth as described above. When good growth was reached (OD_{600nm} = about 1.0) the cells were collected from 8 ml culture by centrifugation (3,000 × g for 15 min). The cell pellets were suspended in 500 µl of THMS (30 mM Tris.HCl, pH 8.0; 3 mM $MgCl_2$; 25% [wt/vol] sucrose) containing 1 mg lysozyme. The suspensions were incubated for 30 min at 37 °C to form protoplasts which were recovered by centrifugation and lysed by the addition of 500 µl TES (50 mM Tris.HCl, pH 8.0; 50 mM NaCl; 5 mM EDTA) containing 1% sodium dodecyl sulphate (SDS). The DNA was further purified by phenol-chloroform extraction and precipitation by adding 0.1 volume of 3 M sodium acetate and 2 volumes of 96% ethanol (*Sambrook* et al., 1989). The DNA pellet was washed with 70% ethanol, dried and finally dissolved in 200 µl TE buffer (10 mM Tris.HCl, pH 8.0; 1 mM EDTA) and stored at 4 °C. The yield of the DNA isolation was checked by agarose gel electrophoresis (*Sambrook* et al., 1989).

Dot blot hybridization. For dot blot hybridization, about 25 µl of the DNA samples were denaturated by the addition of 25 µl 0.5 M NaOH and then transferred to GeneScreen Plus membrane (DuPont, Boston, USA) using a dot blot manifold (Minifold; Schleicher & Schuell, Inc., Keene, N.H.). After blotting, the membranes were neutralized in 0.5 M Tris.HCl (pH 8.0) and air dried. Prehybridization and hybridization were performed in a 0.5 M sodium phosphate buffer (pH 7.2) containing 3% SDS and 1% bovine serum albumin. After 30 min prehybridization at 55 °C the probe, that had been 5'-ended labelled using [γ-^{32}P]ATP (Radiochemical Centre, Amersham, UK), was added and the incubation continued for 4 hours. The blots were washed with 0.3 M NaCl and 0.03 M sodium citrate at 60 °C until a clear signal was found and then exposed to Kodak X-ray films.

DNA-DNA hybridization. DNA-DNA hybridizations were performed by filter hybridization according to *Johnson* et al. (1981). 5 µg DNA was labelled with [γ-^{32}P]ATP by nick translation (*Sambrook* et al., 1989). This amount is ten times the amount that was fixed to the nylon membrane (GenescreenPlus, Dupont). The hybridization was performed in a 0.5 M sodium phosphate buffer (pH = 7.2) with 1% bovine serum albumine and 3% SDS under relaxed conditions at 56 °C (± 25 °C below the Tm). The filters were washed stringently in 0.03 M NaCl and 0.003 M sodium citrate with 1% SDS at 60 °C (± 10 °C below the Tm). The radioactivity was measured with a phosphor screen and

the data were analyzed with ImageQuant software (Molecular Dynamics, Zoetermeer). The percentage of binding was determined relative to the signal found in the homologous hybridizations.

Sequencing of variable regions of 16S rRNA. Two methods based on PCR amplification (*Saiki* et al., 1988) were used for sequencing the V2 and V6 region of 16S rRNA (*Neefs* et al., 1990). In the first method parts of the 16S rRNA were amplified by using the following PCR primers based on conserved sequences; P1: [GCGGCGTGCCTAATACATGC] (position 41 to 60 in the *E. coli* numbering system), P2: [ATCTACGCATTTCACCGCTAC] (complementary to position 685 to 705 in the *E. coli* numbering system) and P3: [GGGTTGCGCTCGTTGCGGGGA] (complementary to position 1094 to 1114 in the *E. coli* numbering system) (*Brosius* et al., 1978). To amplify the V2 region P1 and P2 were used and for the amplification of the V6 region P1 and P3 were used. The same primers were subsequently used in the direct sequencing of both strands of the amplified DNA. The PCR amplifications were performed by using a Biomed Thermocycler (BioMed, Amstelstad, Netherlands). The reactions were carried out in sterile Multimax seal tubes with cap locks (1.5 ml), which contained 50 µl of the following buffer: 10 mM Tris.HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM NaCl, deoxynucleoside triphosphates at 2.5 mM, 100 ng of each primer and 1 unit of Taq polymerase. Template DNA (500 to 1000 ng) was added after being heated to 95 °C to eliminate all protease activity. The amplification was done in 30 cycles by melting the DNA at 93 °C for 1 min, annealing at 54 °C for 1.5 min and elongation at 72 °C for 2.5 min.

After PCR amplification the DNA was purified by electrophoresis on 1.5% agarose gel and the appropriate band isolated from the gel using GeneClean (Bio101 Inc., La Jolla, California). This purified DNA (about 0.2 µg) was then sequenced according to the procedure of *Casanova* et al. (1990), using 0.5% Nonidet P-40 to reduce renaturation of the template DNA. The second method was used to sequence two regions of the 16S rRNA gene from *C. tyrobutyricum, C. butyricum* and *C. sporogenes*. A first set of PCR primers, primer A2: [CGAACGCTGGCGGC] (position 30 to 43 in the *E. coli* numbering system) and primer B2 [GCCTCCCGTAGGAGT] (complementary to position 337 to 351 in the *E. coli* numbering system), was used to amplify a sequence spanning the V1 and V2 regions. A second set of primers, primer A6: [AGCGTGGGGAGCAAA] (position 767 to 781 in the *E. coli* numbering system) and B6: [CACGAGCTGACGACA] (complementary to position 337 to 351 in the *E. coli* numbering system), was used to amplify a fragment comprising the V5 and V6 regions. In each PCR amplification one of the primers was 5'-biotinylated. Two PCR reactions were performed with each set of primers, differing in the primer being biotinylated. The PCR amplifications were performed in a Thermojet thermocycler (Eurogentec, Seraing, Belgium) for 30 cycles with the following setting: denaturation at 94 °C for 1 min, annealing at 45 °C (for V1 and V2 regions) or at 43 °C (for V5 and V6 regions) and elongation at 72 °C for 1.5 min. The reaction mix contained 20 pmoles of the biotinylated primer, 100 pmoles of the second primer, 50–200 ng of template DNA and 1 unit of Taq DNA polymerase in PCR solution (20 mM Tris.HCl, pH 8.3, 1.5 mM $MgCl_2$, 25 mM KCl, 0.05% Tween-20, 0.1 mg/ml gelatin and 200 µM of each dNTP). After amplification, PCR samples were phenol-chloroform extracted and the final volume adjusted to 100 µl with water. Streptavidin coated magnetic beads (100 µl suspension from Promega, Madison, USA) were washed in a salt solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 1 mg/ml bovine serum albumin, pH 7.3) and then added to the PCR sample. NaCl was added to a final concentration of 0.2 M. After 5 min incubation at room temperature with occasional shaking, the beads were collected using the Promega magnetic stand and washed successively in 200 µl of TE buffer, 100 µl of 150 mM NaOH and 150 µl of TE buffer. The biotinylated strand was sequenced using the non-biotinylated primer of the corresponding PCR after addition of fluorescein at the 5'-end. Sequencing reactions were performed using the Eurogentec sequencing kit for automatic sequencer according to the manufacturer's instructions. Fluorescence-based DNA sequence analyses were performed with a Pharmacia Automated Laser Fluorescent DNA Sequencer using a 7% polyacrylamide gel.

Results and Discussion

Probe development

By comparing published sequences of 16S rRNA of a variety of clostridia (*Kane* et al., 1991; *Weisburg* et al., 1989) with the program CLUSTAL (*Higgins* and *Sharp*, 1988) the variable regions V2 and V6 were identified as containing the highest variability.

Subsequently the sequences of these regions of four strains of *C. acetobutylicum, C. beijerinckii, C. butyricum, C. sporogenes* and *C. tyrobutyricum* were determined (see Fig. 1). With the exception of *C. acetobutylicum*, all strains used were isolated from dairy and farm samples. Furthermore, the selected strains of *C. tyrobutyricum* were known for their ability to cause butyric acid fermentation in cheese. Based on the observed differences in the sequence of the V6 region specific DNA probes could be designed for *C. acetobutylicum, C. beijerinckii, C. butyricum, C. tyrobutyricum* and *C. sporogenes* (see the underlined sequences in the V6 region in Fig. 1). It should be noted that the sequence we found for *C. acetobutylicum* in the V2 and V6 region of the 16S rRNA differs from that published by *Collins* et al. (1992), which was identical to the sequence we determined for the V2 and V6 region of 16S rRNA of *C. beijerinckii* ATCC 25752 (Fig. 1).

Fermentation patterns and acid production

To assist in validating the developed probes we classified a collection of strains, partly isolated from dairy products or the farm environment and partly reference strains from culture collections (see Table 2 for a selection of these strains), using conventional techniques. Sporulation was observed with all strains. Based on the API 20 A system (Table 1) at least three groups of clostridia could be clearly distinguished, comprising a) *C. tyrobutyricum*; b) *C. acetobutylicum, C. beijerinckii* and *C. butyricum*; and c) *C. sporogenes* and *C. bifermentans*. The results corresponded rather well with the reactions shown in the API index as far as these are currently available. The fermentation pattern of *C. tyrobutyricum*, which is not given in the API index, is very characteristic and enabled us to distinguish this species very well from the other clostridia, as was also shown by *Rapp* et al. (1987). The phenotypic similarity between *C. acetobutylicum, C. beijerinckii* and *C. butyricum* has previously been described by *Cato* et al. (1986) and by *Magot* et al. (1983).

Results of additional tests, i.e. pattern of acids produced or converted in AC broth and PAN broth and reaction in litmus milk are presented in Figure 2 and Table 3,

V2-region:

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tyrobutyricum   AAGCCAA-GTTTCACATGGAATTTGGATG-AAAGGAGTAA-TTCGCT-TTGAGATGGACCCGCGGCGCATTA
sporogenes      ACATAAGAGAATCGCATGATNTTCTTATC-AAAG-ATTTA--TT-GCT-TTGAGATGGACCCGCGGCGCATTA
butyricum       AGATTGTAGTACCGCATGGTACAAGCAATTAAAGGAGTAA--TCCGCT-ATGAGATGGACCCGCGTCGCATTA
acetobutylicum  ATACTCGAGAATCGCATGATTCTTG-AGCCAAAGGATTTA-TTCCGCT-ATGAGATGGACCCGCGTCGCATTA
beijerincjii    AGATTGTAGTGCCGCATGGCATAGC-AATTAAAGGAGTAA--TCCGCT-ATGAGATGGACCCGCGTCGCATTA
                       ******     *   **** * *   *** ************** *******
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V6-region

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tyrobutyricum   ACCTTACCTGGACTTGACATCCCC-TGAATAACCTAGAGATAGGCGAA-CC-CTTCGG-GGC-AGGGAGACAGGTGGTGCATGGTTGTC
sporogenes      ACCTTACCTGGACTTGACATCCCT-TGCATAGCCTAGAGATAGGTNAA--CCCTTCGG-GGC-AAGGAGACAGGTGGTGCATGGTTGTC
butyricum       ACCTTACCTAGACTTGACATCCTC-TGAATTACTCTGTAATGGAGNAAGCCACTTGGCTGGC-AGGAAGACAGGTGGTGCATGGTTGTC
acetobutylicum  ACCTTACCTAGACTTGACATCTCC-TGAATTAGTCCGTAATGGATNAAGTC-CTTCGGGGGC-ACAGAGACAGGTGGTGCATGGTTGTC
beijerincjii    ACCTTACCTAGACTTGACATCTCC-TGAATTACCCT-TAATCGGGGAAG---CTTCGGTGGC-AGGAAGACAGGTGGTGCATGGTTGTC
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Fig. 1. Alignment of the sequence of the V2 and the V6 region of the 16S rRNA of five *Clostridium* species. N: unknown nucleotide. –: no nucleotide. Asterisk (*): identical nucleotide in all sequences. The following strains have been sequenced:
C. acetobutylicum LMG 5710^T, LMG 5711, LMG 5712 and ATCC 4259
C. beijerinckii ATCC 25752^T, ATCC 858 and ATCC 14950.
C. butyricum ATCC 19398^T, ATCC 859, CNRZ 530[1] and NIZO B526[1].
C. sporogenes ATCC 3584^T, NIZO B544, NIZO B565.
C. tyrobutyricum ATCC 25755^T, NIZO B570 (NIZO 51)[2], NIZO B577 and CEGT 01.
[1]: Based on the obtained sequence these strains appeared to belong to the species *C. beijerinckii*.
[2]: Received from *van der Meer* et al. (1993).
^{T}: Type strain.

