Characterisation of lactococci isolated from natural niches and their role in flavour formation of cheese

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Propositions / Stellingen

1. It is not conclusively shown that aminotransferase activity is the bottle neck in the formation of flavour compounds in cheese.

   - **Yvon, M., Berthelot, S., & Gripon, J.C.** (1998) Adding α-ketoglutarate to semi-hard cheese curd highly enhances the conversion of amino acids to aroma compounds. *International Dairy Journal* **8**, 889-898.
   - This thesis.

2. In contrast to what is generally described, not all lactococci are auxotrophic for at least six amino acids.

   - **Reiter, B., & Oram, J.D.** (1962) Nutritional studies on cheese starters. *Journal of Dairy Research* **29**, 63-77.
   - **Chopin, A.** (1993) Organization and regulation of genes for amino acid biosynthesis in lactic acid bacteria. *FEMS Microbiology Reviews* **12**, 21-38.
   - This thesis.

3. The existing classification of lactococci on subspecies level *cremoris* and *lactis* is seriously affected by studying lactococci isolated from natural niches.

   - **Bergey's** *Manual of Systematic Bacteriology* (1984) Baltimore: Williams and Wilkins.
   - **Klijn, N., Weerkamp, A., & De Vos, W.M.** (1995) Detection and characterization of lactose-utilizing *Lactococcus* spp. in natural ecosystem. *Applied and Environmental Microbiology* **61**, 788-792.
   - This thesis.

4. Testing in model systems is a useful tool in selecting strains with potentially interesting properties as starter cultures.

   - This thesis.

5. Bacterial strains isolated from nature are not only valuable for practical applications today, but in the future they may also provide an expanded gene pool for designing genetic modified strains with improved traits.

6. Every flavour is an off-flavour.

7. Biological agriculture is only a partial solution towards the ongoing crisis in today agriculture.

8. There is a cheese for every taste preference and a taste preference for every cheese.

9. Cheese producers in the ancient Egypt indirectly initiated the work described in this thesis.

10. Windmills, tulips and cheese in The Netherlands are at least as impressive for Egyptians as are the pyramids for the Dutch in Egypt.

11. A tree is known by its fruit. A cheese is known by its flavour.

Propositions belonging to the thesis entitled
'Characterisation of lactococci isolated from natural niches
and their role in flavour formation of cheese'
Eman H.E. Ayad
Wageningen, 1 June 2001

"Living in direct contact with nature helped man to understand common occurrences inherent to his life. He learned how to preserve his food and discovered various ways that provide him a desired meal with specific organoleptic properties. He should search more, nature is still full of secrets"

to my mother
in memory of my father
to Ahmed, Sarah & Salma

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Contents

General introduction

General introduction

Fermented milk products are among the most prominent types of food in the world, because they are not only highly nutritious but, concomitantly, they are attractive for the consumer in the respect to flavour and texture. These products have one feature in common: fermentation by lactic acid bacteria (LAB) is an integral part of their manufacture. In one group of fermented products, the cheeses, a variety of LAB as well as other microorganisms such as micrococci, propionibacteria, brevibacteria, molds and yeasts are used in the production of a wide variety of types. This group of fermented dairy products is the most important from an economical point of view. The world production of cheese is approximately 14 million tons per year according to the International Dairy Federation (1995) and is even growing. Assuming that the milk for cheese making is inoculated with 0.5-1% (v/v) of starter, it can be calculated that more than 10^9 liters of starter bacteria are produced annually. Strains belonging to the species of *Lactococcus lactis* are the most important starters in the manufacture of cheese. The primary role of starter cultures in the manufacture of dairy products is to provide microbiologically safe products with defined organoleptic and structural properties in an efficient and reproducible way. In the last century, industrial starter cultures were first isolated from dairy manufactures. The selection has mainly been based on the desired specific product properties of a limited number of large-scale produced products and on the performance of the starter during the manufacturing process such as acidification rate, proteolysis, antimicrobial activity, phage insensitivity and flavour production (Marshall, 1991). These cultures were maintained by subculturing in milk, a procedure that decreases the number of different strains in the cultures, during the years, leading to a loss in variety of (flavour) characteristics in cheese made with these starters (Gilliland, 1971; Limsowtin *et al.*, 1978) (see below).

Flavour is one of the most important attributes of cheese for the consumer. Cheese flavour is derived from milk components (protein, fat, lactose and citrate) by enzymatic activities of milk, rennet and microorganisms (Urbach, 1993). Research has been focused on the role of starters in cheese ripening (Visser, 1993; Fox *et al.*, 1996a) and has revealed that the formation of many flavour compounds is believed to result from the action of proteases, peptidases and various amino acids convertases from the starter cultures (Broome & Limsowtin, 1998; Yvon *et al.*, 1998; Smit *et al.*, 2000).

Nowadays, consumers demand a large variation in flavour of cheese besides consistency in overall quality. The need for new products requires the use of new microbial strains with novel properties. This has led to a request for novel strains for the innovation and diversification of dairy products. These novel strains can be achieved either by genetic modification of known production strains (Fitzgerald *et al.*, 1993) or by exploring the biodiversity within natural strains from various ecological niches.

The literature survey below gives an introduction to history of the genus *Lactococcus* (its taxonomy) and of the cheese making process. A fair amount of background information on dairy starters, their typology, their nutritional requirements and their properties essential for dairy fermentation is subsequently given. Finally, the flavour formation in cheese during ripening is elaborately addressed. All these topics relate to the content of this thesis, i.e., the potential use of novel lactococci as starter in the manufacture of semi-hard Dutch cheese types.

1. The genus *Lactococcus* - its history and taxonomy

The characterisation of lactic lactococci started when the microbiologist Joseph Lister isolated a pure culture in 1878, which he called *Bacterium lactis* (lactis is the latin for "of milk"). Later, in 1919, Orla Jensen isolated and described *Streptococcus cremoris* (cremoris is the latin for 'of cream'). Comprehensive taxonomic studies performed have led to the transfer of this *Streptococcus* and related streptococci to a new genus *Lactococcus* (Schleifer *et al.*, 1985; Pot *et al.*, 1994; Cogan, 1996). *Lactococcus* species have frequently been isolated from the milk environments. An early investigation (Esten, 1909) identified the cow and the milking equipment as the source of *Lactococcus lactis* in raw milk. This could not always be confirmed by others (Jones, 1921; Stark & Sherman, 1935), since at that time the investigations were rather controversial due to unreliable identification methods. However, the isolation of *Lactococcus* species from sources other than raw milk has been reported as well (Sandine *et al.*, 1972; Schultz & Breznak, 1978; Collins *et al.*, 1983; Schleifer *et al.*, 1985; Williams *et al.*, 1990; Elliott *et al.*, 1991), indicating that these organisms are widespread in the environment and not strictly dairy related.

In the past decade, significant progress has been made in the identification of strains. Especially, the developments of molecular microbiological tools had a significant effect on this progress. The identification of strains is nowadays based on modern methods such as cell wall component determination, DNA-DNA hybridisation and 16S rRNA analysis (Salama *et al.*, 1991; Klijn *et al.*, 1991; Godon *et al.*, 1992; Cogan, 1996).

The genus *Lactococcus* belongs to the group of lactic acid bacteria (LAB), which includes several genera: *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Axelsson, 1998). These genera are commonly defined as Gram-positive, non-sporulating, catalase-negative, anaerobic but aerotolerant, acid tolerant, nutritionally fastidious, strictly fermentative organisms that lack cytochromes and produce lactic acid as the major end-product of carbohydrate metabolism (Axelsson, 1998). A phylogenetic tree of the LAB (as a group) is shown in Fig. 1.

According to the later development in bacterial taxonomy, all technologically useful dairy starter LAB are found only in the genera: *Lactococcus, Leuconostoc, Streptococcus, Lactobacillus* and *Pediococcus*, although *Enterococcus* might also have a good potential. They provide an effective form of 'natural' preservation and moreover, they determine the flavour, texture and frequently the nutritional attributes of the products as well as enhance the variety of food available to use (Daly *et al.*, 1996; Teuber, 2000). The genus *Lactococcus* currently comprises five species: *L. lactis, L. graviae, L. plantarum, L. piscium*, and *L. raffinolactis* (Schleifer *et al.*, 1992, Cogan, 1996). The only important species in starter cultures is *L. lactis* that can be divided into two subspecies *L. lactis* subsp. *lactis* and *L. lactis* subsp *cremoris*. *Lactococcus* spp. are cocci that occur singly, in pairs or in chains, they ferment sugars homofermentatively, producing L- lactate, and grow at 10°C but not at 45°C. Some lactococci, e.g., *L. lactis* subsp. *lactis* biovar *diacetylactis*, are found to be able to metabolize citrate. These organisms are phenotypically and genotypically indistinguishable from *L. lactis* subsp. *lactis* and do not produce as much acid in milk as other *L. lactis* spp. They are regarded as taxonomically the same as *L. lactis* and hence are not given separate species or subspecies identity.

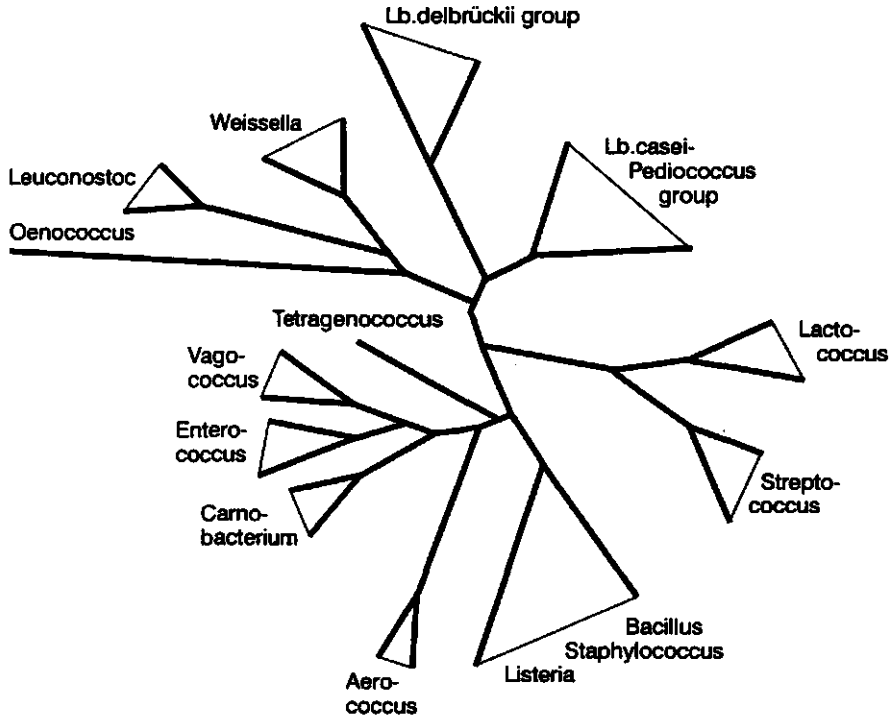

Fig. 1: The phylogenetic relationship between the genera of LAB (Axelsson, 1998).

2. Cheese making process

The production of cheese from milk is an ancient process. Hieroglyphics depicting cheese making have been found in the tombs of the pharaohs. It is believed that cheese originated in the Middle East, where milk stored in the prevailing warm climates would have acidified due to the growth of naturally occurring LAB. Such acid-coagulated milk, when disturbed, would have separated into curds and whey, the former being used as a food, the latter as drink. Cheese manufacture accompanied the spread of civilisation throughout the Middle East, Egypt and Greece, and was well established in the Roman Empire (Cogan, 1996; Fox *et al.*, 1996a). Cheese making remained localised in specific regions due to limited communications. Hence, several varieties of cheese evolved, most of which are still produced locally although the principal varieties as Dutch (Gouda and Edam), Cheddar, Camembert and Fromage Frais types are now produced internationally. More information on the history of cheese can be found in Scott (1986) and Fox (1993).

Within the world today there is an extensive list of cheese varieties. It has been claimed that as many as 900 types exist. However, many of these are based on very similar technologies, differing only in name, production area, size or packaging. Different classification systems have been proposed for the world's cheese varieties based on milk species, technology, moisture content, appearance, type of milk and microflora (Burkhalter, 1981; Scott, 1981). Considering all of this, cheese is the most diverse, scientifically interesting and challenging group of dairy products.

Cheese manufacture is essential a dehydration process in which the casein and fat of milk are concentrated 6 to 12 folds, depending on the variety. It is initiated by a combination of microbial fermentation and an enzymatic hydrolysis of the milk protein (κ-casein) by rennet (chymosin). This process comprises those operations performed during the first 24 hours. Although the protocols for various varieties differ in detail, the basic steps are common for most varieties, i.e., acidification, coagulation, dehydration (cutting the coagulum, cooking, stirring, pressing, and other operations that promote gel syneresis), shaping (kneading, moulding, pressing) and salting (Fox *et al.*, 1996a).

The differences between cheese varieties are partially determined by variations in procedures utilised for curd syneresis, cooking and salting (Hill, 1995). In Gouda cheese production, the curd is cut and stirred to promote syneresis and further treated with hot water (the whey is partially, for 25-40% replaced, with hot water to give a scald temperature of 35-37°C). Curds are lightly pressed under the whey for a short period, moulded, pressed and salted by keeping it in a brine solution. For a detailed description of Gouda-type cheese manufacturing see references Walstra *et al.* (1987; 1993). The pH of this cheese is around 5.7-5.9 after 4 hours from the start of manufacture then lowered to the desired value of

approximately 5.3-5.5 after 6 hours during brining due to lactose conversion, and finally reaches a value of 5.2 in the mature cheese.

The next stage in the production of most cheese types is ripening. Many cheeses are ripened for periods ranging from a few weeks to 2 years or longer at predetermined temperature and humidity, e.g., Gouda cheese is ripened up to 18 months at 12-15°C at 85-90% relative humidity. During this period and under these conditions, many biochemical changes take place, which are essential for flavour development. The role of starter cultures in these changes during the cheese ripening process and in the flavour formation will be discussed in the next section of this chapter. Fig. 2 gives an example of the main steps of the cheese making process.