Table 1. Results of API 20 A test, combined per species[a].

Species		ind	ure	glu	man	lac	sac	mal	sal	xyl	ara	gel	esc	gly	cel	mne	mlz	raf	sor	rha	tre
C. acetobutylicum	NIZO (6)	0	17	100	100	100	100	100	100	83	100	0	100	50	67	67	83	50	33	33	33
C. beijerinckii	NIZO (4)	0	0	100	100	100	100	100	100	100	100	25	100	75	100	100	75	100	100	100	75
	API	5	0	99	20	94	99	99	99	97	82	29	75	75	99	99	5	95	15	5	88
C. bifermentans	NIZO (5)	20	0	100	0	0	0	100	0	0	0	100	100	20	0	100	0	0	0	0	0
	API	99	1	92	0	0	0	64	10	0	0	90	5	3	0	55	0	0	4	0	0
C. butyricum	NIZO (6)	0	0	100	100	100	100	100	100	83	83	50	100	67	100	100	67	83	100	83	100
	API	5	0	99	20	94	99	99	99	97	82	29	75	75	99	99	5	95	15	5	88
C. sporogenes	NIZO (20)	0	0	95	0	5	5	90	0	0	0	100	100	0	0	0	0	5	0	0	15
	API	0	0	99	0	0	0	84	0	0	0	99	58	0	0	0	0	0	0	0	58
C. tyrobutyricum	NIZO (30)	0	0	100	97	7	10	0	0	20	10	7	0	0	0	100	0	0	0	7	3

NIZO: percentage of positive reactions found in this study. [a]: Indicated is the percentage of positive reactions. The number of strains per species is shown in parentheses. For comparison the profiles from the API index are shown. Ind: indole formation; ure: urease; glu, man, lac, sac, mal, sal, xyl, and ara: acid formation from glucose, mannitol, lactose, saccharose, maltose, salicin, xylose, and arbinose, respectively; gel: gelatin hydrolysis; esc: esculin hydrolysis; gly, cel, mne, mlz, raf, sor, rha, and tre: acid formation from glycerol, cellobiose, mannose, melezitose, raffinose, sorbitol, rhamnose, and trehalose, respectively.

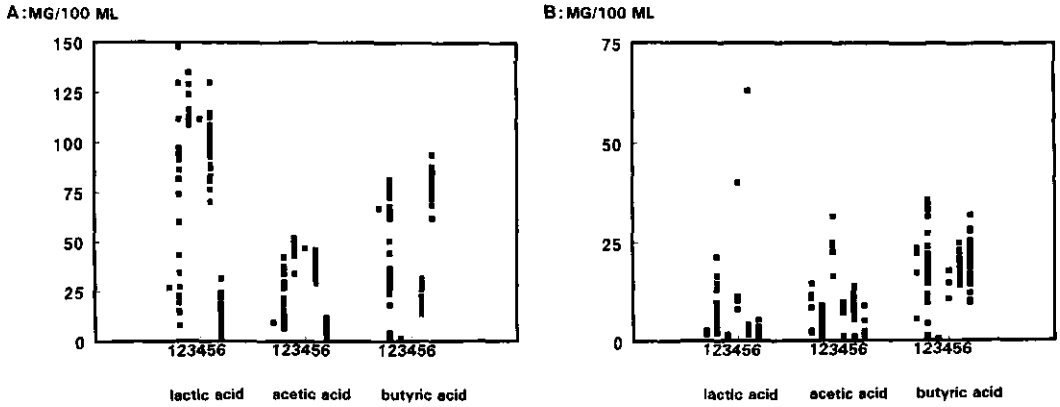

Fig. 2. Concentrations of lactic, acetic and butyric acid in mg per 100 ml. Squares represent individual strains tested. A: after growth in PAN broth by strains[1] of 1: *C. acetobutylicum* (1), 2: *C. beijerinckii* (24), 3: *C. bifermentans* (7), 4: *C. butyricum* (1), 5: *C. sporogenes* (25) and 6: *C. tyrobutyricum* (30). B: After growth in AC broth by strains[1] of 1: *C. acetobutylicum* (5), 2: *C. beijerinckii* (22), 3: *C. bifermentans* (6), 4: *C. butyricum* (5), 5: *C. sporogenes* (18) and 6: *C. tyrobutyricum* (25). The division in the different species was based on the identification with the specific DNA probes.
[1]: Only strains which had grown in AC broth or PAN broth were analyzed (in parentheses the number of analyzed strains).

Table 2. Results of DNA-DNA hybridizations with a selection of 26 strains belonging to six clostridial species.
The amount of homology is expressed in percentage. The type strains are underlined. (LMG 5710 = ATCC 824 = NCIMB 8052)
In parentheses () the names under which the strains were received are shown.

Strain		ATCC 19398	ATCC 25752	LMG 5710	ATCC 25755	ATCC 3584	ATCC 638
C. butyricum	ATCC 19398^T	100	2	5	2	2	4
	ATCC 859	98	3	3	5	4	2
	ATCC 860	97	4	5	6	2	2
	ATCC 3627	85	1	< 1	2	1	1
C. beijerinckii	ATCC 25752^T	7	100	5	7	6	3
(C. butyricum)	CNRZ 530	2	94	5	3	2	4
(C. butyricum)	NIZO B526	4	99	2	3	2	5
(C. butyricum)	ATCC 8260	6	80	1	< 1	< 1	< 1
(C. butyricum)	ATCC 17791	4	95	2	2	3	2
	ATCC 858	7	97	5	3	6	2
(C. acetobutylicum)	ATCC 10132	4	91	3	4	2	2
	ATCC 14950	3	90	4	1	1	< 1
C. acetobutylicum	LMG 5710^T	2	2	100	7	2	4
	LMG 5711 (t1)[1]	6	5	89	6	4	4
	LMG 5711 (t2)[1]	1	2	99	3	3	2
	LMG 5712	2	3	99	2	< 1	< 1
	ATCC 4259	3	5	87	3	5	7
C. tyrobutyricum	ATCC 25755^T	4	4	2	100	7	5
	NIZO B570	8	5	7	94	5	8
	CEGT 01	9	5	7	97	2	6
	NIZO B577	4	3	2	90	< 1	< 1
C. sporogenes	ATCC 3584^T	2	2	< 1	< 1	100	2
	NIZO B544	5	6	6	6	87	5
	NIZO B565	5	5	4	4	86	4
C. bifermentans	ATCC 638^T	6	4	3	3	5	100
	NIZO B528	2	2	< 1	< 1	< 1	98

^{T}: Type strain. [1]: t1 + t2: two morphologically different colony types of the same strain.

Table 3. Reaction of several species of clostridia in litmus milk. In parentheses: number of strains which had grown in milk and could be analyzed.

Species	Reaction in litmus milk
C. beijerinckii (13)	red +++ (11) red +++ and pept (1) red +++ and acid (1)
C. bifermentans (6)	pept (6)
C. butyricum (1)	coag (1)
C. sporogenes (23)	pept (23)
C. tyrobutyricum (33)	red (33)
C. acetobutylicum (6)	red +++ (1) red +++ and pept (5)

Acid: acid formation; coag: coagulation; pept: peptonization; red: reduction of litmus in upper part of tube; red +++: strong reduction in whole tubes.

respectively. Although recognizable patterns can be seen, no significant differences were observed for the various species. The fermentation patterns of *C. bifermentans* strains show less diversity, but the patterns in production levels of lactic, acetic and butyric acid found with strains belonging to the other species show no consistency on the species level. It can be concluded that both the production of organic acids and the reaction in litmusmilk (see Table 3) can give some information on the identity of the strain but not a reliable identification.

Probe specificity and DNA-DNA hybridization

In general there was a good correlation between the identification based on phenotypic properties and the identification based on the specific DNA probes. However some strains that were received as *C. butyricum* and *C. acetobutylicum*, species that are difficult to identify based on their phenotypic properties, gave a signal with the probe specific for *C. beijerinckii*. The strains *C. butyricum* CNRZ 530, M1, ATCC 8260 and ATCC 17791 and *C. acetobutylicum* ATCC 10132 appear to be wrongly classified and should be renamed as *C. beijerinckii*. The misidentification of *C. butyricum* strains CNRZ 530 en ATCC 8260 was previously shown by *Magot* et al. (1983) and *Matteuzi* et al. (1977). In order to test the applicability of the probes, the cross-reaction was investigated with a range of other micro-organisms which are commonly found in raw milk and the farm environment. No signal was found in hybridizations of the amplified V6 regions of 4 *Lactobacillus*, 5 *Lactococcus*, 3 *Streptococcus* and 2 *Bacillus* strains with any of the five specific DNA probes (data not shown).

To further corroborate the identifications obtained with the specific probes DNA-DNA hybridizations were performed with a selection of strains. Labelled total chromosomal DNA of the type strains was hybridized with membrane-fixed total chromosomal DNA of a selection of 26 strains (Table 2). High homology was observed between strains assigned to the same species and very low homologies between the species, confirming the grouping based on the hybridization of the chromosomal DNA with the species-specific DNA probes (Fig. 3). The low values of interspecies homology (< 1% – 8%) probably result from the very stringent washing conditions and the sensitivity of the measurement of radioactive signal obtained with a phosphor screen.

The results presented in our study show that sequencing the 16S rRNA of only one reference strain (usually the type strain) is not sufficient to obtain a reliable, representative sequence for a species, especially when species are phenotypically difficult to distinguish. Differences were found between the sequence of the V2 and V6 region of the 16S rRNA obtained in this study for *C. acetobutylicum* LMG 5710 (and three other *C. acetobutylicum* strains) and the published sequence for *C. acetobutylicum* NCIMB 8052 by *Collins* et al. (1992). The differences in the V2 region (49 bases) and the V6 region (33 bases) were respectively 20 and 11 bases (see Fig. 1). LM 5710 and ATCC 824 should be the same strain according to the culture collection manuals (LMG 5710 = ATCC 824 = NCIMB 8052). From the fact that *Wilkinson* et al. (1993) have found differences in genome size between the strains NCIMB 8052 and ATCC 824, it can be concluded that probably in the NCIMB culture collection a mix up has been made between the original strain and a *C. beijerinckii* strain. A switch between strains of these species is hardly noticeable based on phenotypical properties.

It is concluded from this study that specific DNA probes based on the V6 region of the 16S rRNA are suitable to identify and to differentiate the species of the genus *Clostridium* that are important in the spoilage of fermented milk products. Such probes may be particularly useful to study the relation between the presence of *C. tyrobutyricum* and other clostridial species and the occurence of late blowing in cheese, since they allow the rapid, sensitive and specific detection of the spoilage organisms at the species level.