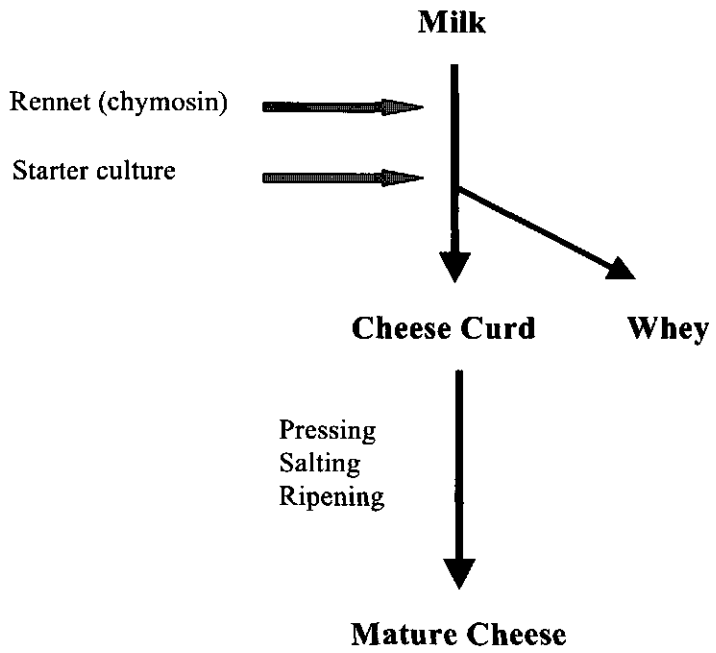

Fig. 2. Summary of the cheese making process.

3. Dairy starter culture

The use of starter cultures in the manufacture of fermented dairy products had been practised already for a long time before knowning that bacteria were actually involved at all.

Backslopping with some of previous day's product was the major source of the inoculum. It is only over the past 100 years that the microbiological basis of these fermentations has been elucidated. The discovery of bacteria naturally present in raw milk paved the way for their isolation, characterization and exploitation. The dairy starter culture industry was born and developed since then into the technically sophisticated industry of today. The historical development of dairy starter cultures is summarised in Table 1 (Cogan, 1996; Stanley, 1998).

Table 1. Historical review of development of dairy starters.

Pre - 1900	Natural souring of milk stored overnight	
1782	Scheele (Sweden):	- Lactic acid as chemical compound responsible for souring milk
1857	Pasteur (France):	- Lactic fermentation due to bacteria - Concept of pasteurisation
1878	Lister (England):	- Discovery of *Bacterium lactis* (now *Lactococcus lactis*)
1890s	Weigmann (Germany): Hansen (Denmark):	- Deliberate inoculation to produce sour cream - First starters
1906	Marschall (USA):	-First commercial undefined mixed strain starters obtained from raw milk
1910	Metchinkoff (Russia):	- Yoghurt bacteria and health, Longevity
1919	Orla-Jensen (Denmark): Storch (Denmark), Hammer & Bailey (USA), Boekhout & Ott de Vries (Holland):	- Nature of starter cultures - Identification of strains for flavour production
1930s – 1940s	Whitehead & Cox (New Zealand):	- Identification of bacteriophages - Defined strain starter system
1950s	Lewis (England):	- Protected system for industrial starter propagation
1960s – 1970s		- Concentrated deep-frozen bulk set starter cultures - Phage-free cultivation
1980s – 1990s		- Direct vat inoculation commercialised - Genetics of LAB

3.1. Types of starter cultures

In the dairy industry, starters can be divided into two broad groups: undefined (artisanal and mixed-strain starters) and defined starters. Artisanal or 'natural' starter cultures are derived from the practice of backslopping, i.e, using a previous batch of product to inculate a

new batch. These traditional undefined mixtures of strains, derived from raw milk, are still used in small-scale factories in southern Europe, especially Spain, Portugal, Greece and Italy. Their use is easy, cheap and closely intertwined with the traditional production of cheeses. This artisanal use of starters is still practised in the manufacture of hard cheese (Swiss types such as Sbrinz and Gruyére; Italian types such as Grana, Pecorino Romano, Provolone, and Montasio) and soft and semisoft cheese (Italian Mozzarella and Italico). The common features of the cultures involved are (1) they are produced each day at the cheese plant and (2) they generally rely on the selective pressure, competition and antagonism among the components of the original microflora present in the raw milk, which is often used for the production of these cheeses, to select the desired microorganisms. Their composition is complex, relatively variable and often poorly defined (complex undefined starter). However, they are a potential source of strains carrying 'novel' or interesting characters (production of inhibitors, phage resistance, etc.). They are reproduced in the presence of phages and are apparently less sensitive for attacks by phages (Limsowtin *et al.*, 1996; Cogan, 1996; Mäyrä-Mäkinen & Bigret, 1998).

Mixed-strain starters (MSS) are undefined mixtures of starters, evolved from artisanal cultures that produced good-quality cheese, they were propagated in the laboratory under controlled conditions for use as inocula for commercial MSS. The starter cultures used in the manufacture of fermented dairy products are commonly divided on the basis of their optimum growth temperature. Mesophilic LAB starter cultures grow at temperatures of 10-40°C with an optimum around 30°C. Mesophilic MSS are commonly used in dairy plants in northern Europe, especially in Scandinavia, Germany and The Netherlands. Thermophilic artisanal-derived starters from Italy, Switzerland and France are also recognised as mixed strain starters (Accolas & Auclair, 1983). The Dutch system of MSS, coming from dairies or/ and butter plants, are applied based on the noticed difference in phage sensitivity between the starters propagated in the laboratory and in practice. The cultures used in practice are propagated, without isolation, in order to keep a composition as close as possible to that of the original culture. When they are propagated under asceptic conditions, the very few bacteriophages attacks are generally unnoticed. The Netherlands Dairy Research Institute (currently NIZO food research) undertook a major research project to clarify the basic ecology of mesophilic MSS cultures. The aim of this research was to achieve consistent starter performance in Gouda cheese manufacture (Stadhouders & Leenders, 1984; Limsowtin *et al.*, 1996; Mäyrä-Mäkinen & Bigret, 1998).

The MSS for the manufacture of Gouda cheese are composed of acid-forming lactococci (*L. lactis* subsp. *lactis* and subsp. *cremoris*) and flavour producers (citrate utilizing strains). Depending on the nature of the citrate utilizing strains, mesophilic starter cultures are separated into: (1) D-types with *L. lactis* subsp. *lactis* biovar *diacetylactis* (2) L-types with *Leuconostoc* spp. (3) DL-types with both citrate utilizing *L. lactis* and *Leuconostoc* spp. (4)

O-type that contain no citrate utilizing strains. Mesophilic starters are used in production of many other cheese varieties, fermented milk products and ripened butter cream (Petersson, 1988). Thermophilic starter cultures have their optimum growth temperature between 40-50°C, they are used for yoghurt as well as for cheese varieties with high cooking temperatures. The most commonly used thermophilic starters contain strains of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus helveticus*.

MSS are thus composed of complex mixtures of strains from several species of LAB, forming a bacterial population that would change in composition depending on the incubation temperature, growth medium, and frequency of subculturing (Hugenholtz, 1986). In modern practice, subculturing is minimized and cultures are preserved by freezing or lyophilization. They are propagated in the dairy only once before inoculation in the cheese milk. Nowadays, most commercial suppliers market MSS for direct inoculation as well. This eliminates unnecessary subculturing within the factory and reduces many difficulties associated with it (Sandine, 1996).

Defined-strain starters (DSS) are blends of two or more strains and can be mesophilic, thermophilic, or mixtures of the two types (single-strain DSS are now rarely used because of the risk of phages). DSS cultures are obtainable from commercial suppliers and dairy research institutes worldwide. In New Zealand multiple-strain cultures are used for the production of Cheddar cheese; these DSS are composed of a small number of defined strains of *L. lactis*. Either the same culture, containing two to six strains, is used alone for a long time or several cultures are used in rotation in order to prevent bacteriophage attacks. In the latter case, the cultures have to have different bacteriophage sensitivity profiles. The starter system most commonly used in New Zealand cheese plants is a single triplet starter containing three defined selected strains (Pearce, 1969; Heap & Lawrence, 1976; Limsowtin *et al.*, 1977; Heap & Lawrence, 1988; Limsowtin *et al.*, 1996; Heap, 1998). The Australian DSS system consists of a limited numbers of strains, which are replaced as soon as possible in case of a bacteriophage attack. From the sensitive strain screened in the factory, a secondary resistant strain is derived to replace the original strain. A combination of these two latter systems has been successfully used in the United States and Ireland by selecting secondary resistant strains and including them afterwards in multiple-strain cultures (Hull, 1983; Thunell *et al.*, 1981; Daniell & Sandine, 1981; Timmons *et al.*, 1988; Limsowtin *et al.*, 1996; Mäyrä-Mäkinen & Bigret, 1998). In many countries where Cheddar and similar types of cheese are manufactured, similar forms of DSS systems have been installed.

3.2. Nutritional requirements and biosynthetic capabilities of lactococci strains

Lactococcus strains have complex nutritional requirements for growth. In addition to nucleotides and several vitamins, they require a number of amino acids and other substrates

(Mitchell *et al.*, 1941; Reiter & Oram, 1962; Otto, 1981). Lactococci appear to have a limited biosynthetic capacity for synthesising amino acids, the number of essential amino acids is strain-dependent and is found to vary between 6 and 14. *Lactococcus lactis* subsp. *cremoris* strains have more requirements than those belonging to *Lactococcus lactis* subsp. *lactis* (Rieter & Oram 1962; Otto, 1981; Chopin, 1993). The requirement for amino acids can result from either the absence of functional specific biosynthetic genes or from specific regulatory mechanisms (Chopin, 1993). Interestingly, genome sequencing results indicate that in principle gene homologes of the various amino acid biosynthesis pathways are annotated (Bolotin *et al.*, 1999). Obviously, the inability of *Lactococcus lactis* to synthesize many amino acids make them dependent on an exogenous supply of amino acids and small peptides. As a consequence, the optimal growth of the strains depends on the amino acid availability in the environment. The enzymic constitution of a cell is influenced by its environment and therefore metabolic differences undoubtedly exist between cells grown in milk and those grown in other media (Rieter & Oram 1962).

Lactococcus lactis starters used in dairy fermentations are believed to be derived from plant strains, which are introduced in a relatively rich ecological niche, milk (Sandine *et al.*, 1972). These dairy strains seem to have acquired features which adapted them to milk, such as capacity to utilize lactose via a phosphotransferase system (De Vos & Gasson, 1989) and to degrade casein by a cell wall protease (Kok, 1990). In parallel, they have lost other functions, including the ability to synthetise a number of amino acids, since amino acids are readily available (Anderson & Elliker, 1953; Rieter & Oram 1962; Farrow, 1980; Deguchi & Morishita, 1992). This indicates that these strains are auxotrophic for some of the amino acids (Godon *et al.*, 1993; Cocaign-Bousquet *et al.*, 1995). This lead to limitation in the biological diversity of their amino acids converting enzymes which are known to play a role in the flavour formation.

3.3. Functional properties of industrial starter cultures

For dairy industries, it is important to produce the desired product with the same high quality and stability every time it is made, which are mainly dependent on starter cultures used. Therefore, the characterisation and maintenance of starter strains is essential to ensure a good performance of a culture. It is interesting to note that the industrial mesophilic LAB starters for the majority of the world's cheeses and other dairy fermented products are based on one single species, namely *Lactococcus lactis*. There are countless numbers of strains of this species exhibiting different characteristics in their growth rate, metabolic rate, phage interactions, proteolytic activities, flavour promotion, etc. The management of these differences, together with the different cheese technologies employed, makes it possible to have a multitude of industrial applications (Stanley, 1998). Research has been focused on the

role of the starter and its required properties for the dairy manufacture (Crow *et al.*, 1993; Desmazeaud & Cogan, 1996; Heap, 1998; Mäyrä-Mäkinen & Bigret, 1998). Several groups of microorganisms participate in the manufacture and ripening of fermented milk products. Starters with other biochemical attributes than producing lactic acid are also needed to achieve the characteristic properties of each type (Table 2; Limsowtin *et al.*, 1996; Johnson & Steele, 1997). Basically, the most important attributes of starters are related to their acid producing activity in milk, their effect on flavour development, their phage insensitivity and their ability to produce inhibitory compounds. The flavour forming abilities of lactococci will be discussed in the next section of this chapter.

Table 2. Starters used in the manufacture of cheeses.

Cheeses	Principal acid producers	(Intentionally introduced) secondary microflora
Feta, Quarg, Cottage, Cream cheese	*L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis*	*L. lactis* subsp. *lactis* biovar *diacetylactis*, *Leuconostoc* sp.
Mozzarella, Provolone, Romano	*Streptococcus thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. helveticus*	*Lactobacillus* species
Camembert	*L. l.* subsp. *cremoris*, *L. l.* subsp. *lactis*	*Penicillium camemberti*, *Brevibacterium linens*
Blue cheeses, Roquefort, Stilton	*L. l.* subsp. *cremoris*, *L. l.* subsp. *lactis*	*Penicillium roqueforti*
Brick, Limburger, Tilsiter, Kernhem	*L. l.* subsp. *cremoris*, *L. l.* subsp. *lactis*	*Geotrichum candidum*, *B. linens*, *Micrococcus* sp., *Staphylococcus* sp.
Gouda, Edam	*L. l.* subsp. *cremoris*, *L. l.* subsp. *lactis*	*Leuconostoc* sp., *L. l.* subsp. *lactis* biovar *diacetylactis*
Proosdij	*L. l.* subsp. *cremoris*, *L. l.* subsp. *lactis*	*Lb. helveticus*, *S. thermophilus*
Maasdam	*L. l.* subsp. *cremoris*, *L. l.* subsp. *lactis*	*Propionibacterium freudenreichii*
Cheddar	*L. l.* subsp. *cremoris*, *L .l.* subsp. *lactis*	*Lactobacillus* sp.
Parmesan	*S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. Helveticus*	*Lactobacillus* sp.
Emmental	*S. thermophilus*, *Lb. helveticus*, *Lb. delbrueckii* subsp. *bulgaricus*	*Propionibacterium freudenreichii*

3.3.1. Acidification activity

Acid production is the major character regularly associated with a LAB starter culture, which results from the metabolism of milk lactose to lactic acid. The resultant lowering in pH is important in determining the preservative, flavour and textural qualities of the end product. A low-pH environment significantly inhibits the growth of pathogens and food-spoilage

microorganisms. Acidification is important for the clotting phenomenon and also enhances the expulsion of whey from the curd during the cheese making process, reducing moisture levels and further promoting the preservative effect. Generally, dairy starter bacteria (LAB) have evolved two main fermentation pathways, the homolactic pathway in which lactic acid is the major end-product and the heterolactic pathway in which other compounds such as acetic acid, ethanol and CO_2 are produced in addition to lactic acid (Monnet *et al.*, 1996).