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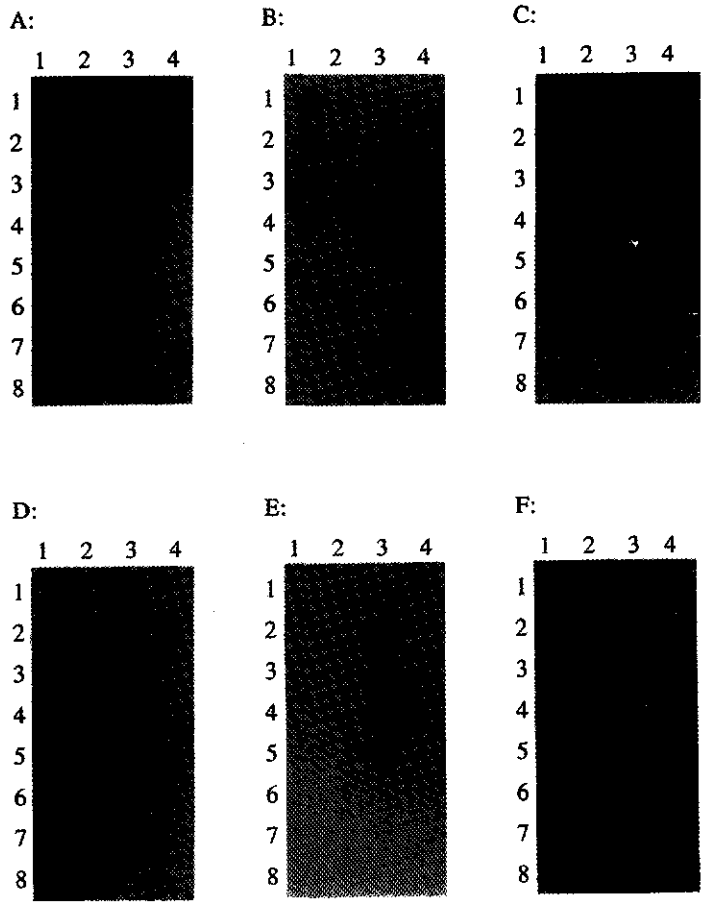

Fig. 3. Hybridization of 26 selected strains with species specific probes. The strains are spotted in the same order as they are placed in Table 2 (starting at the first column with 4 strains of *C. butyricum* and 8 strains of *C. beijerinkii*, at the fifth vow of the second column 5 strains of *C. acetobutylium, at the second row of the third column 4 strains of C. tyrobutyricum* followed by 3 *C. sporogenes* and 2 *C. bifermentans* strains). Total chromosomal DNA was blottet on nylon membranes and hybridized with the following probes:

A: general 16S rRNA probe	[GTATTACCGCGGCTGCT]
B: *C. butyricum*	[CTCCATTACAGAGTAATTCAGGAG]
C: *C. beijerinckii*	[CCCCGATTAAGGGTAATTCAGGAG]
D: *C. acetobutylicum*	[GACTTCATCCATTACGGACTAA]
E: *C. tyrobutyricum*	[CGCCTATCTCTAGGTTATTCAGGG]
F: *C. sporogenes*	[ACCTATCTCTAGGCTATGCAAGGG]

All probes are anti-sense to the coding region.

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Chapter 6

Identification of *Clostridium tyrobutyricum* as the causative agent of late blowing in cheese by species-specific PCR amplification

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Identification of *Clostridium tyrobutyricum* as the Causative Agent of Late Blowing in Cheese by Species-Specific PCR Amplification

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Butyric acid fermentation, the late-blowing defect in cheese, caused by the outgrowth of clostridial spores present in raw milk, can create considerable loss of product, especially in the production of semihard cheeses like Gouda cheese, but also in grana and Gruyère cheeses. To demonstrate the causative relationship between *Clostridium tyrobutyricum* and late blowing in cheese, many cheesemaking experiments were performed to provoke this defect by using spores from several strains of the major dairy-related clostridia. A method of PCR amplification of a part of the 16S rRNA gene in combination with hybridization with species-specific DNA probes was developed to allow the specific detection of clostridial sequences in DNAs extracted from cheeses. The sensitivity was increased by using nested PCR. Late blowing was provoked in experimental cheeses with 28 of the 32 *C. tyrobutyricum* strains tested, whereas experimental cheeses made with spores from *C. beijerinckii*, *C. butyricum*, and *C. sporogenes* showed no signs of butyric acid fermentation. In all experimental and commercial cheeses with obvious signs of late blowing, DNA from *C. tyrobutyricum* was detected; in some cheeses, signals for *C. beijerinckii* were also found. It was concluded that only *C. tyrobutyricum* strains are able to cause butyric acid fermentation in cheese.

Butyric acid fermentation in cheese (late blowing), caused by the outgrowth of clostridial spores present in raw milk and most commonly originating from silage, can create considerable loss of product, especially in the production of semihard cheeses like Gouda cheese, but also in grana and Gruyère cheeses (2, 3, 5, 20). Although *Clostridium tyrobutyricum* is the most frequently isolated strain from late-blown cheeses (3, 20), spores of other clostridia, particularly *C. beijerinckii*, *C. butyricum*, and *C. sporogenes*, have also been isolated from natural and processed cheeses and raw milk (5, 6, 21). Since all of these clostridia are capable of forming butyric acid and hydrogen in various media (19), the correlation between the appearance of this defect and the presence of a specific clostridial species is difficult.

For the production of semihard cheeses like Gouda cheese, it is very important to limit the number of spores of bacteria capable of causing late blowing in the cheese milk to fewer than 1 spore per 10 ml. Currently available methods to detect and to enumerate these spores in milk are inaccurate and nonspecific and take at least several days before the results are available (2). To improve these methods, it is crucial to know whether this defect is caused by a single *Clostridium* species or whether several dairy-related clostridia are able to cause butyric acid fermentation in cheese. The aim of this work was to demonstrate the causal relationship between *C. tyrobutyricum* and butyric acid fermentation in semihard Gouda cheese. This was done by performing many cheesemaking experiments with spores from many different clostridial strains. In addition, the presence of specific clostridial cells in many cheeses with the late-blowing defect obtained from commercial sources was analyzed.

Conventional methods for the isolation of clostridial cells from cheeses with late-blowing symptoms are very complicated and usually result in a mixture of isolates belonging to different clostridial species (3, 13). The identification of isolates is problematic since specific media to discriminate between the clostridial species mentioned above do not exist and phenotypic discrimination is almost impossible (4, 9, 12). For this reason, we previously developed identification methods that use specific DNA probes (9). The use of these probes on the basis of sequence variability in specific regions of the 16S rRNA gene (1, 14) allows reliable identification of *C. acetobutylicum*, *C. beijerinckii*, *C. butyricum*, *C. tyrobutyricum*, and *C. sporogenes*.

To overcome the problems associated with the isolation of clostridial cells from cheeses, we chose the strategy of directly detecting species-specific sequences in DNAs isolated from cheeses with specific probes. A method of PCR amplification (17) of a part of the 16S rRNA gene in combination with hybridization with species-specific probes (10) was developed. To further increase the sensitivity, this method was optimized by using specific probes in nested PCR. This nested PCR method was applied to study the relation between the presence of *C. tyrobutyricum* and other clostridial species and the occurrence of late blowing in commercial and experimental cheeses.

MATERIALS AND METHODS

Collection of strains. Sixty-seven clostridial isolates obtained from different culture collections were used in cheesemaking experiments and are listed in Table 1. Most of these strains were originally isolated in association with dairy products or from farm environments. All strains were classified by the methods described by Klijn et al. (9), which are based mainly on the API 20A system (15) and hybridization with species-specific DNA probes. Some strains were originally received as unidentified strains or appeared to have been classified incorrectly. Strains were maintained by inoculation in AC broth (Difco, Detroit, Mich.) in glass ampoules which were evacuated and sealed. Following pasteurization for 15 min at 75°C, ampoules were incubated for a minimum of 4 days at 30°C or until good growth was obtained and were then stored at 4°C. Clostridia were grown at 30°C in anaerobic jars equipped with Anaerocult A (Merck, Darmstadt, Germany) packs unless otherwise stated.

Production of spores. Spores were produced by growing cultures for 1 week at 30°C under anaerobic conditions in three liquid media, i.e., AC broth, PAN broth [tryptone, 5 g; L-(+)-sodium lactate syrup (60%; wt/wt), 25 g (or sodium lactate, 15 g); yeast extract, 10 g; sodium acetate 3.aq, 8 g; water, 1 liter; sterilized for 15 min at 121°C; final pH, 6.5 ± 0.2], and RCM (Merck). To produce sufficient

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TABLE 1. Strains used in cheesemaking experiments

Species	Strain(s)[a]
C. tyrobutyricum	ADRIA T860, ADRIA T932, ADRIA T103, ATCC 25755^T, DSM 663, BLVM 1519/A, CEGT01, CNRZ 500, CNRZ 505, CNRZ 564, CNRZ 569, CNRZ 596, CNRZ 611, EFAM 1528, EFAM 1553 EFAM 1554, EFAM 1556, EFAM 1558, EFAM 1559, EFAM 1600, EFAM 1602, EFAM 1519, EFAM 1527, IVVO V24, IVVO S42, NIZO BZ15, NIZO BZ18, NIZO S46, NIZO 51, NIZO FL104, NIZO BZ2, NIZO BZ6
C. butyricum	ATCC 19398^T
C. beijerinckii	ADRIA 6B3 ADRIA 25L17, ADRIA 27L17, ATCC 25752^T, ATCC 6014, ATCC 14823, ATCC 14949, ATCC 14950, ATCC 17791[b], BAM M1[b], BAM M2[b], CNRZ 530[b]
C. sporogenes	ADRIA SC25-4, ADRIA S882, ATCC 3584^T, BLVM 1363/A, BLVM 1527, EFAM 1356, EFAM 1534, EFAM 1552, EFAM 1601, IVVO V12A, IVVO V27, IVVO V30, IVVO V31, IVVO S39, IVVO V71, IVVO V80, IVVO V110, NIZO pII, NIZO S2, NIZO N2, NIZO 22.5, NIZO 24.1, NIZO 889

[a] Collections abbreviated as follows: ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; EFAM, Eidgenössiche Forschungsanstalt für Milchwirtschaft, Bern, Switzerland; BLVM, Bundesanstalt für Milchwirtschaft, Wolfpassing, Austria; CNRZ, Centre National de Recherches Zootechniques, Jouy-en-Josas, France; IVVO, Instituut voor Veevoedingsonderzoek, Lelystad, The Netherlands; ADRIA, Association pour le Dévelopement de la Recherche Appliquée, Quimper, France; NIZO, Netherlands Institute for Dairy Research.

[b] Received as *C. butyricum*.

quantities of spores, 100- to 1,000-ml cultures were centrifuged (15 min at about 3,000 × *g*), and spores were suspended in 10 ml of sterile skim milk and stored in small portions at −20°C.

After at least 1 week of storage, the number of CFU was estimated, both directly and after pasteurization for 15 min at 75°C, by plating on RCM agar and anaerobic incubation for 4 days at 30°C.

Cheesemaking. Gouda cheeses were made by standard procedures with bactofuged (spores eliminated by centrifugation) milk to reduce the initial levels of clostridial spores (22). From 800 kg of bactofuged cheese milk, 12 cheeses were produced in four cheese vats, one of which was used for the production of control cheeses. In the other three cheese vats, clostridial spores (100 to 1,000/ml) were added to the cheese milk. Spore suspensions were freshly thawed and used as such or pasteurized. To facilitate butyric acid fermentation, some adaptations of the standard procedures were applied. The pH was normal (5.3 at 14 days), the salt concentration (2.6% on dry matter at 14 days rather than 3.5%) and nitrate concentration (5 g of sodium nitrate per 100 liters of milk rather than 15 g) were lower, and the cheeses were ripened after being brined at 19°C instead of 13°C.

Detection of late blowing. During the ripening of cheeses, gas formation was regularly monitored by palpitation of cheeses by an experienced cheesemaker. After 6 and 12 weeks, cheeses were analyzed. Butyric acid fermentation in cheeses was detected both by visual inspection after the cheese had been cut and by high-performance liquid chromatography (HPLC) analysis of butyric acid. During visual inspections, the formation of holes by hydrogen gas produced during butyric acid fermentation (Fig. 1) and the presence of typical colonies of clostridia were observed. Such colonies are seen as dark round hard spots in cheese (standard diameter ± 1 mm). For butyric acid analysis, 10 g of ground cheese was mixed with 30 ml of 1 M perchloric acid and homogenized with a stomacher (Seward, London, United Kingdom) for 5 minutes. After settling for 1 h, the supernatant fluid was filtered through a Millex-GV membrane (0.22-μm pore size; Millipore). Samples were stored at 4°C before HPLC analysis. HPLC analysis (injection volume, 25 μl) was performed at 20°C on an Aminex ion-exclusion column (HPX-87H; 300 by 7.8 mm; Bio-Rad), with 0.01 N sulfuric acid (flow rate, 0.6 ml/min) as the eluent and a refractive index detector (Erma-7510). A butyric acid content of more than 100 mg per kg of cheese (in 12-week-old cheeses) was considered to be indicative of butyric acid fermentation.