3.3.2. Citrate fermentation

Metabolism of citrate is an important property of some mesophilic cultures and technologically significant in fermentation of milk. In some fermented dairy products, additional bacteria, referred to as secondary microflora, are needed to influence flavour and alter texture of the final product. Two LAB starters, D-type and L-type, are capable to metabolize citric acid. The products of citrate metabolism are acetate, diacetyl, acetoin, 2,3-butanediol, and CO_2. Diacetyl is an important flavour component of cultured buttermilk, ripened cream butter, sour cream, fromage frais and quarg. Acetate also plays a role in flavour and CO_2 is responsible for eye formation in cheeses. CO_2 may also cause undesirable split or crack formation in cheese (Akkerman *et al.*, 1989; Johnson *et al.*, 1998). Many reviews have covered most of the aspects of citrate metabolism (Cogan, 1985; Hugenholtz *et al.*, 1993; Monnet *et al.*, 1996).

3.3.3. Phage insensitivity

In the dairy industry, phages of LAB are of considerable economic importance, because they represent one of the main causes of fermentation failure. They may lead to a decrease or complete inhibition of starter culture activity. This has a major impact on the manufacture of fermented products, because the main biochemical and technological functions of the starter are affected. On the other hand, slow acidification may lead to a good proliferation condition for undesirable contaminant bacteria. All together, manufacturing schedules may be disrupted and the resulting products may be of lower quality and lower economic value.

The phages of the lactococci have been investigated in detail and classified into 12 species, based on their morphology, protein composition and DNA structure (Jarvis *et al.*, 1991). They are differentiated into virulent (lytic) and temperate phages, which reflect different growth responses in the bacterial host. The lytic cycle is the propagation of a virulent phage (phage multiplication), the lysogenic cycle is an alternative pathway of phage replication. The latter concerns an integration of the DNA of a temperate phage in the host chromosome and its subsequent liberation can occur either spontaneously or be induced by UV light or by treatment with mutagenic agents such as mitomycin (Neve, 1996).

Selective environmental pressure placed on lactococci by bacteriophages over the years has resulted in strains that contain bacteriophage defense mechanisms. Extensive reviews of bacteriophage and bacteriophage resistance in LAB are written (Hill, 1993; Klaenhammer & Fitzgerald, 1994; Dinsmore & Klaenhammer, 1995; Garvey *et al.*, 1995; Daly *et al.*, 1996; Allison & Klaenhammer, 1998; Forde & Fitzgerald, 1999). Lactococci harbour numerous plasmids, and plasmid DNA has been linked to a number of phage resistance mechanisms. Four distinct groups of naturally occurring host-directed phage resistance mechanisms have been currently identified: (1) adsorption inhibition; (2) phage DNA injection blocking; (3) restriction/modification (R/M) and (4) abortive infection (Abi), each targeting different aspects of the phage life cycle (Djordjevic & Klaenhammer, 1997; Josephsen & Neve, 1998; Moineau, 1999). The discovery of those natural phage resistance mechanisms has provided additional new approaches to countering phages and these mechanisms have been exploited in strain improvement programmes or/ strategy (Coakley *et al.*, 1997; O'Sullivan *et al.*, 1998). The proper handling (sanitation) for phage control and the use of starter bacteria with total or at least high phage insensitivity is desirable for suitable dairy fermentations.

3.3.4. Antimicrobial production

The ability of LAB to produce antimicrobial substances has long been used to preserve food, and is mainly connected with the formation of lactic acid and the concurrent reduction of the pH during their metabolic activities. These bacteria are known to produce also inhibitory substances other than organic acids (acetate and lactate) like hydrogen peroxide, diacetyl and bacteriocins which are antagonistic towards spoilage and pathogenic organisms (Klaenhammer, 1988; Daeschel, 1989; Bolm & Morvedt, 1991; Ray & Daeschel, 1992; Piard & Desmazeaud, 1992). Because of the increasing public interest in food safety including demands for less artificial additives, research attention is focused on the use of naturally occuring metabolites produced by food-grade bacteria. Bacteriocins produced by LAB may be very promising for use as biological food preservatives. This relates to the fact that they are 'natural' compounds, produced by GRAS (generally recognized as safe) bacteria, which have been associated with the production of food for millenia. These compounds were defined by Tagg *et al.* (1976) as proteinaceous compounds that are bactericidal to strains closely related to the producer strain, and also more recently to different strains of the same species as the producer. Biochemical and genetic studies allow to divide these compounds into distinct classes including (I) lantibiotics, small peptides (<5 Kda); (II) small hydrophobic heat-stable peptides, non lanthionine-containing, (<10 kDa); (III) large heat-labile proteins (>30 kDa), and (IV) complex proteins whose activity requires the association of carbohydrate or lipid moieties (Klaenhammer, 1993; Nes *et al.*, 1996; Ouwehand, 1998). Although a wide variety of bacteriocins is produced by *Lactococcus* strains (see reviews, Ray & Daeschel, 1994; De

Vuyst, 1994), the only bacteriocin that is licensed for use as a food additive and granted a GRAS state by the FDA is nisin (Anonymous, 1988; Delves-Broughton, 1990). Nisin is a 34-residue antibacterial peptide that is produced by several strains of *L. lactis* subsp. *lactis* and belongs to class I bacteriocins. Two natural occurring variants of nisin have been identified, nisin A and nisin Z (De Vuyst & Vandamme, 1994; Delves-Broughton & Gasson, 1994; Delves-Broughton *et al.*, 1996).

In dairy technology the production of bacteriocins by starter bacteria will have a strong influence on the composition of these cultures. The first technological consequence is the possible dominance of the producing strain in a mixed-strain culture (see chapter 3). The second technological consequence is the possibility of designing new starters that inhibit undesirable bacteria (Desmazeaud, 1996; chapter 5). Such an approach has been successful in controlling butyric swelling in Edam and Gouda-type cheeses (Lipinska, 1973; Delves-Broughton *et al.*, 1996). Ideal bacteriocins should have the following characteristics for use in cheese: (1) a broad spectrum of activity against both gram-negative and gram-positive bacteria; (2) a bactericidal rather than a bacteriostatic action; (3) no inhibition of other starter microorganisms used; (4) good activity and stability at the pH values and temperatures during manufacture and ripening and (5) a high consumer safety margin, especially the absence of allergic reactions to the products themselves or their hydrolysis products (Desmazeaud, 1996).

3.3.5. Proteolytic activity

The proteolytic system of lactococci is essential for the bacterial growth in milk (nitrogen metabolism) and it is involved in the development of organoleptic properties of different fermented milk products. It is generally believed that lactococci are fastidious organisms. The concentration of free amino acids and peptides in milk is only sufficient to allow for up to 25% of the total cell mass of a normal fully grown culture (Thomas & Mills, 1981). Consequently, the ability of lactococcal cells to grow to high cell density in milk is dependent on their protein degrading capabilities that can liberate essential amino acids from casein derived peptides. Casein degradation and utilisation of the degradation products requires a complex proteolytic system (Thomas & Pritchard, 1987). According to Bockelman (1995) and Mulholland (1997), the proteolytic system of dairy lactococci (as shown in Fig. 3) is composed of, three main components. (a) Cell wall bound proteinase (PrtP) which is hydrolysing casein to oligopeptides. This proteinase is anchored to the cell membrane and protrudes through the cell wall, and therefore is also named cell-envelope proteinase (CEP) (Pelissier, 1984). Two main types of proteinase in lactococci (PI and PIII) have been recognised which differ in their specificity towards caseins (Law & Haandrikman, 1997). (b) Intracellular peptidases which hydrolyse the large peptides into small peptides and amino acids (Monnet *et al.*, 1993; Kok & De Vos, 1994; Visser, 1998). Several peptidases with

different specificities have been identified in lactococci, to date all peptidases have been found to be intracellular (Juillard *et al.*, 1995; Axelsson, 1998). (c) Uptake mechanisms (transport systems) which are involved in the transport of these small peptides and amino acids into the bacterial cell (Kunji *et al.*, 1995; Konings *et al.*, 1997). Amino acid transport systems (Konings *et al.*, 1989), two di- and tri-peptide transport systems (DtpT and DtpP) (Smid *et al.*, 1989; Foucaud *et al.*, 1995) and an oligopeptide transport system (Opp) accepting four to eight residue peptides (Tynkkynen *et al.*, 1993) are present in lactococci. Long oligopeptides, not transported into the cells, can be a source for the liberation of bioactive peptides in fermented milk products when further degraded, for example by intracellular peptidases within the bacterial cell after cell lysis (Law & Haandrikman, 1997). The production of high-quality fermented dairy products is dependent on proteolytic systems of starter bacteria, since peptides and amino acids formed impact flavour directly or serve as flavour precursors in these products (see below).

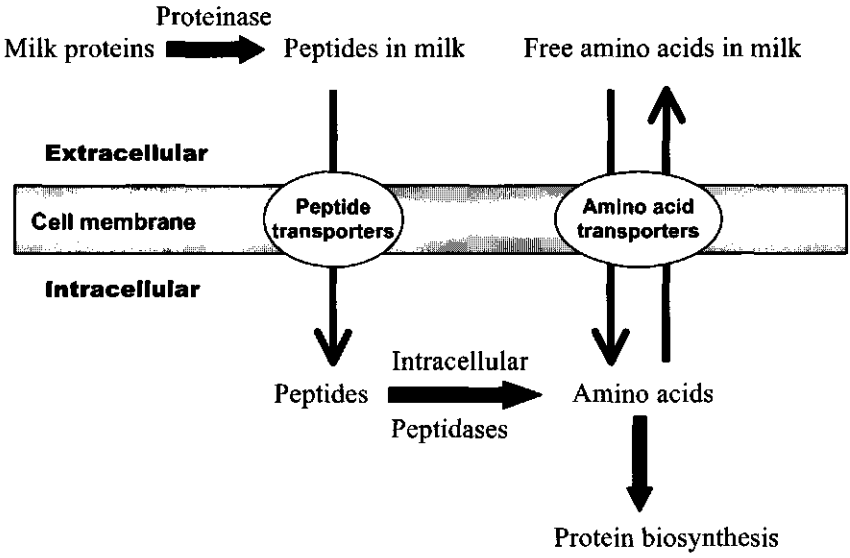

Fig. 3. Proteolytic system of lactococci for growth in milk.

The proteolytic system of dairy LAB, in particular that of lactococcus, is extensively studied; there are several reviews covering this topic in depth (Pritchard & Coolbear, 1993; Poolman *et al.*, 1995; Juillard *et al.*, 1996; Christensen *et al.*, 1999).

3.3.6. Lysis (autolysis) of starter bacteria

The ability of lactococcal strains to lyse and the subsequent release of intracellular (proteolytic) enzymes can be a very desirable trait in some matrices of dairy products such as cheese. Various studies demonstrate the association of starter cell lysis with increased proteolysis and/or flavour development in cheese (Crow *et al.*, 1995; Wilkinson *et al.*, 1994; Morgan *et al.*, 1995). A number of surveys compare autolysis of lactococcal strains and the autolysis systems of several strains have been characterized (Ostlie *et al.*, 1995; Riepe *et al.*, 1997). Different factors, such as pH, temperature, carbon source, and salt concentration, appear to be important for the autolytic process (Ostlie *et al.*, 1995). The degree of autolysis is strain dependent and the structure and components of the cell wall are important contributory factors (Mou *et al.*, 1976). Generally *Lactococcus lactis* subsp. *cremoris* cultures lyse faster than *Lactococcus lactis* subsp. *lactis* (Chapot-Chartier *et al.*, 1994; Wilkinson *et al.*, 1994).

3.3.7. Exopolysaccharide formation

Many strains of LAB produce exopolysaccharides (EPS), that can be as a capsule, closely attached to the bacterial cell, or loosely attached or excreted as slime (Sutherland, 1977; Cerning, 1990). Utilization of slime forming LAB has been used more widely in the dairy industry as a natural biothickner. Strains of lactococci producing EPS are used in Scandinavian fermented milks, e.g., Viili, Taetemilk, Latte, etc. (Macura & Townsley, 1984). These help to thicken the fermented product and give it additional important textural characteristics over those imparted by acid production (Cerning, 1990).

4. Flavour formation in cheese

The acceptability of cheese depends on its appearance and sensory properties (colour, texture, flavour, etc.). Among these, flavour is the most important attribute for the consumer. The characteristics of flavour and texture of the individual cheese varieties is developed during ripening process.

4.1. Cheese ripening process

During ripening, cheeses undergo numerous biochemical changes leading to the development of the appropriate flavour. The ripening agents catalysing these changes generally originate from five sources; the coagulant, the milk, starter bacteria, secondary or adjunct starter bacteria and non-starter bacteria (Fox *et al.*, 1996a). The biochemical changes during cheese ripening are very complex, involving three primary processes, glycolysis,

lipolysis and proteolysis (Fig. 4). The glycolytic event, the conversion of lactose to lactate and subsequently to propionic acid and also the fermentation of citrate to diacetyl, acetaldehyde and ethanol, is mediated by the starter culture. Lipolysis is the first step in the hydrolysis of fat to free fatty acids, which may act as precursors for other flavour compounds (Welsh *et al.*, 1989). The degree of lipolysis varies widely between cheese varieties. It is extensive in mould-ripened and Italian cheese varieties. Proteolysis is the most important of these biochemical processes for texture and flavour formation in hard and semi-hard type cheeses (Adda, 1986; Visser, 1993). The extent of proteolysis ranges from limited (e.g,. Mozzarella) to extensive (e.g. blue mould-ripened cheeses) (McSweeney & Fox, 1997). LAB possess a proteinase and a wide range of peptidases which are principally responsible for the formation of small peptides and amino acids in cheese (see Fig. 3). During cheese manufacture the primary proteolysis of casein is due to the action of the added rennet to the cheese milk, which leads to several peptides (soluble-nitrogen formation in cheese, SN). The secondary proteolysis of these casein-derived peptides into small peptides and amino acids is due to the proteolytic enzymes of the starter (leading to the amino-nitrogen, AN). The general pathway for the breakdown of casein and enzymes involved during cheese manufacture and ripening are shown in Fig. 4.

The physiological state of the starter population in cheese is important for proteolysis and consequently for flavour formation. In Gouda cheese, for instance, the starter bacteria grow rapidly in cheese milk and they are mechanically included in the curd to around 10^8 colony forming units CFU g^{-1} cheese. They grow to 10^9 CFU g^{-1} cheese before salting, which means that they divide only a few times during manufacturing (Walstra *et al.*, 1987). Subsequently, the viable number begins to decrease over a period of several weeks, reaching approximately 1% of their maximum after one month. As they die and lyse the natural barrier of the cell membrane is disrupted. The rate of lysis is reported to be strain-dependent (Wilkinson, 1992). This behaviour and thus the fate of the actual proteinase/peptidase complement of starter cells can partly explain the differentiation in cheese maturing patterns observed between different starter strains. Depending on the enzymes released from the starter cultures used, various flavours can be produced.

Cheese ripening is a slow, and hence an expensive, process especially in hard, low moisture varieties which ripen for at least 18 months. Therefore, there is an economic incentive for the acceleration of cheese ripening. Several methods are used to enhance ripening and flavour formation in cheese by either increasing the levels of putative key enzymes or providing more favourable conditions for the activity of endogenous enzymes in cheese. Such conditions may include increasing the ripening temperature, addition of exogenous enzymes, use of modified starters and cheese slurries. However, in some cases, the use of such approaches causes off-flavours. This topic has been reviewed extensively in the literature (e.g., Law, 1984; 1987; Fox, 1988-1989; El-Soda, 1993; Fox *et al.*, 1996b).

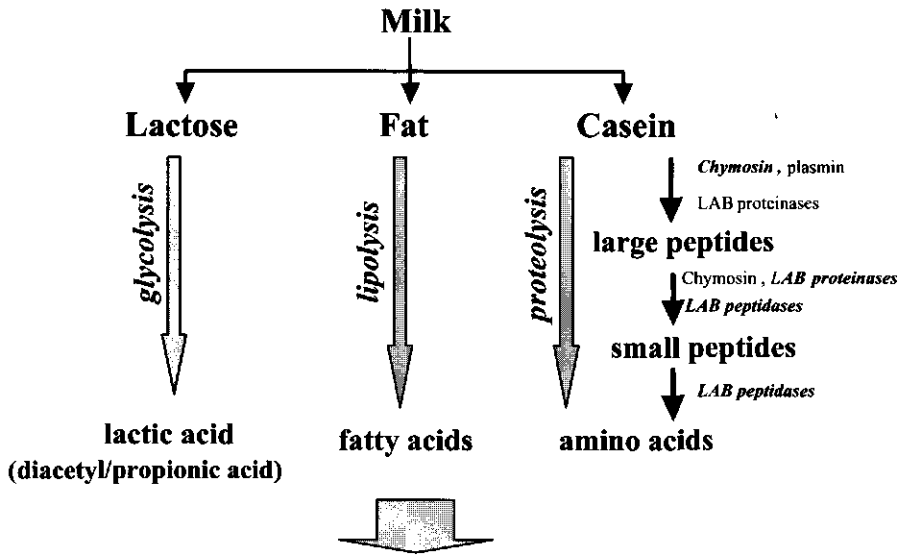

Fig. 4. The major conversion processes leading to flavour formation in ripened cheese. The enzymes considered to play important roles in proteolysis are presented in italics.

4.2. Cheese flavour

Cheese flavour is believed to be caused by a balance of a number of components resulting from enzymatic reactions rather than from chemical interactions (Delahunty & Piggott, 1995). The major flavour forming pathways in cheese (Fig. 4) give rise to a series of volatile and non-volatile compounds, which contribute to cheese flavour. During the last decade, the extensive literature on cheese flavour has been reviewed (Olson, 1990; Urbach, 1993; Fox, 1994; Urbach, 1997). The study on the mechanism of flavour formation has been focussed on the degradation of caseins. The proteolysis products, peptides and amino acids, are flavour compounds by themselves or act as precursors of flavour compounds during the actual cheese flavour formation.

For the development of an acceptable cheese flavour, a well-balanced breakdown of the curd protein into peptides and amino acids, is necessary (Law, 1982). Without the right balance in proteolysis, taste defects may occur due to the accumulation of bitter-tasting

peptides (Fox *et al.*, 1995). Attention has been paid to study this defect, the most common off-flavour in cheese (Visser *et al.*, 1983; Lemieux *et al.*, 1989; Darwish *et al.*, 1994). Recently, it has been shown that bitterness in cheese can be controlled by the use of highly debittering cultures (Smit *et al.*, 1998; 2000).

Amino acids are actually the key precursors in the development of basic cheese flavour, their catabolism during ripening is a source of many flavour compounds (Hemme *et al.*, 1982; Law, 1987; Crow *et al.*, 1993; Urbach, 1993; Urbach, 1995). The amino acids are converted through the action of amino acid converting enzymes; the first stage involves decarboxylation, deamination, transamination, desulfuration, or perhaps hydrolysis of the amino acid side chains. The second stage involves conversion of the resulting compounds (amines and α-ketoacids), as well as amino acids themselves, to aldehydes, primarily by the action of deaminases on amines. The final level of amino acid catabolism is the reduction of the aldehydes to alcohols or their oxidation to acids (Hemme *et al.*, 1982). Sulfur-containing amino acids can undergo an extensive conversion, leading to the formation of a number of sulfur compounds (Engels, 1997, Yvon *et al.*, 1998; Smit *et al.*, 2000). According to the recent modification developments for the conversion of amino acids (Christensen *et al.*, 1999), general pathways of amino acids catabolism are shown in Fig. 5 (G. Smit, personal comunication).

The conversion of each individual amino acid leads to the liberation of a specific volatile compound in cheese. For instance, the conversion of leucine and isoleucine results in the formation of 3- and 2-methylbutanal, respectively. These aldehydes were found to be key-flavour components in some cheese types (Neeter *et al.*, 1996; Engels, 1997, Christensen *et al.*, 1999). The breakdown of methionine, results in the formation of methanethiol which itself is a very potent flavour compound (onion, cheese) and a precursor for subsequent conversion to other sulphur components, e.g., dimethylsulphide and dimethyldisulphide (Lindsay & Rippe, 1986). Dimethylsulphide has been recognized as a very important flavour compound with a relative low odour threshold in cheeses such as Limburger, Chedder and Gouda (Urbach, 1993; Engels *et al.*, 1997). The enzyme activities in such conversions should be well balanced to avoid excessive production of flavour compounds and thus off-flavour formation.

Specific enzymes of mesophilic starter lactococci, used in the manufacture of Gouda cheese, are involved in the conversion of amino acids to aroma compounds (Engels & Visser, 1996). In recent years, a number of these enzymes have been identified and characterised (Alting *et al.*, 1995; Yvon *et al.*, 1997; Roudot-Algaron & Yvon, 1998; Yvon *et al.*, 1998; Engels *et al.*, 2000). Non-starter organisms present in cheese and the indigenous flora of raw milk may also contribute considerably to the formation of flavour compounds.

Based on sensory evaluation and chemical analysis of cheeses, various groups of volatile compounds have been identified as being responsible for the final taste and aroma of cheese. These compounds comprise fatty acids, esters, aldehydes, alcohols, ketones, sulphur

compounds and various other components. All these components occur in most or all cheeses, although a great diversity occurs in their relative concentrations among the cheese varieties (Maarse & Vischer, 1989; Badings, 1991; Bosset & Gauch, 1993; Urbach, 1995; Engels *et al.*, 1997). The differences in flavour between the types of cheese indeed correspond greatly with the distinctive starter used. Therefore, the flavour production is highly strain specific (Smit *et al.*, 2000).

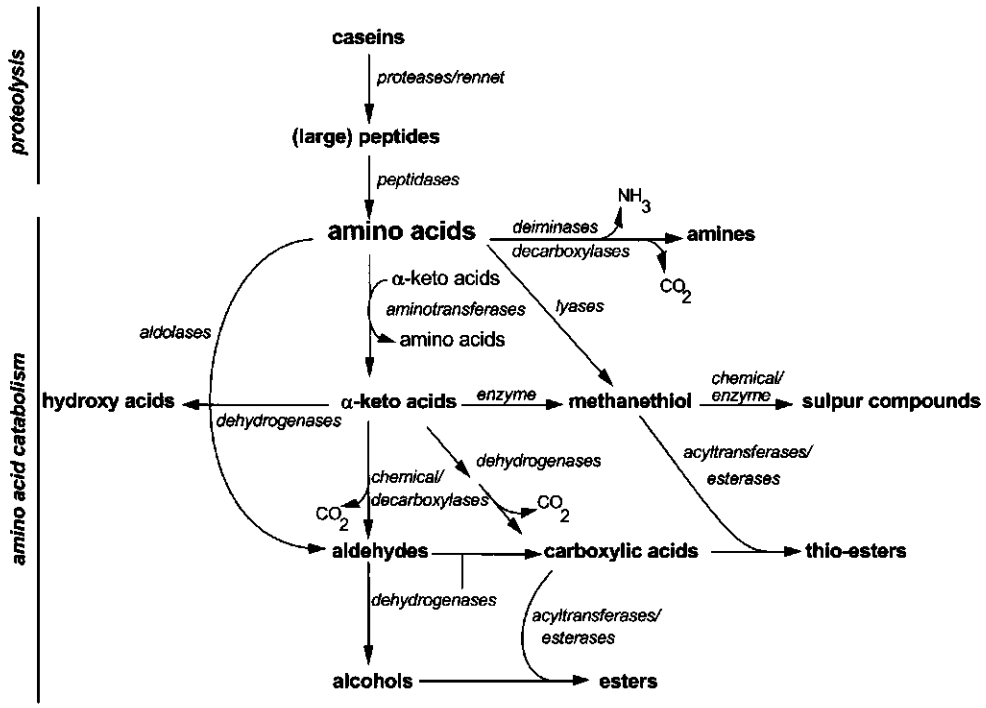

Fig. 5. Pathways of amino acid conversions by LAB leading to flavour compounds.

4.3. Flavour formation by using 'wild' lactococci

As is shown in Table 2, the mixed-strain starter cultures of LAB most commonly used for the production of Gouda, Edam, Proosdij and Maasdam-type cheeses are primarily composed of *Lactococcus lactis.* subsp. *cremoris* and *L. lactis* subsp. *lactis* as acid producing organisms. Other important starters in these cultures are the citric acid fermenting organisms *L. lactis* subsp. *lactis* biovar *diacetylactis* and/or *Leuconostoc* sp., which are important for the

formation of eyes in cheese. In Proosdij-type cheeses, an extra mixed culture containing several strains of the thermophilic species *Streptococcus thermophilus* and *Lactobacillus helveticus* are added for specific flavour development. In Maasdam-type cheeses (Swiss-like cheeses) propionic acid bacteria are added for additional CO_2 production and formation of larger eyes.

Without exception, the mesophilic lactococci in the starters for these cheeses are essential not only for the acidification but also for the flavour formation (see above). The use of standardised starters, strictly hygienic processing conditions and well controlled ripening circumstances, has had an enormously positive effect on the cheese quality. However, as a consequence of the constant quality, the diversity of flavour varieties in one cheese type has been diminished. The consumer of today demands a larger diversification of cheeses and for their innovation novel strains of lactococci are required. For this, the biodiversity within natural ecological niches should be exploited.

Recently it has been demonstrated that strains of LAB can be isolated from different milk environments (Mayo *et al.*, 1990; Centeno *et al.*, 1996; Weerkamp *et al.*, 1996; Cogan *et al.*, 1997) and non-dairy sources such as plants, animals and soil (Collins *et al* 1983; Williams & Collins, 1990; Klijn *et al.*, 1995). These strains referred to as 'wild' strains, have in common that they have so far not been used as starter organisms. In an international project funded by the European Community (EC-ECLAIR contract AGRE-0064) many strains of LAB were isolated from 24 different sources of artisanal products e.g., cheese and fermented milk from a number of European countries. The isolates were identified and partially characterised.

The first studies on the wild *Lactococcus lactis* strains, showed that these isolates differ in a number of phenotypical properties from strains commonly present in industrial starter cultures. The classical differentiation between the most frequently used subspecies *lactis* and *cremoris*, is based on phenotypical differences. For instance, *L. lactis* subsp. *lactis* strains are characterized by their ability to hydrolyse arginine, to metabolize a number of sugars, and to grow at 40°C and in the presence of 4% NaCl, whereas *L. lactis* subsp. *cremoris* strains are not able to grow at 40°C and in the presence of 4% NaCl, and to hydrolyse arginine (Bergey's manual, 1984; Salama *et al.*, 1991; Cogan, 1996). Although in most cases, complete agreement was obtained between phenotype and genotype, some phenotypically distinguishing characteristics were becoming a little blurred, since some strains that were phenotypically *L. lactis* subsp. *lactis* appeared genotypically *L. lactis* subsp. *cremoris* and *vice versa* (Salama *et al.*, 1991; Godon *et al.*, 1992; Salama *et al.*, 1993; Klijn *et al.*, 1995). The use of probes in the identification of strains at the subspecies level has been very effective in distinguishing different subspecies from each other, since they are different in specific DNA sequences including those encoding 16S rRNA (Godon *et al.*, 1992). It has been found also that these wild lactococcal isolates are able to survive outside the dairy environment in contrast to industrially produced lactococci tested. This indicated that they clearly differ from

industrial starter strains (Klijn *et al.*, 1995; Weerkamp *et al.*, 1996). These findings implicate that a natural biodiversity exists in lactococci strains, which might harbour an important potential for new starters to be used for making fermented dairy products.

Although numerous studies have been focused on the characterisation of mesophilic LAB from starters, still relatively little is known about these wild lactococci, with regard to their potential application, their flavour formation and their stability in dairy products. This is especially true for lactococci isolated from non-dairy origins.

5. Outline of the thesis

The aim of the research presented in this thesis is to exploit the biodiversity of lactococci isolated from various natural niches for flavour formation in cheese as a basis for product innovation. In order to achieve this goal, various characteristics of the strains as well as the mutual interactions between the strains had to be studied.