DNA extraction from cheese. For DNA isolation from cheese, 5 g of ground cheese was homogenized in 50 ml of sodium citrate solution (2% [wt/vol] trisodium citrate dihydrate) at 45°C with a stomacher. From this suspension, 2 ml was incubated for 30 min at 37°C after the addition of 200 μl of proteinase K (final concentration, about 2 mg/ml). After centrifugation for 10 min at 2,750 × *g*, the fat layer was removed with a cotton tip and 1 ml of supernatant fluid was also removed with as much fat as possible. The remaining 1 ml, including the pellet, was incubated with 1 ml of THMS (30 mM Tris-HCl [pH 8.0], 3 mM $MgCl_2$, 25% [wt/vol] sucrose) and 100 μl of lysozyme (final concentration, 1 mg/ml) for 30 min at 37°C. Then 100 μl of 10% sodium dodecyl sulfate (SDS) solution (final concentration, 0.5%) was added and again incubated for 10 min at 60°C. Subsequently, 2 ml of phenol-TE (phenol equilibrated to TE [10 mM Tris-HCl, pH 8.0; 1 mM EDTA]) was added, as described by Sambrook et al. (18), mixed with a Vortex mixer, and centrifuged for 20 min at 2,750 × *g*. The supernatant fluid was transferred to a clean tube, and 1 ml of phenol-TE and 1 ml of chloroform were added. After being mixed with a Vortex mixer and centrifuged for 20 min at 2,750 × *g*, the top layer was transferred to a clean tube, and 2 ml of chloroform was added and mixed on a Vortex mixer. The tube was centrifuged for 3 min at 2,750 × *g*. The top layer was transferred to a clean tube, and 100 μl of 3 M sodium acetate (pH 5.5) and 5 ml of 96% ethanol (−20°C) were added. The tube was turned over a few times and stored overnight at −20°C. Then the tube was centrifuged for 20 min at 2,750 × *g*, and the liquid was removed as completely as possible. Two milliliters of 70% ethanol was added, briefly mixed with a Vortex mixer, and centrifuged for 10 min at 2,750 × *g*. The liquid was removed as completely as possible, and the pellet was dried in a vacuum exsiccator (about 15 min) and dissolved in 0.5 ml of TE.

PCR amplification. PCRs for specific amplification of the V6 region of the 16S rRNA gene were performed by using a Thermocycler 480 (Perkin-Elmer, Gouda, The Netherlands). The reactions were carried out in sterile 0.5-ml tubes which contained 50 μl of the following buffer: 10 mM Tris-HCl (pH 8.8), 3.0 mM $MgCl_2$, 50 mM NaCl, 2.5 mM (each) deoxynucleoside triphosphates, 1 U of *Taq* polymerase (Ampli-Taq; Perkin-Elmer), and 15 ng (each) of primers P3 and P4 (Table 2). After being heated to 95°C to eliminate all protease activity, 5 μl of template DNA was added. Amplification was done in 30 cycles of melting DNA at 94°C for 1 min, annealing at 55°C for 1.5 min, and elongation at 72°C for 2.5 min.

Nested PCR. Nested PCR was performed by first amplifying a part of the 16S rRNA gene (nucleotides 41 to 1114) with primers P1 and P2 according to the protocol described above for 45 cycles. The resulting PCR product was diluted 10-fold to decrease the remaining concentrations of primers P1 and P2. Then 5 μl of this dilution was used as the template for the second PCR amplification with one of the specific primers and P5 (Table 2). In this second step, the conditions were adapted with respect to the annealing temperature (63 or 72°C) and the number of cycles (20 or 25). When the annealing temperature was closer to the elongation temperature (72°C), the number of cycles was decreased (see Table 3).

Hybridization with specific DNA probes. For hybridization with specific probes, the PCR product (the amplified V6 region) was denatured by the addition of 100 μl of 0.5 M NaOH and then transferred to GeneScreen Plus membranes (DuPont, Boston, Mass.) by using a dot blot manifold (Minifold; Schleicher & Schuell, Inc., Keene, N.H.). After being blotted, membranes were neutralized in 0.5 M Tris-HCl (pH 8.0) and air dried. Prehybridization and hybridization were performed with 0.5 M sodium phosphate buffer (pH 7.2) containing 3% SDS and 1% bovine serum albumin. After 30 min of prehybridization at 55°C, the probe that had been 5′ end labelled with [γ-^{32}P]ATP (Amersham, Buckinghamshire, United Kingdom) was added, and incubation continued for 4 h. Blots were washed with 0.3 M NaCl and 0.03 M sodium citrate at 60°C until a clear signal was found and were then exposed to Kodak X-ray films.

RESULTS

Cheesemaking experiments. The strains used for contamination of experimental cheeses are listed in Table 1. None of the control cheeses showed any signs of late blowing after 12 weeks. During these experiments, it appeared that spores cultivated from pure cultures were less successful in provoking late blowing than were spores present in milk from natural contamination. Therefore, several cheese productions were carried out for a number of strains and large infective doses (1,000 spores per ml of cheese milk) were applied. The presence of late blowing was determined both by visual inspection (Fig. 1) and by analysis of butyric acid contents (more than 100 mg/kg of 12-week-old cheese). For all cheesemaking experiments, 28 of the 32 *C. tyrobutyricum* strains tested were shown to cause butyric acid fermentation in Gouda cheese. *C. tyrobutyricum* NIZO FL104, NIZO S46, EFAM 1527, and CNRZ 611 did not cause butyric acid fermentation in experimental cheeses. In parallel experiments with spores of 35 strains of *C. beijerinckii*, *C. butyricum*, and *C. sporogenes*, late blowing was never detected. Only one strain of *C. butyricum* was tested,

FIG. 1. Cheeses with (top) and without (bottom) butyric acid fermentation. Cheeses 67077 and 67080 were made of cheese milk contaminated with spores of *C. tyrobutyricum* NIZO 51 and ATCC 25755^T. Cheeses 67071 and 67074 were controls.

since it was not possible to obtain sufficient spores from the other *C. butyricum* strains in our collection.

PCR amplification and specific hybridization. Extraction from cheese of DNA that can be successfully used in PCR amplification is reported to be difficult (18, 19). The procedure developed here yielded relatively small amounts of DNA that were often not visible on agarose gels stained with ethidium bromide, especially from older cheeses. However, by using high magnesium concentrations in PCR mixtures together with the optimization of the dilution series, positive results were obtained in PCR amplifications.

The DNAs isolated from six experimental cheeses were used in PCR amplification of the V6 area of the 16S rRNA gene with general primers P3 and P4 (based on the conserved sequences flanking the V6 region) (Table 2). The reaction product was blotted on a nylon membrane and hybridized with P3 (as a control for PCR efficiency) and the species-specific DNA probes for *C. tyrobutyricum* and *C. sporogenes* (Table 2) (9). Specific signals were obtained with the *C. tyrobutyricum*-specific DNA probe for cheeses made with milk inoculated with *C. tyrobutyricum* spores and showing symptoms of late blowing (Table 3), as indicated by high levels of butyric acid content and excessive holes formed in the cheese matrix. No specific signals were found for control cheeses, cheeses not showing symptoms of late blowing although they were made of milk contaminated with spores of *C. tyrobutyricum* NIZO BZ18, or cheeses made of milk contaminated with spores of *C. sporogenes* IVVO V27. Amplified DNA from the last cheese also showed no signal when the *C. sporogenes*-specific DNA probe was used. To increase the sensitivity of this method, we developed a method involving nested PCR of isolated DNA.

Nested PCR. In the first step of nested PCR, part of the 16S rRNA gene (nucleotides 40 to 1114 [conforming to the *Escherichia coli* nomenclature]) was amplified with primers P1 and P2. The efficiency of this PCR amplification was checked on an agarose gel stained with ethidium bromide (Fig. 2A). After the first step was found to be successful, the second amplification was performed on the diluted product of the first step with a specific primer and P5 (Table 3). The specificities of the primers used in PCRs were validated with DNAs isolated from pure cultures of the different species used in this study (Fig. 2B to F). The products resulting from the second amplification step were analyzed on an agarose gel stained with ethidium bromide. This step can result in several amplification products because of the carryover of primers from the first PCR step. However, the concentration is relatively low, compared with that of the specific primer and P5 (15 ng/μl), as a result of incorporation into the product formed in the first PCR step and dilution between the two PCR steps. The largest fragment (1,070 bp) is the amplification product of P1 and P2 (the same product that is formed in the first PCR step), the next largest (750 bp) is the amplification product of P2 and P5, and the

TABLE 2. Primers used in this study

Primer and specificity (sense)[a]	Sequence	Annealing temp (°C)	*E. coli* numbering	Location
P1 (S)	GCGGCGTGCCTAATACATGC	55	41–60	Conserved region
P2 (A)	GGGTTGCGCTCGTTGCGGGA	55	1094–1114	Conserved region
P3 (S)	GCAACGCGAAGAACCTTACC	55	966–985	Conserved region
P4 (A)	ATCTCACGACACGAGCTGAC	55	1064–1083	Conserved region
P5 (S)	GGAATCTTCCACAATGGGCG	55–72	361–380	Conserved region
Pac, *C. acetobutylicum* (A)	GGACTTCATCCATTACGGACTAAC	63	≈1000–1050	V6 region
Pbe, *C. beijerinckii* (A)	CTTCCCCGATTAAGGGTAATTCAG	72	≈1000–1050	V6 region
Pbu, *C. butyricum* (A)	GTGGCTTGCTCCATTACAGAGTAA	72	≈1000–1050	V6 region
Pty, *C. tyrobutyricum* (A)	CGCCTATCTCTAGGTTATTCAG	63	≈1000–1050	V6 region
Psp, *C. sporogenes* (A)	CACCTATCTCTAGGCTATGCAA	63	≈1000–1050	V6 region

[a] S, sense; A, antisense.

TABLE 3. Comparison of detection methods for *C. tyrobutyricum* in cheese (amplification of the V6 region followed by hybridization with a specific DNA probe and nested PCR)

Inoculation[a]	Concn of butyric acid (mg/kg)[b]	Reaction result[c]				
		Amplification of V6 region	Hybridization		Nested PCR	
			C. tyrobutyricum[d]	*C. sporogenes*[e]	*C. tyrobutyricum*	*C. sporogenes*
No spores		+	−	−	−	−
C. tyrobutyricum NIZO BZ6	264	+	+	−	+	−
C. tyrobutyricum NIZO BZ6	953	+	++	−	+	−
C. tyrobutyricum NIZO BZ6	1,167	+	++	−	+	−
C. sporogenes IVVO V27	36	+	−	+	−	±
C. tyrobutyricum NIZO BZ18	66	+	−	−	+	−

[a] 1,000 spores per ml were added to the cheese milk as indicated.
[b] A concentration of >100 mg/kg is considered indicative of late blowing.
[c] ++, strongly positive reaction; +, positive reaction; ±, weakly positive reaction; −, no reaction.
[d] Hybridization probe, 5'-CCCCTGAATAACCTAGAGATAGGCG-3'.
[e] Hybridization probe, 5'-CCCTTGCATAGCCTAGAGATAAGG-3'.

smallest fragment (660 bp) is the amplification product of the specific primer and P5 (Fig. 2).