Chapter 2 gives a description on the functional properties of a large number of wild *Lactococcus* strains isolated from various natural environments as compared to those of industrial strains with the focus on flavour forming abilities. In addition, the amino acid requirements for growth of the strains were investigated, since amino acid biosynthesising and converting enzymes are believed to play a role in the formation of flavour compounds.

In **Chapter 3** the first results of the potential application of wild strains as starters in pilot plant cheese making, either individually or together with industrial strains, are presented. Attention was paid to their technological characteristics and to their aroma forming abilities during cheese ripening. The ability of the strains to survive in simple defined strain starter cultures during the cheese making process was another point of interest. Wild strains showed various interactions with industrial strains, some appeared even to inhibit the growth of industrial strains due to the production of bacteriocins.

Chapter 4 deals with a further characterisation of antimicrobial producing wild *Lactococcus* strains among the selection of strains described in chapter 2. In order to apply them successfully as a part of tailor-made starter cultures for the manufacture of cheese, specific requirements were examined and determined.

In **Chapter 5** the stability of wild strains and their behaviour in a complex defined mixture of starter cultures is described for conditions prevailing in the manufacture of Gouda-type cheese. The emphasis was focussed on the development of flavour and other properties typical for this cheese. The results of this study opened new possibilities for designing tailor-made starter cultures for cheese diversification. However, the results also reflected unwanted interactions that can occur between strains in defined strain starters, such as the negative effect on the growth of *L. lactis* subsp. *lactis* biovar *diacetylactis* strains. To obtain insight in

such interactions in some defined starter cultures, **Chapter 6** presents an investigation on their possible mechanisms.

The interactions found within mixtures of cultures not only affected the population dynamics in the cultures but might also have an impact on flavour formation. **Chapter 7** describes the mechanism of the complementary interaction between different strains with respect to flavour production. These findings open new avenues to enhance flavour formation by tailor-made cultures.

In **Chapter 8** the combination of knowledge of flavour formation and other functional characteristics of the selected *Lactococcus* strains is applied to improve the flavour of Proosdij-type cheese in a directive manner.

A summary together with concluding remarks is given in **Chapter 9**.

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Chapter 2

Flavour forming abilities and amino acid requirements of *Lactococcus lactis* strains isolated from artisanal and non-dairy origin

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ABSTRACT

Wild lactococci from raw and fermented milk and from non-dairy origin were individually used in milk and in a cheese model to screen for their flavour forming abilities. Organoleptic evaluation revealed that wild strains generally produce specific flavours distinct from those produced by industrial strains. Analysis of volatiles by gas chromatography mass spectrometry (GC-MS) showed that several wild strains produced relatively high levels of primary alcohols and branched aldehydes in these model systems, most likely originating from amino acid degradation. A good correlation between GC-MS data and organoleptic descriptions was perceived. Using the single omission technique, it was shown that wild strains required between 1 and 4 amino acids for growth, whereas the industrial strains generally needed 9-10 amino acids for growth. This indicates that wild strains are more dependent on their own synthesis of amino acids. Concomitantly, these strains probably possess more active amino acid convertases, which could explain their ability to produce unusual flavours.

INTRODUCTION

Strains belonging to the species of *Lactococcus lactis* are the most important starters in the manufacture of semi-hard cheeses such as Gouda, Edam and Cheddar. The main function of the starter bacteria during cheese manufacture is the production of lactic acid at an appropriate rate. In addition, these bacteria make important contributions to proteolysis during ripening and to the development of cheese flavour (Smid *et al.*, 1991; Limsowtin *et al.*, 1995; Lynch *et al.*, 1997).

Almost all cheeses in industrialised European countries, Australia, Asia and America are produced using industrial starter cultures. The dairy industry has selected these cultures primarily on the basis of their performance during milk fermentation with most attention being paid to characteristics as acidification rate and phage insensitivity (Marshall, 1991). Cheese flavour is the result of the breakdown of milk protein, fat, lactose and citrate due to enzymes from milk, rennet and micro-organisms. However, the formation of many compounds essential for cheese flavour is believed to result from the action of enzymes from the starter cultures (Urbach, 1993; Broome & Limsowtin, 1998). During the years, the diversity in (flavour) characteristics of starter cultures has been strongly reduced as a consequence of the desire to deliver consistency in product quality for certain markets requiring certain flavours. Those strains seem to have acquired features linked to the adaptation to milk, such as the capacity to utilize lactose via a phosphotransferase system (De Vos & Gasson, 1989) and to degrade casein by a cell wall protease (Kok, 1990). In parallel,

certain enzymes involved in amino acid biosynthesis seem to be disrupted in these strains (Reiter & Oram, 1962; Farrow, 1980; Deguchi & Morishita, 1992). Nowadays, consumers demand, in addition to consistency and quality, for large variations in flavour of cheeses. This has initiated a need for the availability of strains (from different species of lactic acid bacteria) with novel properties for use in the dairy industry. Recently, it has been demonstrated that new strains of lactic acid bacteria, so-called 'wild strains', can be isolated from different milk environments (Weerkamp *et al.*, 1996; Cogan *et al.*, 1997) and other non-dairy sources such as plants, animals and soil (Sandine *et al.*, 1972; Williams & Collins, 1990; Collins *et al.*, 1993; Klijn *et al.*, 1995).

Based on the assumption that wild lactococci are likely to be more dependent on their own synthesis of amino acids than industrial strains, and the knowledge that amino acid conversion enzymes of lactococci play an important role in flavour formation, the focus of this work was to characterise functional properties of selected wild lactococci in relation to industrial cultures with special emphasis on flavour forming abilities.

MATERIALS AND METHODS

Origin of strains and growth conditions

Three types of strains were used in this study, (i) industrial strains derived from commercial starter cultures, (ii) dairy wild strains (DWS) originating from fermented raw milk of goats, sheep and cows from farms with artisanal production of dairy products, and (iii) non-dairy wild strains (NDWS), which come from various sources other than milk such as soil, grass, silage, milk machine, saliva of cow and udder. All industrial strains, all DWS and 9 NDWS were obtained from the culture collection of NIZO food research, Ede, The Netherlands. All these strains were identified previously and either belong to the species *Lactococcus lactis*. subsp. *lactis* or *L. lactis* subsp. *cremoris*. In addition, 55 NDWS with unknown identity were employed, which were previously isolated by Dr. Nicolette Klijn from NIZO food research.

Industrial starter strains and DWS were stored at -40°C in litmus milk with $CaCO_3$ and 0.5 % yeast extract (Difco Laboratories, Detroit, MI). NDWS that were previously isolated by Nicolette Klijn were acquired by culturing overnight at 30°C on M17 medium (Oxoid, Hampshire, UK) containing 5 g L^{-1} lactose (LM17) and subsequently individual colonies were streaked on LM17 agar plates. Cultures were stored as frozen stocks at -80°C in M17 broth with 15 % (v/v) glycerol during the time of this study. For permanent storage, cultures were stored at -135°C.

Identification of NDWS isolates

NDWS isolates (55 in total) were initially identified on the basis of cell morphology. Subsequently, cocci were genetically identified using genus-specific DNA probes for *L. lactis*. *Lactococcus* strains which were able to produce unusual flavours were further characterised at the (sub) species level with specific probes by using polymerase chain reaction amplified variable regions of 16S rRNA and specific DNA probes as described (Klijn *et al.*, 1991; Te Giffel *et al.*, 1997).

Flavour production in milk cultures and cheese model

Individual strains were pre-grown for 16 h at 30°C in sterilised milk containing 0.5 % yeast extract. Subsequently, 1% of each culture was added to 100 mL skimmed UHT milk. Sensory evaluation of the milk cultures was carried out after incubation at 30°C for 48 h. Strains which gave clearly different flavour in milk in comparison with industrial strains were selected and tested in a cheese paste model (Smit *et al.*, 1995) to characterise flavour production in a cheese-like environment. For this purpose, the cheese paste was heated to 55°C for 2 h to regain a liquid consistency and after cooling to 30°C a bacterial culture was added to a final concentration of $1x10^8$ - $5x10^8$ bacteria per g. To this end, bacteria were grown for 16 h at 30°C in 100 mL whey-permeate containing 0.5% (w/v) yeast extract and 15% (v/v) skimmed milk, centrifuged (10 min, 12,100 x *g*) and resuspended in 10 mL distilled water. The inoculated cheese paste samples were incubated in the dark at 17°C for three weeks before sensory evaluation. Milk cultures and cheese pastes were graded by an experienced panel consisting of at least 6 judges. The sensory data were subjected to principle component analysis (PCA) using the Statistica (Statsoft) package (O'Mahony, 1985).

General characterisation of strains

The ability of the strains to grow at 20, 30, and 40°C was examined in LM17. Growth was followed by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Ultrospec 3000, Pharmacia Biotech., UK). The sensitivity to salt of the strains was determined by following growth of strains in LM17 medium at 30°C in the presence of 1, 2 and 4% NaCl. The ability to hydrolyse casein was determined by using plates containing 10% skimmed milk, 1.9% β-glycerophosphate (pH 6.9), 0.001% bromocresolpurple and 1.3% agar (GMA-agar plates) (Limsowtin & Terzaghi, 1976; Hugenholtz *et al.*, 1987) and the ability to hydrolyse arginine was assessed as described previously (Weerkamp *et al.*, 1996).

Amino acids requirements

The amino acid requirements of the strains were determined with the single omission technique (Cocaign-Bousquet *et al.*, 1995) in a chemically defined medium (CDM) as described by Otto *et al.* (1983) and modified by Poolman and Konings (1988). To avoid carry-over of essential nutrients, pre-cultures were grown overnight on LM17 agar, individual colonies were picked up and resuspended in sterile physiological salt solution. Subsequently, cells were washed twice and inoculated at 1% in CDM. Bacterial growth was followed during 72 h at 30°C by spectrophotometric measurements at 600 nm in the presence or absence of each amino acid and in the presence of all amino acids as a reference medium. For every strain the maximum growth rate (μ_{max}) in the complete medium was determined (control). All conditions were examined in a minimum of three identical cultures. The percentage of μ_{max} obtained on CDM after omission of an amino acid was calculated for each amino acid. Amino acids were recorded as (a) essential if in their absence the growth rate was less than 3% of μ_{max} (b) stimulating if in their absence the growth rate was between 3 and 10% of μ_{max} (c) non-essential if in their absence the percentage of μ_{max} was more than 10%.

Analysis of volatile compounds

Volatile compounds formed by the cultures in milk and in cheese paste were identified using purge-and-trap thermal desorption cold-trap (TDCT) gas chromatography mass spectrometry (GC-MS) (Neeter & De Jong, 1992). For the analyses of milk cultures, 10 mL of the cultures was used directly. For the analyses of cheese paste, 20 mL of a cheese paste obtained by homogenization of a mixture of the cheese and double-distilled water (1:3 w/v) was prepared and used immediately after the preparation. The samples were purged with 150 mL min^{-1} helium gas for 30 min at room temperature in case of milk and at 42°C in case of cheese paste and volatile components were trapped on an absorbent trap containing carbotrap (80 mg, 20-40 mesh, Supelco) and carbosieve SIII (10 mg, 60-80 mesh, Supelco). The trapped compounds were transferred on to a capillary column of a gas chromatograph using the Chrompack PII injector (Chrompack, The Netherlands) in the TDCT model, by heating the trap for 10 min at 250°C. A narrow injection band was achieved by cryofocusing at -100°C. The conditions for the chromatographic separation and mass spectrometry have been described previously (Engels *et al.*, 1997). Structures were assigned by spectrum interpretation, comparison of the spectra with bibliographic data and comparison of retention times of reference compounds.

Free fatty acids (FFA) formed in cheese paste were determined by GC as described previously (De Jong & Badings, 1990). The amount of a particular FFA was calculated in

relation to its amount in the blank as follows: (FFA in mg kg^{-1} inoculated cheese paste / FFA in mg kg^{-1} blank cheese paste) x 100%.

RESULTS AND DISCUSSION

Identification of non-dairy wild strains isolates

Microscopic examination of the 55 NDWS isolates with unknown identity revealed that 45 of these NDWS were cocci. Subsequently, these 45 strains were genetically identified using genus-specific DNA probes for *L. lactis* and 23 strains were identified as lactococci (data not shown). Based on the flavour production in milk (see below), 7 strains were further identified on the (sub) species level; 6 strains were genotypically identified as *Lactococcus lactis* subsp. *lactis* and one strain was identified as *Lactococcus lactis* subsp. *cremoris* (data not shown). Following identification, these strains were registered in the culture collection of NIZO food research, Ede, The Netherlands. These results indicate that lactococci can be isolated from various environmental sources.

Flavour production

Wild lactococci, 47 DWS (all from the collection of NIZO food research) and 32 NDWS (9 from the collection of NIZO food research and 23 identified as such in the present study) were individually grown in milk to determine their flavour forming abilities as compared to 22 industrial reference strains. About one-third of the wild strains (16 DWS and 6 NDWS) appeared to produce flavours similar to those produced by the reference strains; e.g., yoghurt, sour, creamy and slightly sweet. However, the majority of the wild strains (31 DWS and 26 NDWS) produced specific flavours compared with industrial strains (Table 1). Descriptors such as chocolate, malty, grass, herbs, coarse, sharp, yeasty, sulphur, fruity, fatty acid, farm cheese like, sweety etc. were mentioned by the sensory panel (data not shown). Subsequently, 21 wild strains (13 DWS and 8 NDWS), representative for the broad range of different flavours that were produced in the milk cultures, were selected for further study together with 10 industrial strains. The origin of these selected strains are listed in Table 2. Each of these strains was used in the Ch-easy model (Smit *et al.*, 1995) to analyse the flavour generation properties of the strains under cheese-like conditions. The sensory data were subjected to principal component analysis. Factor analysis was applied to a set of 6 variables (the sensory attributes: creamy, yoghurt, sweet, fatty acids, fruity and chocolate taste). The PCA analysis in milk and cheese paste of a limited number of strains from each group (three DWS: B1152, B1157 and B1158, two NDWS: B1153 and B1156 and two industrial strains: SK110 and

B14) are presented in Fig. 1. This figure clearly shows that the flavour perception of the wild strains is distinct from that produced by the industrial strains (SK110 and B14), and on the other hand, the figure demonstrates a good correlation between the flavours produced in milk and in cheese paste prepared with the same strain.

Table 1. Numbers of strains used in milk culture.

	Industrial strains	Wild strains	
		Dairy	Non-dairy
Total number of strains	22	47	32
Standard flavour	22	16	6
Unusual flavour	-	31	26
Selected strains	10	13	8

Previous studies indicated that cheeses made with *lactis* strains as starter develop an abnormal flavour (fruity, dirty, etc.) as compared with cheeses made with *cremoris* strains (Perry, 1961; Vedamuthu *et al.*, 1964; Bills *et al.*, 1965). Abnormal flavours were indeed encountered with some of the artisanal and non-dairy *lactis* strains in our study. However, also the wild *cremoris* strains B1153 and B1157 included in our work gave rise to abnormal flavour and, moreover, none of the industrial *lactis* strains tested in our study gave rise to a flavour defect in milk or in cheese paste. The previous observations that *lactis* strain cause an abnormal flavour as compared with *cremoris* strains therefore seems not to be a general phenomenon.

Strikingly, no abnormal flavour was perceived with the industrial *lactis* strain B20 (which is the same strain as ML8), which is in contrast with the observations of Perry (1961). This could be due to several subculturing of this strain and supports the idea that industrial strains have lost some characteristics over the years.

Using purge-and-trap TDCT GC-MS, the production of volatile compounds during growth in milk and cheese paste was investigated. The volatile compounds produced by B1152, B1157, B1158 (DWS), B1153, B1156 (NDWS) and SK110 (industrial strain) are listed in Table 3.

Table 2. Phenotypical characteristics of selected *Lactococcus lactis* strains.

Strains	Subspecies	Source[a]	Proteolytic activity[b]	Arginine hydrolysis	Growth at 40°C	Growth With 4% NaCl
Industrial strains						
NIZO B697 (SK110)	*cremoris*	commercial starter	+	-	-	-
NIZO B64 (E8)	*cremoris*	commercial starter	+	-	-	-
NIZO B442 (Hp)	*cremoris*	commercial starter	+	-	-	-
NIZO B48 (AM1)	*cremoris*	commercial starter	+	-	-	-
NIZO B33 (AM2)	*cremoris*	commercial starter	+	-	-	-
NIZO B78	*cremoris*	commercial starter	+	-	-	-
NIZO B14	*lactis*	commercial starter	+	+	+	+
NIZO B20 (ML8)	*lactis*	commercial starter	+	+	+	+
NIZO B21	*lactis*	commercial starter	+	+	+	+
NIZO B22	*lactis*	commercial starter	+	+	+	+
Dairy wild strains						
NIZO B1158	*lactis*	raw goat milk (Fr)	-	+	+	+
NIZO B1162	*lactis*	raw goat milk (Fr)	+	+	+	+
NIZO B1163	*lactis*	raw sheep milk (Sp)	+	+	+	+
NIZO B1152	*lactis*	raw cow milk (Nl)	+	+	+	+
NIZO B1164	*lactis*	raw goat milk (Sp)	+	+	+	+
NIZO B1157	*cremoris*	raw sheep milk (Sp)	-	+	+	+
NIZO B1165	*lactis*	raw cow milk (Nl)	-	+	+	+
NIZO B1155	*lactis*	fermented raw milk (It)	-	+	+	+
NIZO B1166	*lactis*	fermented raw milk (It)	±	+	+	+
NIZO B1167	*lactis*	fermented raw milk (It)	±	+	+	+
NIZO B1168	*lactis*	fermented raw milk (Fr)	+	+	+	+
NIZO B1169	*lactis*	fermented raw milk (It)	±	+	+	+
NIZO B1170	*lactis*	fermented raw milk (Po)	+	+	+	+
Non-dairy wild strains						
NIZO B1156	*lactis*	grass (Be)	-	+	+	+
NIZO B1171	*lactis*	silage (Nl)	-	+	+	+
NIZO B1172	*lactis*	silage (Nl)	-	+	+	+
NIZO B1153	*cremoris*	milk machine (Nl)	-	+	+	+
NIZO B1159	*lactis*	milk machine (Nl)	-	+	+	+
NIZO B1154	*lactis*	soil (Nl)	-	+	+	+
NIZO B1173	*lactis*	silage (Nl)	-	+	+	+
NIZO B1174	*lactis*	silage (Nl)	-	+	+	+

[a] (Sp), Spain; (Fr), France; (Nl), The Netherlands; (Be), Belgium; (It), Italy; (Po), Portugal.
[b] +, proteolytic; -, not proteolytic; ±, weakly proteolytic.

As an example, the GC-MS aroma profiles of cheese paste prepared with B1152 and SK110 are presented in Fig. 2. Milk cultures prepared with strains B1152, B1157 and B1158 contained high levels of 2-methylpropan-1-ol, 3-methylbutan-1-ol and 2-methylbutan-1-ol in comparison with SK110 and B1156. In addition, these three samples contained relative high concentrations of the corresponding aldehydes, 2-methylpropanal, 2-methylbutanal, 3-

methylbutanal and 2-methyl-2-propenal. Methylalcohols and methylaldehydes are most likely derived from the branched-chain amino acids leucine, isoleucine and valine (Morgan, 1976; Molimard & Spinnler, 1996).

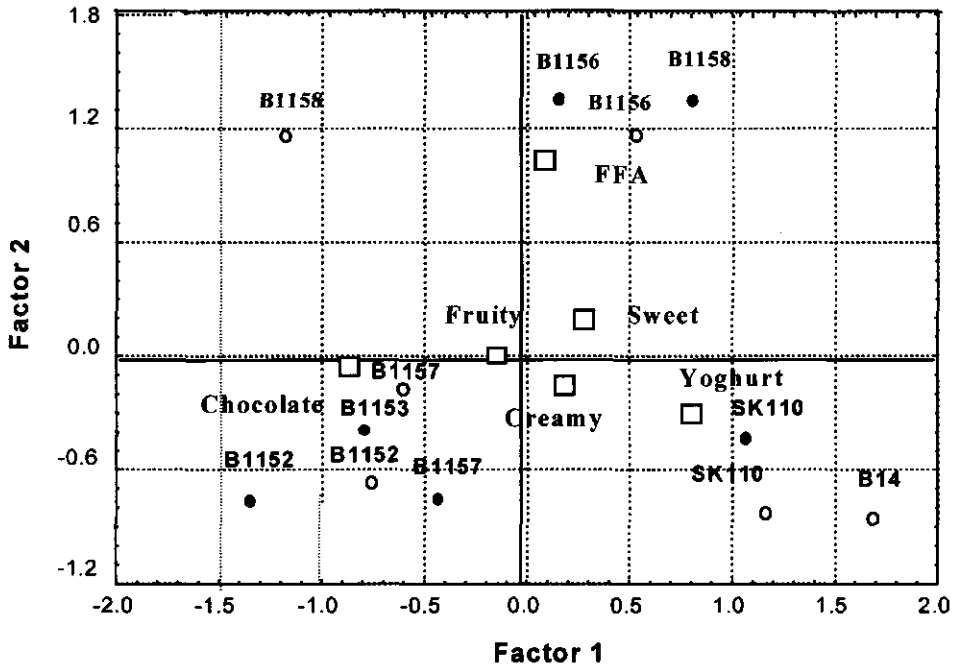

Fig. 1. Sensory profiling of industrial strain SK110, B14 and some selected wild lactococci in milk cultures and cheese model system by principal components analysis. Symbols: ○, strain in milk; ●, strain in cheese paste; □, flavour attributes.

Branched-chain primary alcohols could give rise to a slightly sweet, fresh flavour, however, the contribution of these compounds to the final flavour is thought to be rather low. Methylaldehydes developed in raw milk by the metabolic activity of *L. lactis* subsp. *lactis* biovar. *maltigenes* have been recognized as off-flavours in Cheddar cheese (Morgan, 1976). On the other hand, 3-methylbutanal has been found as major volatile compounds during ripening of Proosdij and Parmesan cheese, which are responsible for a spicy, cocoa flavour (Barbieri *et al.*, 1994). Indeed, a chocolate flavour was encountered during the organoleptic evaluation of milk incubated with B1152, B1157 and B1158, and not with SK110 and B1156 (Fig. 1). Cheese paste samples made with B1152, B1153 and B1157 also contained, in

correspondence with the organoleptic evaluation, relatively high concentration of both methylalcohols and methylaldehydes.

Table 3. GC-MS analysis of volatile compounds in milk culture and cheese paste prepared with commercial starter, dairy and non-dairy wild strains.

Compounds	Relative peak area[a]									
	Milk culture					Cheese paste				
	SK110	B1157	B1158	B1152	B1156	SK110	B1157	B1158	B1152	B1156	B1153
Alcohols											
Ethanol	20,470	53,061	34,981	46,664	48,740	44,281	26,358	20,283	28,506	29,918	63,870
Propan-2-ol	887	289	965	Trace[b]	1469	59	140	394	334	285	755
Propan-1-ol	1165	912	1376	633	2325	759	884	1394	1341	1134	1186
1-Pentanol	ND	ND	ND	ND	ND	499	1399	1761	2407	1246	554
2-Methyl propan-1-ol	494	**9859**[c]	1175	**6093**	618	ND	ND	ND	ND	ND	**1233**
3-Methyl butan-1-ol	123	**13,272**	**15,813**	**62,612**	216	532	**5954**	1480	**12,300**	1069	**5760**
2-Methyl butan-1-ol	98	**26,204**	273	**6698**	197	78	**2587**	303	**3172**	126	**1447**
Aldehydes											
2-Methyl propanal	ND	**209**	**204**	**499**	168	ND	ND	ND	ND	ND	ND
2-Methyl-2- propenal	ND	**142**	**282**	**6251**	ND	Trace	**168**	**132**	**783**	Trace	**259**
2-Methyl butanal	ND	**345**	ND	**404**	ND	ND	ND	ND	ND	ND	ND
3-Methyl butanal	56	**503**	**16,441**	**47,255**	36	487	**12,298**	**2891**	**16,270**	441	**10,513**
Pentanal	114	143	129	ND	155	429	861	1070	1571	582	639
Hexanal	91	387	98	101	191	257	320	429	358	235	255
Benzaldehyde	242	453	226	449	309	753	767	1384	1362	1193	556
Nonanal	539	**1675**	534	938	**2145**	1334	383	940	424	1410	548
Decanal	ND	ND	ND	ND	ND	1417	116	724	134	1534	86
Ketones											
Acetone	17516	23960	21402	19012	26833	2032	2139	3114	3096	4067	**8427**
Diacetyl	1532	**7306**	1728	2448	**3722**	1667	1096	1291	1408	1145	1172
Butanone	4258	3715	4148	3613	5643	448	101	1500	**2811**	1092	**2612**
2-Pentanone	569	225	591	316	784	819	1127	1747	1405	962	1200
2,3-Pentanedione	37	Trace	**773**	**656**	**1524**	ND	ND	ND	ND	ND	ND
2-Heptanone	1097	739	1343	794	1484	2798	2571	3421	2907	2510	3834
2-Nonanone	844	267	693	246	614	680	594	904	740	617	1134
Esters											
Ethyl acetate	**1296**	**2986**	1403	**4124**	1794	251	**1181**	**1091**	**2934**	400	631
Ethyl butanoate	ND	Trace	ND	ND	ND	130	147	217	160	127	510
Ethyl-3-methylbutanoate	ND	Trace	52	1214	ND	ND	ND	ND	ND	ND	ND
3-Methylbutyl acetate	ND	Trace	63	**319**	ND	51	154	91	**394**	70	82
Sulphur compounds											
Dimethyldisulphide	43	Trace	Trace	ND	Trace	37	45	**127**	63	45	76

[a] Relative peak areas of the identified peaks divided by the area obtained for the internal standard methylbutanoate expressed in arbitrary units.

[b] Trace: area ≤ 25. ND: not detected.

[c] Relatively high concentration are indicated in bold.

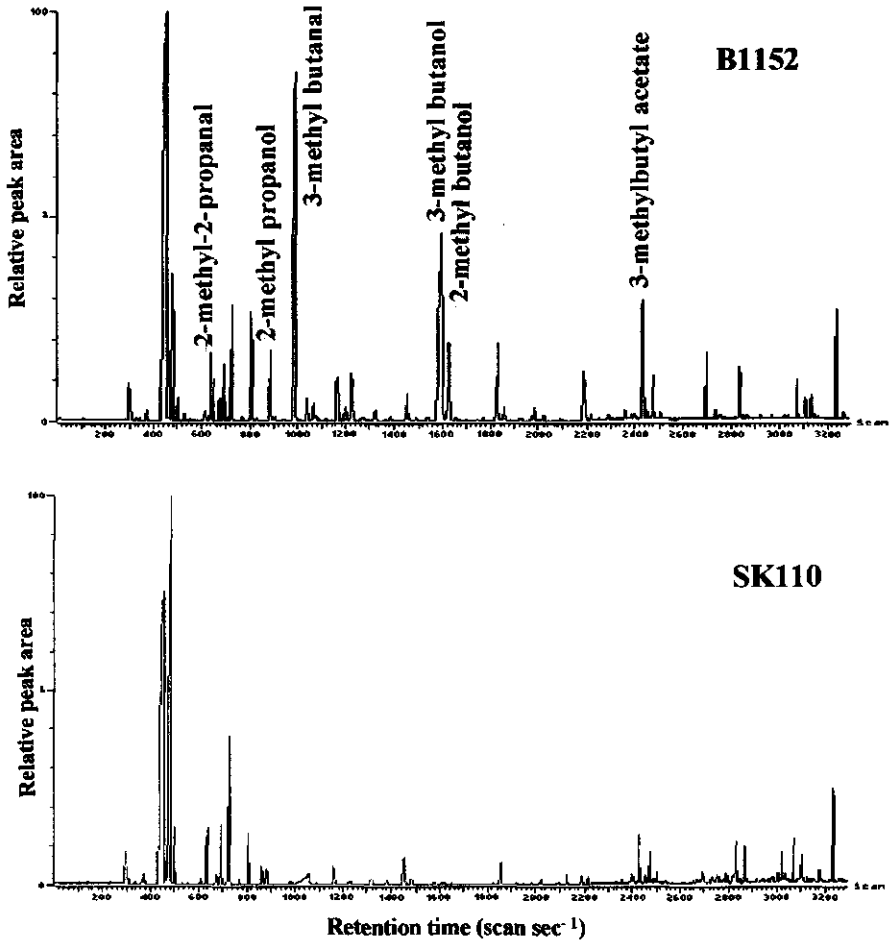

Fig. 2. GC-MS aroma profiles of volatile compounds purged from Ch-easy model prepared with wild dairy wild strain *L. lactis* B1152 and industrial strain *L. lactis* SK110. Relative peak areas are expressed in arbitrary units, normalised to largest peak.