Specific detection of clostridia in cheese by nested PCR. The same cheeses used for direct hybridization of PCR products were analyzed by nested PCR (Table 3). This technique yielded positive reactions with the *C. tyrobutyricum*-specific primer in all cheeses contaminated with *C. tyrobutyricum* spores. In addition, nested PCR with the *C. sporogenes*-specific primer resulted in a specific signal in the cheese contaminated with *C. sporogenes* IVVO V27 spores, which had been negative by direct hybridization of the amplified V6 region from the 16S rRNA gene. To get an estimate of the relative sensitivity of this method, some cheeses contaminated with *C. tyrobutyricum* spores were monitored over time. After 3 weeks, clear signals were found by species-specific nested PCR (Table 4). At the same time, elevated concentrations of butyric acid were detected in these cheeses. The experiment showed that detection by nested PCR requires at least the germination and possibly outgrowth of spores, since no signals were found in cheeses during the first 2 weeks.

All experimental cheeses which showed no significant symptoms of late blowing were analyzed by species-specific nested PCR. In most cheeses inoculated with *C. tyrobutyricum*, weak signals were found, indicating that either the germination of spores or the growth of cells was retarded. In some cheeses contaminated with *C. beijerinckii*, *C. butyricum*, and *C. sporogenes*, clear signals were found by homologous species-specific nested PCR.

Nested PCR was subsequently used to analyze 23 cheeses from commercial sources (with and without late blowing symptoms) with primers specific for *C. acetobutylicum*, *C. beijerinckii*, *C. butyricum*, *C. tyrobutyricum*, and *C. sporogenes*. In the 11 commercial cheeses that did not show any symptoms of late blowing, no signal was obtained with any specific primer by species-specific nested PCR. In all commercial cheeses with

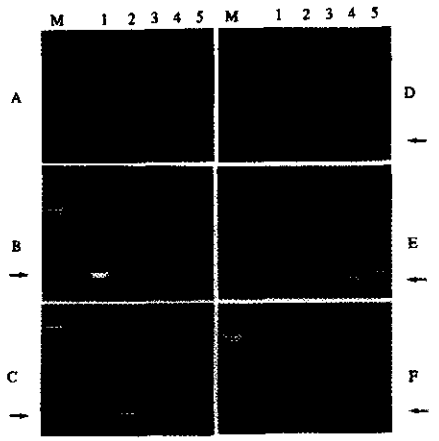

FIG. 2. Specificities of the species-specific primers used in nested PCR. DNAs extracted from pure cultures of the five type strains *C. acetobutylicum* LMG 5710 (lanes 1), *C. beijerinckii* ATCC 25752 (lanes 2), *C. butyricum* ATCC 19398 (lanes 3), *C. tyrobutyricum* ATCC 25755 (lanes 4), and *C. sporogenes* ATCC 3584 (lanes 5) were used in this nested PCR to demonstrate the specificities of the primers listed in Table 2. Lanes M, lambda *HindIII*. Shown are agarose (1.5%) gels stained with ethidium bromide and containing the products of PCR amplifications with primers P1 and P2 (A), Pac and P5 (B), Pbe and P5 (C), Pbu and P5 (D), Pty and P5 (E), and Psp and P5 (F). (B to F) Products of second PCR step. Arrows indicate the specific product (about 660 bp).

TABLE 4. Specific detection by nested PCR with the *C. tyrobutyricum*-specific primer in cheeses made of milk inoculated with *C. tyrobutyricum* NIZO 51[a]

Age of cheese (wk)	Concn of butyric acid (mg/kg)	Result	
		Visual inspection[b]	Specific PCR[c]
1	ND[d]	−	−
2	ND	−	−
3	267	+	+
4	677	+	+
5	970	+	+
6	969	+	+
7	956	++	+
8	1,226	++	+
9	1,893	++	+
10	1,676	++	+
11	1,841	++	+
6 (control)	ND	−	−
11 (control)	ND	−	−

[a] 1,000 spores per ml.
[b] −, no holes; +, isolated holes; ++, excessive hole formation and colonies present.
[c] −, negative reaction; +, positive reaction; ++, strongly positive reaction.
[d] ND, not detected (detection limit is about 50 mg/kg).

TABLE 5. Specific detection by nested PCR of five clostridial species in cheeses from commercial sources suspected of butyric acid fermentation[a]

Cheese code	Concn of butyric acid (mg/kg)	Reaction result[b]					
		General PCR	Specific PCR for:				
			C. tyrobutyricum	*C. sporogenes*	*C. butyricum*	*C. beijerinckii*	*C. acetobutylicum*
1	463	+	+	−	−	−	−
2	179	+	+	−	−	−	−
3	1,790	+	++	−	−	−	−
4	624	+	+	−	−	±	−
5	189	+	++	−	−	+	−
6	ND[c]	+	±	−	−	−	−
7	ND	+	±	−	−	−	−
8	109	+	+	−	−	−	−
9	96	+	±	−	−	−	−
10	119	+	+	−	−	±	−
11	195	+	+	−	−	±	−
12	307	+	+	−	−	−	−

[a] Eleven cheeses without symptoms of late blowing were negative in all specific PCR assays.
[b] ++, strongly positive; +, positive; ±, weakly positive; −, negative.
[c] ND, not detected (detection limit is about 50 mg/kg).

obvious gas production (holes in the cheese matrix) and elevated concentrations of butyric acid, specific nested PCR signals for *C. tyrobutyricum* were found, but some cheeses yielded an additional signal for *C. beijerinckii* (Table 5).

DISCUSSION

Extraction of DNA from cheese and other food matrices for subsequent use in PCRs is often considered to be a problem (16, 23, 24). Rossen et al. (16) have shown that cheese matrices, various food substances, and chemicals used to extract DNA from foodstuffs can inhibit PCR. Our work has shown that DNA extracted from cheese can be successfully used as template DNA in PCR. Potential problems were overcome by increasing the magnesium concentration in the PCR mix and optimizing the dilution of the cheese extract (generally 25- to 50-fold). Another method involving a two-phase extraction procedure to eliminate inhibitory substances from cheese extracts has recently been described by Lantz et al. (11).

A nested two-step PCR method was successfully developed to detect the presence of clostridial DNA in cheese. To obtain optimal performance in this nested PCR, previously described primers for *C. tyrobutyricum*, *C. sporogenes*, *C. butyricum*, and *C. beijerinckii* (9) had to be redesigned because the specificity of a PCR primer depends largely on the terminal bases at the 3′ end (7). These primers proved to be reliable in specific identifications of these various species (Fig. 2).

The procedure developed, which involves the extraction of DNA from cheese followed by nested PCR with specific primers, allowed the detection of *C. tyrobutyricum* in cheeses at adequate levels, since a clear signal was found in all cheeses with obvious symptoms of late blowing. The results of experiments in which the occurrence of specific DNA sequences in cheeses was monitored over time suggest that DNA is not extracted from spores and that germination and possibly outgrowth of cells are required to obtain a signal. Recently, a method of bead-bead extraction was reported to allow extraction of DNA from *Bacillus anthracis* spores (8). Adaptation of such a method to clostridial spores may provide additional information.

Our results show that with pure isolates, only *C. tyrobutyricum* is able to provoke late blowing; this species is present in all naturally contaminated cheeses that show this defect. This strongly suggests that the presence of *C. tyrobutyricum* is a prerequisite for the occurrence of butyric acid fermentation in cheese. However, it appeared that in cheeses artificially contaminated with cultivated spores of this species, late blowing was not always easily provoked. This could have been due to impaired germination of cultivated spores or to a decrease in the ability of germinated spores to form butyric acid and hydrogen gas, compared with that of natural milk contaminants.

In some experimental cheeses contaminated with spores of *C. sporogenes* or *C. beijerinckii*, specific signals were obtained by nested PCR, and signals were also found for *C. beijerinckii* in some commercial cheeses with late blowing. This shows that these clostridia are at least able to germinate and possibly to grow in cheese. Nevertheless, even if they do grow, these species apparently do not produce hydrogen gas and butyric acid under these conditions, even though they have the ability to do so in various culture media (19). Most anaerobic spores found in raw milk belong to the species *C. beijerinckii* (6). Since commercial cheeses with late blowing are obviously made of cheese milk with a high level of natural contamination of spores, the presence of *C. beijerinckii* can be expected.

To improve currently used methods for routine detection of clostridial spores in milk, the causative agent of late blowing in cheese had to be identified. This study unequivocally proves the causal relationship between *C. tyrobutyricum* and butyric acid fermentation in cheese and rules out the involvement of other dairy-related clostridial species. Currently, PCR amplification is not a technique that can be implemented as a routine detection method in the cheese industry. However, the results of this study indicate that a routine molecular detection method for *C. tyrobutyricum* spores in cheese milk with a species-specific DNA probe would be of great value to the dairy industry to avoid considerable losses during cheese production.

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Summary and concluding remarks

Summary and concluding remarks

The research described in this thesis concerns the development and application in food microbiology of molecular identification and detection techniques based on 16S rRNA sequences. The technologies developed were applied to study the microbial ecology of two groups of bacteria, namely starter cultures and sporeforming spoilage bacteria, that are of importance to the food industry and in particular the dairy industry.

In this Chapter the results are summarized and discussed in relation to recent developments. Moreover, the impact of the results obtained on quality control systems and risk assessment of the use of genetically modified microorganisms in food will be discussed.

Role of ribosomal RNA sequences in the identification and detection of microorganisms

In Chapter 1 several identification and detection techniques based upon rRNA sequences are described. They are based upon the structural and sequence differences within the ribosomal operon, like ribotyping, analysis of the spacer regions, or restriction legnth polymorphism of amplified parts. Some of the techniques strictly involve specific sequence differences within the rRNA genes, which consist of alternating conserved and more variable regions. The rRNA genes are uniquely suited for the reliable identification of microorganisms and the rational probe design for the specific detection of microorganisms. In particular 16S rRNA sequences have become to play an important role in microbial taxonomy. Many microorganisms have been reclassified or renamed based on new insights in their phylogenetic relationships based on 16S rRNA sequence homology. Within the constraints of the current data-bases it is now possible to identify new isolates quickly and reliably by sequencing their 16S rRNA. The use of rRNA sequencing in the identification and detection of microorganisms has indicated that many organisms are still unknown and not characterized, comprising both culturable and non-culturable microorganisms (25).

16S rRNA-derived DNA probes can either be based on conserved regions containing group- and/or genus-specific sequences or more variable regions containing species- and/or subspecies-specific sequences. These probes can be applied in hybridizations to DNA or RNA fixed to a membrane or present in *in situ* fixed cells. In order to increase the specificity and the sensitivity of a detection method, such probes can also be applied in specific DNA amplifications. Direct amplification of specific 16S rRNA sequences from, for instance, a food matrix like cheese, is the most straightforward and sensitive detection method. Unfortunately, most of these food matrices, and also other environmental samples like soil and faecal material, contain components that may inhibit the PCR reaction. This necessitates complex DNA extraction protocols or the use of enrichment cultures in order to obtain nucleic acid extracts that can be applied in PCR amplifications successfully.

It is possible to identify an isolate reliably at the species or subspecies level based on ribosomal sequences (Chapters 2 and 5). However, it is possible to distinguish isolates at the strain level with other techniques, like the frequently applied Random Amplified Polymorphic DNA (RAPD) technique, in which strain specific patterns are formed by the amplification with arbitrarily chosen primers of approximately ten nucleotides (1). This technique is often used in addition to 16S rRNA-based identifica-

tion methods, as has been shown recently by the discrimination of different serotypes of *Listeria monocytogenes* (2). The combination of both approaches can be very useful, in particular in epidemiologic studies (24). Moreover, RAPD patterns can be used for the development of strain-specific DNA probes as well (8). This can also be a very successful approach for obtaining species-specific DNA probes for microorganisms, such as *Campylobacter jejuni, Campylobacter coli* and *Campylobacter lari*, that are difficult to design on the basis of ribosomal sequences (11). It has been described that some genera, like *Rhizobium*, include species that contain an almost identical 16S rRNA but share almost no DNA-DNA homology. In order to discriminate between these species, other techniques have to be applied, of which RAPD or other chromosomal fingerprinting methods are used most frequently (5).