Remarkably, strain B1158 produced less cocoa flavour in cheese paste than in milk (Fig. 1 and Table 3). The farm cheese like flavour noticed during organoleptic evaluation of this paste (data not shown) could be attributable to dimethyldisulphide (Table 3). Dimethyldisulphide, which is thought to originate from methionine breakdown, has been recognized as a very important flavour compound (with a relatively low odour threshold) in cheeses such as Limburger, Cheddar and Gouda (Parliament *et al.*, 1982; Urbach, 1993).

Less prominent differences were noticed in the other classes of volatiles. For example, some variations in the straight-chain aldehydes, particularly nonanal and decanal, were found. These aldehydes are formed during β-oxidation of unsaturated fatty acids and are characterised by green, herbaceous flavours (Moio *et al.*, 1993). The concentration of the different ketones also varied slightly between the different samples. Methylketones, which are well known for their contribution to the flavour of blue mould-ripened cheeses, are formed by enzymic oxidative decarboxylation of fatty acids. Diacetyl comes from citrate conversion and is responsible for a creamy flavour (Welsh *et al.*, 1989). Some differences in levels of ethylesters were also encountered. These compounds, which are formed by an enzymic or chemical reaction of fatty acids with primary alcohols, give a fruity and sweet character to cheese (Barbieri *et al.*, 1994).

Considering the fatty acid flavour that was perceived with quite a number of strains, the FFA content of cheese paste samples prepared with B1152, B1157, B1158 and B1156 and SK110 were analysed by GC (Fig. 3). The results indicate that B1156 produced the highest level of FFA in cheese paste, which corresponds with its organoleptic characterisation (Fig. 1). The cheese paste of B1158, which also received a fatty acid description in the organoleptic evaluation, contained fatty acid levels that were higher than those of the sample with reference strain SK110. The amount of FFA in the cheese paste incubated with B1152 was comparable with the amount in the one incubated with SK110, which fits with the sensory analysis (Fig. 1). The FFA levels in cheese paste incubated with strain B1157, were slightly higher than those in the cheese paste incubated with SK110, although this sample was not organoleptically judged as such (Fig. 1).

Phenotypical characteristics of selected strains

Selected strains were tested for various phenotypical properties (Table 2). Nine of the 13 selected DWS were able to hydrolyse milk proteins upon culturing on GMA-agar; three out of these nine strains showed a relatively low hydrolytic activity towards casein (indicated as ± in Table 2). None of the NDWS showed proteolytic activity. This will have important implications for application of these cultures in cheese making, i.e., they have to be combined with industrial strains to guarantee sufficient acidification of the milk during cheese making.

Strikingly, all wild strains were able to hydrolyse arginine, including those identified on the basis of 16S rRNA as subsp. *cremoris*. The capacity to hydrolyse arginine is a generally known characteristic for strains phenotypically identified as *L. lactis* subsp. *lactis* (Salama *et al.*, 1991; Godon *et al.*, 1992). As anticipated, all industrial strains of the *cremoris* phenotype were unable to hydrolyse arginine (Table 2).

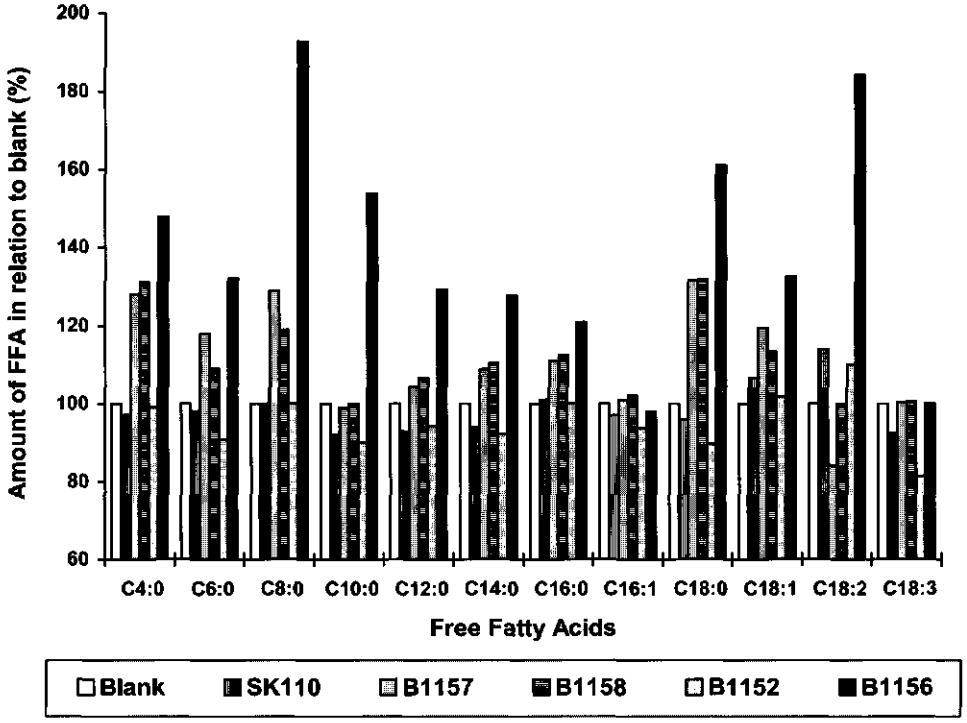

Fig. 3. Free fatty acids (FFA) extracted from Ch-easy model prepared with commercial starter SK110, dairy strains (B1157, B1158, B1152) and non-dairy wild strain (B1156).

All wild strains (subsp. *cremoris* and *lactis*) were able to grow at 40°C and in the presence of 4% NaCl in contrast to the industrial starters. So, the arginine-hydrolysing subsp. *cremoris* strains also possess other phenotypic characteristics of the subspecies *lactis*. This agrees with previous findings and seems to indicate that the phenotypical characteristics of *L. lactis* subsp. *cremoris* strains is confined to industrial starter cultures (Klijn *et al.*, 1995; Weerkamp *et al.*,1996). The ability of the wild strains to grow at 40°C and in the presence of 4% NaCl, could be functional for application in certain cheeses which are cooked to high temperatures and contain relatively high salt concentrations (e.g., Cheddar), respectively.

Amino acid requirements

Since several wild strains produced relatively high levels of primary alcohols and branched aldehydes in the model systems, which most likely originate from amino acid degradation, we focussed on the possibility that wild strains have different amino acid requirements. It can be argued that such strains are more dependent on their own synthesis than the industrial strains.

Lactococci have a limited biosynthetic capacity which explains their complex nutritional requirements. Lactococci require a number of amino acids for growth, the number of essential amino acids is strain dependent and varies from six for *L. lactis* subsp. *lactis* up to 14 for certain *L. lactis* subsp. *cremoris* strains (Mittchell *et al.*, 1941; Reiter & Oram, 1962).
The amino acid requirements of eight selected wild strains (arbitrarily choosen) were compared with the amino acid requirements of two industrial strains. In addition, four wild strains producing a standard flavour in milk culture, i.e. similar as the industrial strains, (B26, B72, B1175 and B1176) were studied (Table 4). Maximum growth rates of the strains on CDM in the presence of all amino acids were between 0.1 and 0.23 h^{-1}. The two industrial *L. lactis* subsp. *cremoris* strains tested, SK110 and Wg2, required 9-10 amino acids for growth. These results are in agreement with previous studies (Mittchell *et al.*, 1941; Reiter & Oram, 1962) in which it was concluded that glutamate, valine, methionine, histidine, serine, leucine and isoleucine are essential for most dairy *Lactococcus* strains. Both dairy and non-dairy wild strains appeared to require less amino acids than the industrial strains. The wild *L. lactis* subsp. *cremoris* strains generally required 2-3 amino acids while most of *L. lactis* subsp. *lactis* strains only required 1 or 2 amino acid. In some cases, it was found that omission of a particular amino acid still allowed growth at a very low rate (i.e., 3-10 % of μ_{max}, indicated by ± in Table 4), which suggests that the genes for the biosynthesis of these amino acids are present, but are not very active. The absence of some amino acid biosynthethic pathways in dairy lactococci might be a consequence of their adaptation to dairy products. In milk, amino acids are readily available by the proteolysis of caseins and therefore, the need for such enzymes in starter cultures is limited. Wild strains are not naturally associated with a rich environment such as milk which makes them more dependent on their own synthesis of amino acids compared to industrial strains.

The requirement of a given amino acid can result from either the absence of functional specific biosynthetic genes or from specific regulatory mechanisms (Chopin, 1993). For example, the existence of defects in biosynthesis of histidine and branched-chain amino acids has been established in *L. lactis* strains resulting from accumulated mutations and deletions within the genes coding for the biosynthetic enzymes (Delorme *et al.*, 1993; Godon *et al.*, 1993). The involvement of regulatory mechanisms in amino acid requirements has also been demonstrated in *L. lactis*.

Table 4. Essential amino acids of wild *Lactococcus* strains[a].

Amino acid	Industrial strains				Dairy wild strains						Non-dairy wild strains					
	subsp. *lactis*		subsp. *cremoris*		subsp. *lactis*			subsp. *cremoris*			subsp. *lactis*				subsp. *cremoris*	
	C_2[b]	ML3[b]	SK110	Wg2	B1155	B1152	B1162	B1157	B72	B1176	B1156	B1159	B26	B1154	B1153	B1175
Glutamic acid	+	+	+	+	-	+	+	+	-	+	±	+	+	-	+	±
Leucine	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	+
Valine	+	+	+	+	+	+	+	+	+	+	±	+	+	+	+	+
Isoleucine	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Methionine	+	+	+	+	-	-	-	-	+	+	-	-	-	-	-	+
Histidine	+	+	+	+	-	-	+	±	+	-	-	-	-	-	±	-
Proline	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Phenylalanine	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Tyrosine	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Lysine	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Cysteine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arginine	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Tryptophane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspartic acid	-	-	-	-	-	-	-	±	-	-	-	-	-	-	-	-
Alanine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Serine	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Threonine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrulline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] +, essential amino acid, i.e., growth rate is 0-3 % of μ_{max}; ±, stimulating amino acid, i.e., growth rate is 3-10% of μ_{max}; -, non-essential amino acid, i.e., growth rate is more than 10% of μ_{max}.
[b] Data from Reiter and Oram (1962).

For instance, the biosynthesis of the amino acids of the glutamate family (GLU, GLN, ARG and PRO) is dependent on the synthesis of glutamate itself which, in turn, can be affected by the ammonium ion concentration in the medium. The finding that all tested strains have a requirement for valine (Table 4) cannot result from a defect in a structural gene since all genes required for the valine synthesis are also required for the synthesis of leucine and isoleucine, suggesting a coordinated expression of these genes. Conceivably, an intermediate of the branched-chain amino acid pathway or another anabolic or catabolic pathway interferes with valine biosynthesis (Godon *et al.*, 1993).

The *L. lactis* subsp. *lactis* biovar. *maltigenes* strain described by Morgan (1976), that is thought to be responsible for production of the 'malty aldehyde' 3-methylbutanal in raw milk, requires leucine, isoleucine and valine for multiplication in a completely synthetic medium. However, the three strains that produced high amounts of 3-methylbutanal in cheese paste in our study, i.e. *L. lactis* subsp. *lactis* B1152, *L. lactis* subsp. *cremoris* B1153 and *L. lactis* subsp. *cremoris* B1157 (Table 3), do not require leucine for growth. It is therefore conceivable that these strains are able to produce leucine or the intermediate α-ketoisocaproic acid from other metabolites.

The four wild strains producing a standard flavour in milk culture that were tested for amino acid needs (B26, B72, B1175 and B1176) also required only 2-3 amino acids for growth (Table 4). Three of these strains required methionine, whereas the wild strains producing an unusual flavour, i.e., those presented in Fig. 1, were all able to grow without this amino acid. This finding might form the basis for the differences between two groups of wild strains, however, variations in substrate specificities of certain amino acid converting enzymes or regulation pathways may in addition play a role.

Taken together, the results show that the wild strains generally are less demanding regarding the amino acid supply than industrial strains. For this reason, wild strains probably harbour more (active) amino acid convertases which could explain their ability to produce interesting flavours in milk and cheese paste distinct from those produced by industrial strains. Further studies on the complex regulation of cell metabolism of these wild strains will gain insight into the routes of flavour formation in lactococci.

CONCLUSIONS

In milk culture, and under cheese-like conditions, wild strains of *Lactococcus lactis* are able to produce flavours different from those produced by industrial strains. GC-MS analysis revealed that wild strains produced several volatiles in milk and in a cheese model system. The major volatile compounds were methylalcohols and methylaldehydes, which are most

likely derived from branched-chain amino acids. The GC-MS data generally showed a good correlation with the organoleptic descriptions.

Wild strains were found to be prototrophic for most amino acids whereas industrial strains were found auxotrophic for 9-10 amino acids. Since wild strains are more dependent on their own synthesis of amino acids, these strains most likely harbour more amino acid convertases. Therefore, these strains have more active amino acid convertases which matches with their ability to produce unusual flavours. In conclusion, the use of wild strains as starters for the development of new cheeses and/or flavours looks very promising. Further studies with these strains with regard to aspects important in cheese making and elucidation of flavour forming pathways in these strains are underway.