Development and application of molecular, 16S rRNA based, identification methods for Lactococcus *species*

In the food industry in general, but specifically in the dairy industry, lactic acid bacteria are used as starter cultures to initiate specific fermentations. Both for basic research on lactic acid bacteria and for their application in industrial food fermentations, reliable and simple methods for the identification of such bacteria are required. Because little difference exists in phenotypic properties of especially the mesophilic lactic acid bacteria, reliable identification and detection techniques have been developed based on specific sequences in variable regions of 16S rRNA (Chapter 1). Species-specific DNA probes, based on the first variable region (V1) of the 16S rRNA (16), were designed for *Lactococcus garvieae, L. plantarum* and *L. raffinolactis* and subspecies-specific DNA probes for *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. The third subspecies *L. lactis* subsp. *hordnieae* was not distinguishable from *L. lactis* subsp. *lactis* based on 16S rRNA sequences because these differed in only one base pair. In addition, species-specific probes for *Leuconostoc* were developed based on the third variable region (V3) of the 16S rRNA (16).

There is a growing need for new production strains for the innovation of dairy products. These can be obtained either by genetic modification of known production strains or by isolation of new strains from natural ecological niches. Both for the application of genetically modified starter strains and to allow for an efficient search for strains from natural ecosystems, it is important to know if and where a *Lactococcus* strain survives outside the dairy environment. Lactose-utilizing *Lactococcus* isolates from environmental samples taken on cattle farms and in the waste flow of a cheese production plant were identified up to the species level, using amplified variable regions (V1) of the 16S rRNA in combination with species-specific DNA probes (Chapter 3). These isolates were further characterized by using specific PCR amplification of sequences related to *cit*P, *prt*P and *nis*A, coding for citrate permease, protease and prenisin, respectively. It was possible to isolate *Lactococcus* spp. from various environments, indicating that lactococci can survive outside the dairy plant and that some are able to persist in soil, effluent water, on vegetation and on cattle. During the characterization of the environmental lactococcal isolates discrepancies were observed between the 16S rRNA based identification of *L. lactis* strains and identification based on their phenotypical properties. Similar findings were obtained with isolates from spontaneous milk fermentations, collected from several european countries (26). The classical differentiation between *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* is

Table 1. Different phenotypes found for the two ribotypes of *Lactococcus lactis*.

Strain	Source	Ribotype	Phenotype				
			37°C	4% NaCl	Arg	Citrate	Protease
LMG8514	Raw milk	*L. lactis* subsp. *lactis*	+	+	+	-	+
1F48	Farm cheese	*L. lactis* subsp. *lactis*	+	+	-	+	+
S19	Sheep milk	*L. lactis* subsp. *lactis*	+	+	-	-	+
8F35	Farm cheese	*L. lactis* subsp. *cremoris*	+	+	+	-	+
N118	Udder	*L. lactis* subsp. *cremoris*	+	+	+	-	-
SK110	Starter	*L. lactis* subsp. *cremoris*	-	-	-	-	+
B36R	Villi	*L. lactis* subsp. *lactis*	-	-	-	-	-
NCDO712	Starter	*L. lactis* subsp. *cremoris*	+	+	+	-	+

+ or - indicate the capacity or the absence, respectively, of growth at 37 °C, in the presence of 4% NaCl, to hydrolyse Arginine, to metabolize Citrate or to produce Protease.

based on phenotypical differences. *L. lactis* subsp. *cremoris* strains are characterized by their inability to hydrolyse arginine, to metabolize a number of sugars, and to grow at 37 °C and in the presence 4% NaCl . Based on SDS-PAGE of whole-cell proteins both phenotypes are distinguishable and form two seperate clusters (6, 20). However, within the group of environmental isolates, identified as *L. lactis* subsp. *lactis* on the basis of their phenotype, 16S rRNA sequences belonging to both *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* were encountered. Detailed characterization of a large collection of lactococcal isolates has led to the conclusion that within the species *L. lactis* two ribotypes are present, each having a specific 16S rRNA sequence. However, for each ribotype different phenotypes can be found (Table 1). The ribotype of the strain NCDO 712, the parental strain of MG1363 (10), which is frequently used in genetic studies of *L. lactis,* shows that it belongs to *L. lactis* subsp. *cremoris*, confirming the conclusion based on the mapping of its chromosome. It is quite remarkable that this strain shows the *L. lactis* subsp. *cremoris* ribotype, while phenotypically it resembles *L. lactis* subsp. *lactis*.

The phenotype described for *L. lactis* subsp. *cremoris* is only observed with isolates that have been obtained from industrial starter cultures or traditional fermented milk products like villi. Such strains may belong to either ribotype (Table 1). This may suggest that the continuous culturing in milk has resulted in a differentiation of phenotypic properties between "starter" strains and strains originating from environmental sources. Detailed characterisation of *L. lactis* isolates from natural environments indicated that they differ from strains commonly present in starter cultures. This does not only suggest that starter isolates do not survive outside the dairy environment, but also that *L. lactis* strains present within the natural population have potential to be applied in product innovation and differentiation. This applies in particular to isolates that were obtained from spontaneous milk fermentations, which are still used in the southern part of Europe for the production of artisanel cheeses (26). An initial inventory of the biodiversity of such isolates illustrates the large diversity of properties, like aroma

formation, acidification and bacteriocin production, which can be exploited to obtain differentiation in fermented milk products (26).

Molecular detection techniques were not only used for the identification of environmental isolates but also for the monitoring of the survival of *L. lactis* in the human gastrointestinal tract. For the potential application of *L. lactis* as probiotic, as genetically modified starter culture, or as live vaccine, it is important to determine whether these bacteria survive in the gastrointestinal tract after consumption by humans. In Chapter 4, a human feeding study is described in which the fate was monitored of *L. lactis* strain TC165.5, which was genetically marked by insertion of the naturally occurring sucrose-nisin conjugative transposon Tn*5276* and spontaneous chromosomal resistance to rifampicin and streptomycin. A method was developed for the efficient extraction of microbial DNA from human faeces. The passage of strain TC165.5 through the intestinal tract was monitored by selective plating and specific detection of the *nis*A gene by PCR amplification. The study showed that up to 0.1-1% of *L.lactis* cells, consumed in a dairy product, may survive passage of the gastrointestinal tract, provided that they pass within 3 days after consumption. The partial survival of lactococci provides a positive prospective for the use of *Lactococcus* strains as probiotic or in the development of live vaccines.

Biosafety assessment of the use of genetically modified Lactococcus *spp. in fermented food products*

The studies presented in Chapters 3 and 4 were part of an assessment of the biosafety aspects of the use of genetically modified starter cultures, specifically addressing general ecological parameters like survival, dissemination and transfer of genetic information. In order to quantify the survival of starter lactococci, careful monitoring of the waste flow of a cheese production plant was performed. This indicated the absence of typical industrial strains (Chapter 3) suggesting that most starter organisms are not able to persist in non-dairy environments, although natural niches are available. In addition, the numbers of lactococci found in the non-dairy environments were considerably lower than those that are daily released into the environment via the industrial production of fermented milk products. It was therefore concluded that there is no environmental release of viable starter bacteria resulting from the waste flow of the production process of fermented foods.

Another avenue for the release of lactic acid bacteria into the environment is by means of the consumption of fermented milk products by humans. The results of the human feeding trial (Chapter 4) showed that only a small fraction of viable cells of the marked *L. lactis* strain survived passage through the human gastrointestinal tract.

It is well known that *L. lactis* strains possess efficient systems for transfer of genes via plasmids, transposons and phages (10). A number of studies have been published on gene transfer in lactococci under natural conditions, including large-scale fermentation, cheese manufacturing, and passage through the gastro-intestinal tract of mice (7, 9, 12, 22). The results showed that the transfer rates decreased rapidly under natural conditions where cell-to-cell contact and growth are limited. On the other hand, under conditions favourable for cell-to-cell contact, such as on agar plates and intestinal mucosal surfaces, the transfer frequencies are relatively high, up to 10^{-4} (22).

The transfer of genetic information from one strain to another per se is not to be regarded as a potential hazard, since it was demonstrated that lactococci are already

capable of transferring genetic elements. In some cases, however, the properties encoded by new genetic traits may be potentially hazardous in combination with specific strains or in specific ecosystems. If the encoded properties are already present in the ecosystem, no specific new hazard may be expected, since in the past it has not resulted in hazardous biological consequences. By relating the potential hazards of the application of genetically modified lactic acid bacteria to the regular hazards associated with the consumption of fermented dairy products, the potential hazard may be normalized. So both for legislation purposes and for the acceptance by the consumers it is important to identify and normalize the biosafety aspects by relating them to current practice. In this way the potential hazards are more comprehensible and acceptable.

Development and application of molecular detection and identification methods for Clostridium tyrobutyricum

Major spoilage problems in the food industry are caused by sporeforming bacteria, belonging to the genera *Clostridium* and *Bacillus*, which survive heat-treatments that are applied to prolong the shelf-life of the food product. Butyric acid fermentation in cheese (late blowing), caused by the germination of clostridial spores which survive the heat treatment of cheese milk, is still causing considerable loss in the cheese producing industry. For the production of semi-hard cheeses, like Gouda cheese, it is very important to limit the number of spores in the cheese milk of bacteria capable of causing late blowing. Although *Clostridium tyrobutyricum* is regarded as the causative agent of butyric acid fermentation, also spores of other clostridia, such as *C. beijerinckii* and *C. sporogenes* are frequently isolated from late-blown cheeses. The current detection method for *C. tyrobutyricum* is not specific, since also other clostridial species are able to form butyric acid and hydrogen. In Chapter 5 and 6 the development of specific detection methods is described for the *Clostridium* spp. most frequently encountered in dairy environments. Based on specific sequences in the V2 and V6 region of the 16S rRNA, species-specific DNA probes were developed for *C. tyrobutyricum, C. acetobutylicum, C. beijerinckii, C. butyricum* and *C. sporogenes* (Chapter 5).

In Chapter 6 the causative relationship between *C. tyrobutyricum* and late blowing in cheese is demonstrated. Cheese experiments were performed to provoke this defect by using spores from different strains of several dairy-related clostridia. To overcome problems associated with isolation of *Clostridium* spp., specific clostridial sequences were directly detected in DNA isolated from cheese by a two-step specific PCR amplification. Only specific sequences of *C. tyrobutyricum* were detected in both the experimental and in commercially obtained cheeses showing signs of late blowing. This clearly identified *C. tyrobutyricum* as the causative agent of late blowing in cheese.

In order to prevent late blowing in cheese, the number of spores has to be limited to less than 1 per 10 ml of cheese milk. To improve the currently used methods for the routine detection of clostridial spores in milk, a specific and very sensitive method has to be developed. Although the current methods are very aspecific and often give false positive results mainly due to the presence of *C. beijerinckii* spores, it is possible to detect up to 1 spore per 100 ml by MPN-methods (17). Recently, Herman *et al.* have shown that it should be possible to detect one *C. tyrobutyricum* spore per 100 ml raw milk by concentrating the spores by centrifugation, followed by DNA extraction and a nested PCR amplification (13). However, all reports on DNA extraction from spores (21) describe experiments with artificially obtained spore-suspensions. It is not clear if these

methods can also be applied on natural spore populations. Since it is known that during the production of artificial spore suspensions residues of cell material, including DNA, can adhere to the outside of the spores, proper control experiments should be included. Such control experiments have not been described, so far making it impossible to evaluate the efficiency of the extraction methods described.

Relevance of molecular identification and detection techniques based on ribosomal sequences for the food microbiology in general and in particular for the dairy industry

The availability of molecular methods for the detection and identification of microorganisms has several advantages. In many cases the identity of both desired and unwanted microorganisms present in food products is of major importance. Enormous efforts have been invested in the reproducible identification of isolates based upon phenotypic properties such as carbohydrate fermentation, formation of specific metabolites, enzyme activities, and lipid composition. Unfortunately, most of these methods appeared to be irreproducible or not sufficiently discriminative for proper identification. The 16S rRNA-based methods are far more suited for the quick and reliable identification of isolates. However, the 16S rRNA sequencing share one particular limitation with many other identification methods, like those of API or Biolog, namely that it is only possible to obtain a correct identification within the limitations of the database. As many other databases, the RPD-database is not complete and contains only the 16S rRNA sequences of approximately 3000 bacteria (15). Most of the widely distributed species are present, but the 16S rRNA sequences of many bacteria frequently encountered in foods have not yet been determined or deposited. The absence of most *Brevibacterium* spp. and many other relatives of the *Arthrobacter* group, including *Corynebacterium* spp., is very problematic since they are important in the dairy industry as part of the surface microflora of surface-ripened cheeses and as spoilage organisms of pasteurized milk and cream. It appears that the biodiversity within this group is enormous, complicating clear systematic descriptions for these bacteria (19).

Molecular identification methods based on ribosomal sequences have revolutionized the classification and systematics for many bacteria, but also the ability to specifically detect microorganisms has made a great impact on food microbiology. This is particularly the case with respect to the detection and specific quantification of those bacteria that are unculturable, like *Candidatus arthromitus* (23), or those for which no suitable selective media are available, like *Bifidobacterium* spp.(14). In addition, molecular techniques will allow to obtain more detailed information on critical points in the production processes of foods (18). Such information is essential for safeguarding the product quality. For instance, by the use of strain-specific RAPD patterns to monitor the population dynamics of mixed-strain starter bacteria during the fermentation process, better insights could be obtained in which factors are important for the quality of the resulting fermented food product.

Although the potential sensitivity of molecular methods should make them suitable for the reliable detection of pathogens and spoilage organisms in food products and raw materials, there are some major practical problems that still have to be solved. One of the largest problems is the presence of PCR-inhibiting components in several food products, like cheese and meat. This inhibition can be circumvented by applying enrichment procedures before the actual detection with a specific PCR reaction. Even

if these procedures result in a quicker and more reliable detection of the target organism there are some important limitations to such procedures. It is known that a significant problem with the detection of some pathogens, like *Salmonella*, is the unreliability of a successful pre-enrichment in buffered peptone (3). This failure to obtain growth in the pre-enrichment step can be caused by competing flora or the physiological state of the target organisms (4). Still, the detection kits that are currently available, based on DNA-DNA hybridization, can be successfully used as verification methods for *Listeria* and *Salmonella* after a successful pre-enrichment.

Besides the practical problems, the implementation of molecular methods is also complicated by legislation, codes of practice, and regulations for processing and product control. Each adapted detection protocol for bacteria like *Salmonella, Listeria* and other important food pathogens requires extensive tests and validations before it is accepted by regulatory authorities. In addition, the routine application of these methods is still not possible because of the lack of automation for routine analysis of food products. Automated equipment developed for the medical market, is so far not suitable for this purpose and only a limited number of PCR detection kits have been specifically designed for application in the food industry.

In this thesis the usefulness has been demonstrated of molecular methods for the identification and detection of microorganisms relevant for the food industry. These methods have increased the insight in the microbiology of spoilage by sporeforming bacteria and in the ecological aspects of the use of lactic acid bacteria as starter cultures. Results obtained with molecular techniques, like 16S rRNA sequencing, quantification of specific DNA's, and *in situ* hybridisation, are expected to create new insights in the dynamics of complex microbial populations such as exist in starters, ripening flora of cheeses and other fermented foods.

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Samenvatting

Moleculaire methoden voor de identificatie en detectie van micro-organismen in de voedingsmiddelenindustrie

Moleculaire methoden voor de identificatie en detectie van micro-organismen in de voedingsmiddelenindustrie

Dit proefschrift beschrijft de ontwikkeling en toepassing van moleculaire identificatie en detectiemethoden voor micro-organismen die van belang zijn voor de voedingsmiddelenindustrie. Dergelijke methoden zijn noodzakelijk om op betrouwbare wijze onderzoek te kunnen doen naar zowel de gewenste bacteriën, zoals bijvoorbeeld zuurselbacteriën in gefermenteerde produkten, als ongewenste bacteriën, zoals ziekteverwekkers en micro-organismen die bederf van voedsel veroorzaken. De huidige identificatiemethoden, gebaseerd op biochemische eigenschappen zoals suikerfermentatie, enzymatische omzettingen, groeicondities en lipidensamenstelling, zijn vaak slecht reproduceerbaar en afhankelijk van de fysiologische staat van het micro-organisme. Bovendien is het niet altijd mogelijk om op basis van deze eigenschappen voldoende onderscheid te maken tussen verschillende bacteriesoorten.

De ontwikkeling van moleculaire detectiemethoden en hun toepassing bij de studie van de microbiële ecologie van zuurselbacteriën worden beschreven, zowel tijdens de produktbereiding als in de afvalstroom. Bovendien is de specifieke uitgroei van sporevormende bederforganismen in zuivelprodukten, zoals kaas, bestudeerd. In Hoofdstuk 1 wordt een overzicht gegeven van de specifieke rol van 16S rRNA-sequenties in de identificatie en systematiek van micro-organismen. Bovendien worden enkele detectie- en identificatiemethoden beschreven die zijn gebaseerd op specifieke sequenties in het ribosomale operon, waarbij door middel van voorbeelden de toepassingsmogelijkheden worden geïllustreerd.

In Hoofdstuk 2 wordt de ontwikkeling van specifieke identificatiemethoden voor de zuurselbacteriën *Lactococcus* en *Leuconostoc* beschreven. Deze methoden zijn gebaseerd op de amplificatie van variabele regio's in het 16S rRNA (V1 en V3) die vervolgens zijn gebruikt in een hybridisatie met soort-specifieke DNA-probes. Op basis van deze benadering zijn methoden opgezet om op snelle en betrouwbare wijze isolaten uit omgevingsmonsters te kunnen identificeren.

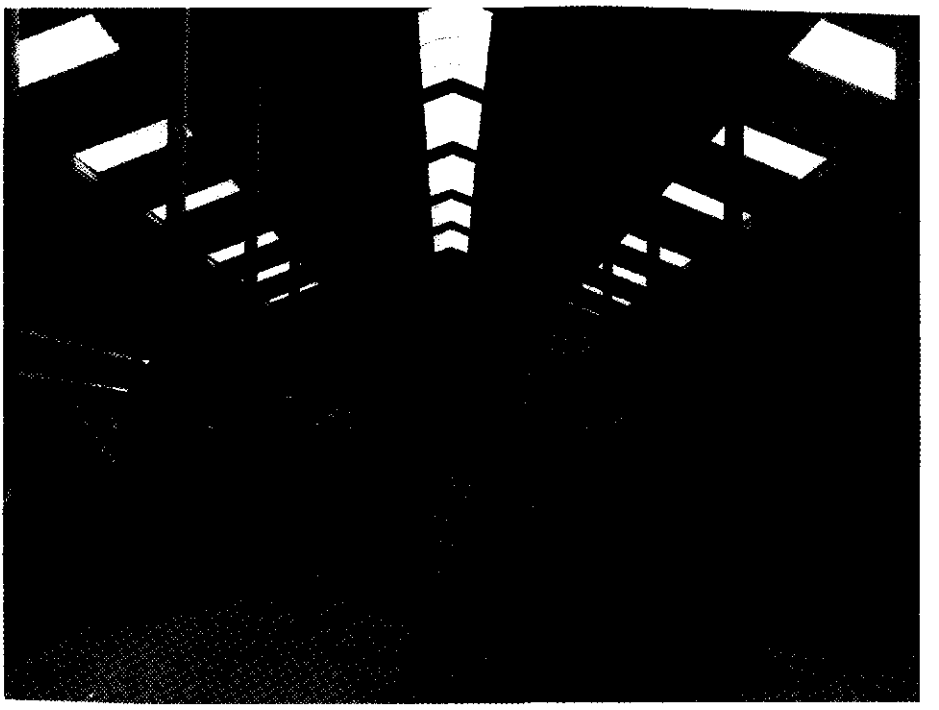
In de loop van de jaren is de biodiversiteit van industrieel toegepaste zuursels afgenomen. Nieuwe stammen met andere eigenschappen dan die van de huidige zuurselbacteriën kunnen mogelijk worden toegepast voor produktinnovatie. Dergelijke stammen kunnen ofwel uit natuurlijke bronnen worden geïsoleerd, of door middel van genetische modificatie worden geconstrueerd. Voor beide benaderingen is het essentieel om de natuurlijke niches en de van zuurselbacteriën te kunnen identificeren. In Hoofdstuk 3 wordt beschreven hoe deze methoden zijn toegepast bij de detectie en identificatie van natuurlijke *Lactococcus*-isolaten. De resultaten geven aan dat lactokokken kunnen worden aangetroffen in de natuurlijke omgeving van runderen. Ze bleken voor te komen op de huid en in het speeksel van runderen, in het voer en op de graslanden. Bovendien werden de lactokokken die aanwezig waren in de afvalstroom van de kaasproduktie, zoals lekwei en afvalwater, geanalyseerd. Een nadere karakterisering van de natuurlijke isolaten en de isolaten uit de afvalstroom van kaasproduktie wees echter uit dat er aanzienlijke fysiologische verschillen bestaan tussen de natuurlijk voorkomende lactokokken en die welke in industriële zuursels aanwezig zijn. Uit verder onderzoek, waarbij meerdere natuurlijk voorkomende isolaten werden geanalyseerd, bleken beide twee ribotypen, nl. *L. lactis* subsp. *lactis* en *L. lactis* subsp. *cremoris* voor te komen. De natuurlijke isolaten van *L. lactis* subsp. *cremoris* hadden echter beduidend andere fenotypische eigenschappen dan *L. lactis* subsp. *cremoris* geïsoleerd uit zuursels.

Dit suggereert dat hoewel er lactokokken in het milieu kunnen worden aangetroffen, de industriële stammen waarschijnlijk niet of nauwelijks buiten de zuivelomgeving kunnen overleven. Kennelijk hebben deze stammen door de jarenlange selectie op bepaalde produkteigenschappen belangrijke eigenschappen met betrekking tot de overleving buiten de zuivelomgeving verloren. Tijdens deze studie zijn echter wel natuurlijk voorkomende stammen aangetroffen die over eigenschappen beschikken die van belang zouden kunnen zijn voor innovatieve produktontwikkeling, zoals het vermogen specifieke proteases en bacteriocine te produceren.

Voor de mogelijke toepassing van lactokokken in probiotische produkten, ter bevordering van de gezondheid van de consument, of als orale vaccins, is het noodzakelijk om te weten of deze organismen na consumptie kunnen overleven in het maag-darmkanaal. Hiervoor is een studie naar het lot van *L. lactis* in het menselijke maag-darmkanaal opgezet, die beschreven staat in Hoofdstuk 4 van dit proefschrift. Uit de resultaten blijkt dat een kleine maar significante fractie (0.1 tot 1%) van de geconsumeerde lactokokken na passage in de faeces is terug te vinden. Binnen 72 uur na consumptie kon de aanwezigheid van de lactokokken in de faeces worden vastgesteld. Gezien het feit dat het gemiddeld acht dagen duurt voordat geconsumeerde bacteriën uit het maag-darmkanaal zijn verdwenen, kan worden gesteld dat *L. lactis*-stammen kunnen overleven, maar dat tijdens de passage ook een gedeeltelijke inactivatie plaatsvindt. Deze overleving biedt perspectieven voor de mogelijke toepassing van lactokokken in gezondheidsbevorderende zuivelprodukten of voor orale vaccins.

Een andere belangrijke doelstelling binnen de voedingsmiddelenindustrie is het beschermen van produkten tegen bederf door uitgroei van met name sporenvormende

bacteriën. Voor deze groep bacteriën, waarvan de sporen de toegepaste hittebehandelingen overleven en vervolgens in het eindprodukt kunnen uitgroeien, zijn gevoelige detectie- en specifieke identificatiemethoden van groot belang voor een adequate bewaking van de kwaliteit van het uiteindelijke voedingsprodukt. Een belangrijk bederfprobleem in kaas is boterzuurgisting. Doordat de sporen van boterzuurvormende bacteriën in de kaas gaan uitgroeien, ontstaan er sterke smaakafwijkingen en door de gelijktijdige gasvorming grote gaten en scheuren in de kaas. Door kaasmakers wordt dit gebrek "laat los" genoemd. Er wordt verondersteld dat met name de uitgroei van *Clostridium tyrobutyricum* dit gebrek in kaas veroorzaakt. Er zijn echter nog een aantal andere *Clostridium*-soorten die in zuivelprodukten kunnen voorkomen en ook in staat zijn boterzuur te vormen, zoals *C. acetobutylicum, C. beijerinckii, C. butyricum* and *C. sporogenes.* De thans toegepaste kweekmethode om het aantal sporen van *C. tyrobutyricum* te bepalen is niet specifiek en geeft, in tot wel 70% van de gevallen, aanleiding tot vals positieve resultaten. Zowel voor de specifiekere detectie van *C. tyrobutyricum* als om de relatie tussen het gebrek "laat los" in kaas en de uitgroei van *C. tyrobutirycum* vast te kunnen stellen zijn specifieke DNA-probes ontwikkeld voor de meest voorkomende clostridia in de zuivelomgeving. In Hoofdstuk 5 wordt de ontwikkeling en validatie van deze probes beschreven. Op basis van deze specifieke DNA-probes voor *C. acetobutylicum, C. beijerinckii, C. butyricum, C. tyrobutyricum* and *C. sporogenes* zijn methoden ontwikkeld waarmee zeer gevoelig de aanwezigheid van het DNA van clostridia in kaas kan worden aangetoond. In Hoofdstuk 6 wordt beschreven hoe deze methoden worden toegepast in een onderzoek om definitief de relatie tussen boterzuurgisting in kaas en de uitgroei van sporen van *C. tyrobutyricum*



vast te stellen. Uit analyse van experimentele kazen, waaraan sporen van verschillende *Clostridium*-soorten waren toegevoegd, als van praktijk-kazen bleek dat in alle gevallen waarin er duidelijke tekenen van het gebrek "laat los" in kazen werden aangetroffen, er ook altijd specifieke DNA sequenties voor *C. tyrobutyricum* werden gesignaleerd. Omdat hiermee de relatie tussen *C. tyrobutyricum* en boterzuurgisting in kaas eenduidig is vastgesteld, wordt de noodzaak voor een gevoelige en specifieke detectiemethode voor sporen van *C. tyrobutyricum* in kaasmelk onderstreept.

Op basis van het in dit proefschrift beschreven onderzoek kan worden vastgesteld dat door middel van moleculaire, op 16S rRNA gebaseerde identificatie- en detectiemethoden het nu mogelijk is snel en betrouwbaar inzicht te krijgen in specifieke aspecten van de microbiologie van voedingsmiddelen waarvoor voorheen geen juiste middelen beschikbaar waren. Dit is met name een vooruitgang voor de specifieke en gevoelige detectie van organismen die in zeer lage concentraties in produkten aanwezig zijn en voor de detectie van moeilijk te differentiëren organismen zoals melkzuurbacteriën.

Nieuwe en betere inzichten werden verkregen in de ecologische aspecten van het gebruik van melkzuurbacteriën in zuursels en in het bederf van zuivelprodukten door sporenvormende bacteriën. Dit onderzoek illustreert de toekomstige toepassingsmogelijkheden voor moleculaire technieken in de analyse van kritische controlepunten binnen een produktieproces. Door een beter en gedetailleerder inzicht in de aanwezigheid, overleving en uitgroei van specifieke micro-organismen kunnen produktieprocessen van gefermenteerde voedingsmiddelen beter worden gecontroleerd. Bovendien zijn deze moleculaire technieken ook essentieel in de bewaking van de veiligheid en kwaliteit van voedingsmiddelen.

Door de toekomstige automatisering van moleculaire identificatie- en detectiemethoden wordt hun implementatie in routinematige controle-laboratoria mogelijk. Hierdoor zal in de toekomst de produktie van veilige produkten met een hoge kwaliteit nog beter kunnen worden gewaarborgd.

Nawoord

Hier ligt het dan! Het proefschrift dat tot stand kwam in samenwerking met collega-microbiologen, de "geneten" en meerdere studenten en stagiaires. Op de eerste oriënterende vragen van Willem of ik wellicht geïnteresseerd was in een promotiebaan, antwoordde ik enigszins terughoudend. Na tweemaal zes jaar studeren wilde ik graag "echt" aan het werk. Eenmaal begonnen met werken bleek de wetenschap toch erg fascinerend. Zeer inspirerend waren de prikkelende discussies met collega-wetenschappers uit de gehele wereld tijdens mijn vele congresbezoeken in het buitenland. Mede hierdoor was ik in staat om enkele van de meest revolutionaire ontwikkelingen in de microbiologie op de voet te volgen, zoals de introductie van de Polymerase Chain Reaction, een grensverleggende technologie, en de ontwikkeling van de moleculaire basis voor de phylogenie van micro-organismen op basis van het 16S-rRNA, waardoor voor het eerst de evolutionaire verbanden tussen micro-organismen duidelijk werden.

Gelukkig bestaat onderzoek doen niet alleen uit wetenschap, maar voor een groot deel ook uit samenwerking in een team met vele collega-onderzoekers. Op NIZO heb ik geleerd dat het beste onderzoek ontstaat door het werken met een zeer gemotiveerde groep onderzoekers, die elk hun specifieke steentje bijdragen in het creëren van de juiste condities voor het doen van succesvolle experimenten. Mede daardoor heb ik het altijd als een voorrecht beschouwd te mogen werken in twee afdelingen, nl. Microbiologie en Genetica/BFC. Bij Microbiologie maakte ik als kersverse ingenieur kennis met de praktijk van de zuivelmicrobiologie. Vooral de combinatie van nieuwe technieken, jeugdig enthousiasme en jarenlange ervaring met zuivelonderzoek heeft geresulteerd in het onderzoek gepresenteerd in dit proefschrift. Met name de relativering van Frans en Leo, gebaseerd op ruime ervaring, weerhielden (en houden!) mij regelmatig van het trekken van al te voorbarige conclusies.

Microbiologie heeft nogal wat veranderingen ondergaan sinds Jeroen en ik begonnen in 1989/1990. Als nieuwelingen op NIZO hebben wij samen het huidige team projectleiders tot stand zien komen. Wanneer de frustraties over mislukte en/of hopeloze experimenten weer eens de boventoon voerde, waren de gesprekken met Jeroen en de meer recente teamleden, Gerrit en Mark, een grote steun en motiveerden steeds weer tot nieuw onderzoek. Onder leiding van Ton is Microbiologie uitgegroeid tot een goed georganiseerde en gemotiveerde onderzoeksgroep waarin fundamenteel onderzoek naar de fysiologie van melkzuurbacteriën wordt gecombineerd met het vakkundig oplossen van microbiologische problemen in de zuivelpraktijk. Veel van mijn publikaties, verslagen en brieven danken hun leesbaarheid aan het onuitputtelijke geduld en de aandacht die Ton aan mijn schriftelijke oefeningen heeft besteed.

Genetica bestaat voor een groot deel uit jonge onderzoekers, allemaal bezig met hun promotie-onderzoek. De samenwerking met lotgenoten zoals Silke, Rutger, Martien, Christ, Peter, Evert, Richard, Pascalle en Wilco was een bron van inspiratie. In de kelder van het NIZO worden uitbundige watersmijtpartijen en practical jokes afgewisseld met gedisciplineerd onderzoek, gebaseerd op collectieve verantwoordelijkheid voor chemicaliën en apparatuur. Mede door Willems inspiratie wordt menige wetenschappelijke uitdaging aangegaan en tot een succesvol einde gebracht. Telkens wanneer mijn zelfvertrouwen tot een dieptepunt was gedaald, wist Willem mij er weer van te overtuigen dat met enig doorzettingsvermogen de gewenste resultaten behaald zouden worden.

In de eerste jaren van mijn onderzoek besteedde ik vele aangename uren achter de laboratoriumtafel waar ik al mijn creativiteit kwijt kon. Maar al gauw, wat mij betreft veel te snel, werd mijn aandacht opgeëist door andere zaken zoals artikelen en verslagen schrijven, projectvoorstellen maken en apparatuur aanschaffen en beheren. De voortgang van het praktische onderzoek is vooral te danken aan zeer gemotiveerde medewerkers, zoals Arjen, Jan en Roger, die vele "simpele" experimenten "even" hebben uitgevoerd. Deze ondersteuning zal zeker in de komende jaren van het grootse belang zijn gezien de nieuwe uitdaging waarvoor NIZO mij gesteld heeft. Als accountmanager zal het een uitdaging zijn om een communicatieve brug te slaan tussen fundamentele wetenschap en industriële toepassing.

Het schrijven van een dankwoord is altijd moeilijk. Het moet niet te zwaarwichtig zijn maar toch serieus, onderhoudend maar niet te luchtig. Ik heb geprobeerd de belangrijkste factoren die bijgedragen hebben aan het totstandkomen van dit proefschrift te beschrijven. Ik wil besluiten met het nogmaals bedanken van het gehele NIZO; de directie, voor het feit dat zij mij in staat stelden mijn promotie-onderzoek tot een goed einde te brengen; alle collega's van Microbiologie, voor de gezelligheid, de goede werksfeer en de vele borrels en andere festiviteiten; de collega's in de andere onderzoeksafdelingen, Analytische Chemie, Biofysische Chemie, Voeding, Technologie en Procestechniek, voor hun bijdrage met kennis en expertise op vele voor mij onbekende gebieden; de operators van de Technologiehal, voor de productie van vele opgeblazen kazen; het secretariaat, voor de administratieve support. Maar vooral de medewerkers van communicatie en fotografie. Zonder het vakkundige werk van Joop, Simon en Jacques zou dit proefschrift er een stuk minder aantrekkelijk uitzien!

Allemaal hartstikke bedankt!

Nicolette

Curriculum vitae

De schrijfster van dit proefschrift werd op 17 november 1965 geboren te Amsterdam, als dochter van F.T.M. Klijn en P.J. van Duijker. Na het behalen van het diploma Gymnasium B aan het Stedelijk Gymnasium te Haarlem in 1984, werd in hetzelfde jaar begonnen met een studie Biologie aan de Landbouwuniversiteit te Wageningen. Op 30 maart 1990 werd het doctoraalexamen behaald met als afstudeervakken Industriële Microbiologie (Prof. dr. J.A.M. de Bont) en Moleculaire Biologie (Prof. dr. A. van Kammen). Een stage werd uitgevoerd bij het Nederlands Instituut voor Zuivelonderzoek (NIZO) te Ede onder leiding van Prof. dr. W.M. de Vos. Vanuit deze stage werd reeds één maand voor het verkrijgen van het ingenieursdiploma begonnen aan een tijdelijk dienstverband bij NIZO, met betrekking tot een studie naar de risicoanalyse van het gebruik van genetisch gemodificeerde melkzuurbacteriën in voedingsmiddelen, gedeeltelijk gefinancierd door de Algemene Commissie Biotechnologie, ministerie van Landbouw en Visserij. Sinds 1 april 1994 is zij als vaste medewerkster van NIZO werkzaam als projectleider binnen de afdeling Microbiologie waar zij onder meer verantwoordelijk is voor de moleculaire detectie en identificatie van micro-organismen. Ze leeft samen met Cor van Aggelen en zij zullen gezamenlijk vanaf oktober 1996 een bescheiden paardenfokkerij beheren aan de Hammerdijk te Lunteren.

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