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Chapter 3

Application of wild starter cultures for flavour development in pilot plant cheese making

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ABSTRACT

A number of wild lactococci from dairy and non-dairy origin which have the ability to produce unusual new flavours in model systems were studied with regard to various characteristics important for cheese making. All strains were found to be non-lysogenic and resistant to phages affecting strains present in commercial starters. Since the overall acidifying activity of many potentially interesting strains is rather low, they were used in combination with commercial starters. Defined strain starter cultures (DSS) were prepared, composed of a combination of wild strains together with industrial strains, and tested in real cheese making (Gouda-type) experiments. The population dynamics of DSS were studied to understand the behaviour of the selected wild strains in the cheese environment. Wild strains showed various interactions with industrial strains in a defined strain starter culture. Some wild strains, which were able to grow well together with industrial strains could be used relatively easily for practical applications. Other strains appeared to inhibit the growth of the industrial strains, due to the production of bacteriocins. In many cases the bacteriocin appeared to be nisin. Sensory evaluation revealed that the selected wild strains also produced typical flavours in a real cheese environment which corroborated the results obtained in model systems. GC/MS data confirmed the results of sensory evaluations.

INTRODUCTION

Starter cultures used in manufacturing cheeses such as Gouda, Edam and Cheddar usually consist of mesophilic lactic acid bacteria (LAB), mainly *Lactococcus lactis* spp. Important characteristics of starter cultures related to cheese making are phage insensitivity, acidification activity, proteolytic activity and flavour production. In Gouda cheese also eye-formation is an important characteristic. Flavour is one of the most important attributes of cheese, therefore it has received much attention (e.g., Urbach, 1997). Cheese flavour development is a very complex process, originating from a combination of microbiological, biochemical and technological aspects. Starter cultures play a key role in the flavour development during ripening of cheese (Urbach, 1993; Broome & Limsowtin, 1998).

New strains of lactic acid bacteria, so-called 'wild strains', can be isolated from different milk environments (Weerkamp *et al.*, 1996; Cogan *et al.*, 1997) and other non-dairy sources such as plants, animals and soil (Sandine *et al.*, 1972; Williams & Collins, 1990; Collins *et al.*, 1993; Klijn *et al.*, 1995). In a previous study (Chapter 2; Ayad *et al.*, 1999), it was shown that such strains have the ability to produce flavours distinctly different from those produced by industrial starter cultures in model systems. Moreover, these wild strains, in contrast to industrial strains, have the capacity to grow at 40°C and in the presence of 4% NaCl, which

could be functional for application in cheeses which are cooked to high temperatures (e.g., Cheddar) and in cheeses containing relatively high salt concentrations. Therefore, these strains may have a good potential for developing new types of cheese.

The present work focusses on using wild lactococci strains individually and in combination with industrial strains in order to test their behaviour in real cheese making. Attention was paid to the technologically important characteristics of the wild strains, the aroma formation and the population dynamics of the mixtures of strains to understand the behaviour of the wild strains during the cheese making process.

MATERIALS AND METHODS

Origin of strains

Strains were obtained from the culture collection of NIZO food research. Industrial strains were derived from commercial starters; dairy wild strains (DWS) originated from fermented raw milk of goats, sheep and cows from farms with artisanal production of dairy products; non-dairy wild strains (NDWS) came from various sources other than milk such as soil, grass and silage (Table 1). All strains tested belonged to the species *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris* (Ayad *et al.*, 1999).

Technological characterisation of strains

Acidification activity was measured by the change in pH after 6 h of incubation in NILAC milk powder (NIZO food research, Ede, The Netherlands) at 30°C (Stadhouders & Hassing, 1981). The ability of the strains to hydrolyse casein was determined by using plates containing 10% skimmed milk, 1.9% β-glycerophosphate (pH 6.9), 0.001% bromocresolpurple and 1.3% agar (GMA-agar plates) (Limsowtin & Terzaghi, 1976; Hugenholtz *et al.*, 1987a). Lysogeny of strains was measured by induction of prophages upon treatment with mytomycin C as described by Neve and Teuber (1991). Briefly, cultures in absence (control) and presence of mytomycin C were incubated at 30°C and growth was followed for 24 h by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Ultrospec 3000, Pharmacia Biotech., UK) and by a shift in the incubation temperature from 30°C to 40°C for 2.0-2.5 h (Feirtag & McKay, 1987). The sensitivity of strains to bacteriophages was tested using a phage enrichment technique as described before (Weerkamp *et al.*, 1996).

Population dynamics

Individual strains were pre-grown for 16 h at 30°C in sterilised milk with 0.5% yeast extract for protease-negative (prt-) strains and without yeast extract for protease positive (prt+) strains. Subsequently, 1% of individual cultures and 1% of defined strain starter cultures, i.e. the wild strain together with the industrial strain (SK110), were combined in different ratios (2:1 and 1:4). Individual and defined strain starter cultures were grown in 100 mL skimmed UHT milk for 48h at 30°C. The population dynamics of cultures were followed by plate counts. Samples were taken after 0, 2, 4, 6, and 24 h, diluted and spread on GM-agar plates and incubated at 30°C under anaerobic conditions for 2-3 days. On GMA plates, wild strains (prt-) form small white colonies during 2-3 days in contrast to the large yellowish colonies of the industrial strain SK110 (prt+). Sensory evaluation of milk cultures was carried out after 48h.

The population dynamics of defined starter cultures were also followed as mentioned above during three subcultivations. To this effect, the cultures were taken after 16 h and inoculated (1%) for the next subculturing in 100 mL skimmed UHT milk.

Cheese trials and analyses

Gouda-type cheese was made from 200 L portions of pasteurised (10 s, 74°C) milk in a manner characteristics for Gouda cheese (Walstra *et al.*, 1987). Two series were made; in each series five starters sets were used for cheese making from one batch of milk. The strains were pre-grown as a single culture for 16 h at 30°C in low-fat milk. The acidifying activity of each strain was determined prior to the experiments and the amounts of culture added to the cheese vats were adjusted accordingly to obtain the activity commonly used for Gouda cheese making. The culture was inoculated directly into processed milk via direct vat inoculation (DVI) (Osborne, 1992; Stanley, 1996). Seven non-proteolytic wild strains (B1157, B1158, B1156, B1159, B1153, B1155 and B1154) which produced different flavours in model systems, were each combined with the industrial strain SK110 in the ratio 2:1 (wild-type : SK110) into defined strain starter cultures (DSS). One cheese was prepared from milk inoculated with 1% of wild strain B1152 without addition of SK110, since this strain has sufficient proteolytic activity for acidification of the cheese milk. Cheeses prepared with strain SK110 (1%) were used as a control in each series. The cheeses were ripened for 6 months at 13°C and analysed at various intervals.

For measuring the population dynamics during cheese making and during ripening of the cheeses, the total number of bacteria in each sample were determined. Cheese samples were diluted 10-times in 2% trisodium citrate solution (w/v), and subsequently, homogenized for 5

min in a stomacher (Lab-Blender 400, Seward, London). Viable counts were enumerated on GM-agar plates.

Compositional analyses for fat, salt, pH and moisture on the cheese two weeks after manufacture were performed according to IDF Standards (1997, 1979, 1989, 1982, respectively). Proteolysis, total nitrogen (TN) soluble nitrogen (SN) and amino acid nitrogen (AN) were determined according to Noomen (1977).

The sensory evaluation was carried out by a panel consisting of five to eight trained cheese graders after 6 weeks, 3 and 6 months of ripening. Each panel member assessed the cheeses separately, taking into account the following features: flavour (odour and taste), consistency and firmness. For assessment of flavour and consistency, the following scale customery for similar work at NIZO were used: 8 = very good; 7 = good; 6 = sufficient; 5 = insufficient; 4 = bad; 3 = very bad. The scale for firmness was: 1 = very soft; 2 = soft; 3 = slightly soft; 4 = normal; 5 = slightly firm; 6 = firm; 7 = very firm. Intensity of flavour attributes was scored on a scale from 0 (absent) to 4 (very strong). The averages of sensory evaluations data with standard deviations were determined.

Analysis of volatile compounds

Volatile compounds in 3-months-old cheeses were identified using purge-and-trap thermal desorption cold-trap (TDCT) gas chromatography mass spectrometry (GC-MS) (Neeter & De Jong, 1992). Briefly, 20 mL of a cheese slurry, obtained by homogenization of a mixture of cheese and double-distilled water (1:2 w/v) was prepared and used immediately after the preparation. The samples were purged with 150 mL min^{-1} helium gas for 30 min at 42°C and volatile components were trapped on an absorbent trap containing carbotrap (80 mg, 20-40 mesh, Supelco) and carbosieve SIII (10 mg, 60-80 mesh, Supelco). The trapped compounds were transferred on to a capillary column of a gas chromatograph using the Chrompack PTI injector (Chrompack, The Netherlands) in the TDCT model, by heating the trap for 10 min at 250°C. A narrow injection band was achieved by cryofocusing at -100°C. The conditions for the chromatographic separation and mass spectrometry have been described previously (Engels *et al.*, 1997). Structures of the volatile compounds were assigned by spectrum interpretation, comparison of the spectra with bibliographic data and comparison of retention times with those of reference compounds.

Volatile sulphur compounds formed in some cheeses were determined by a sensitive and fast method without sample treatment, using a direct static headspace in combination with gas chromatography and flame photometric detection (HS-FPD) as described by De Jong *et al.* (2000).

Bacteriocin production

Antimicrobial activity was determined in a agar well-diffusion assay against two target organisms. Plates were prepared by adding 2 mL from an overnight culture of either *Micrococcus flavus* NIZO B423 or *L. lactis* subsp. *cremoris* SK110 as indicators to 200 mL of M17 agar medium (Oxoid, Hampshire, UK) containing 5 g L^{-1} lactose (LM17) held at 45°C. Approximately 10^5 colony-forming units (cfu) per mL were added. The agar was then immediately dispensed into round sterile 8.5 cm diameter petri dishes and after solidification, wells (diameter 3 mm) were made by removing the agar by a sterile metal borer. Subsequently, 20 μL of the neutralized and filter-sterilized supernatants of culture obtained from overnight cultures of various *L. lactis* strains, grown in LM17 broth at 30°C, were dispensed in individual wells. The plates were incubated for 2 h at 4°C and subsequently overnight at 30°C after which the diameter of the inhibition zones was measured.

Characterization of the antimicrobial activity was obtained by evaluation of the sensitivity to various heat treatments and the susceptibility to different proteolytic enzymes. Active supernatants were heated at 100°C for 5, 10, 20 and up to 30 min or treated with proteolytic enzymes (α-chymotrypsin, trypsin, proteinase K or pepsin) all at a final concentration of 10 mg mL^{-1} in 20 mM phosphate buffer at pH 8.0. The incubations were performed at 30°C for 2 h. To inactivate enzymes, supernatants were heated in a boiling water bath for up to 10 min. The remaining activities of supernatants were analysed by the agar-diffusion test as described above with *L. lactis* subsp. *cremoris* SK110 as the indicator strain.

RESULTS AND DISCUSSION

Technological characteristics of strains

Several wild strains were tested for various technological properties which are important for cheese-making (Table 1). All non-dairy wild strains (NDWS) and about 50% of the dairy wild strains (DWS) showed low acidification activity. Two of the DWS (B1152 and B1170) showed high acidification activity when grown in milk, comparable to the activity of industrial strains of *L. lactis* indicating the presence of a highly active protease in these strains. Nine of the 13 selected DWS were able to hydrolyse milk proteins upon culturing on GM-agar; three of these strains showed a relatively low hydrolytic activity towards casein (Table 1). All NDWS tested showed no proteolytic activity. In general, acid production and proteolytic activity were higher in DWS than in NDWS, which might be due to the fact that NDWS are isolated from environments where casein is not the normal substrate.

Table 1. Technological characteristics of *Lactococcus lactis* strains important for cheese making.

Strains	Subspecies	Sources [a]	Acidification activity (unit°N) [b]	Proteolytic activity [a]	Lysogenic	Phage sensitive
Industrial strains						
NIZO B697 (SK110)	*cremoris*	commercial starter	45	+	+	-
NIZO B64 (E8)	*cremoris*	commercial starter	40	+	-	-
NIZO B14	*lactis*	commercial starter	50	+	-	ND
NIZO B20	*lactis*	commercial starter	52	+	-	ND
NIZO B21	*lactis*	commercial starter	51	+	-	ND
NIZO B22	*lactis*	commercial starter	49	+	-	ND
NIZO B1183	*cremoris*	commercial starter	44	+	+	+
NIZO B1181	*cremoris*	commercial starter	43	+	+	+
NIZO B1182	*cremoris*	commercial starter	45	+	+	+
NIZO B1184	*cremoris*	commercial starter	41	+	+	+
Dairy wild strains						
NIZO B1158	*lactis*	raw goat milk (Fr)	24	-	-	-
NIZO B1162	*lactis*	raw goat milk (Fr)	33	+	-	-
NIZO B1163	*lactis*	raw sheep milk (Sp)	33	+	-	-
NIZO B1152	*lactis*	raw cow milk (Nl)	50	+	-	-
NIZO B1164	*lactis*	raw goat milk (Sp)	38	+	-	-
NIZO B1157	*cremoris*	raw sheep milk (Sp)	21	-	-	-
NIZO B1165	*lactis*	raw cow milk (Nl)	26	-	-	-
NIZO B1155	*lactis*	fermented raw milk (It)	22	-	-	-
NIZO B1166	*lactis*	fermented raw milk (It)	33	±	-	-
NIZO B1167	*lactis*	fermented raw milk (It)	24	±	-	-
NIZO B1168	*lactis*	fermented raw milk (Fr)	35	+	-	-
NIZO B1169	*lactis*	fermented raw milk (It)	24	±	-	-
NIZO B1170	*lactis*	fermented raw milk (Po)	46	+	-	-
					-	-
Non-dairy wild strains						
NIZO B1156	*lactis*	grass (Be)	22	-	-	-
NIZO B1171	*lactis*	silage (Nl)	23	-	-	-
NIZO B1172	*lactis*	silage (Nl)	22	-	-	-
NIZO B1153	*cremoris*	milk machine (Nl)	22	-	-	-
NIZO B1159	*lactis*	milk machine (Nl)	24	-	-	-
NIZO B1154	*lactis*	soil (Nl)	22	-	-	-
NIZO B1173	*lactis*	silage (Nl)	23	-	-	-
NIZO B1174	*lactis*	silage (Nl)	23	-	-	-

[a] (Sp), Spain; (Fr), France; (Nl), The Netherlands; (Be), Belgium; (It), Italy; (Po), Portugal.
[b] The acidity is expresed as degree *N* (the number of mL 0.1 N NaOH to neutralize 100 mL of milk).
[c] +, proteolytic; -, not proteolytic; ±, weakly proteolytic.
ND, not determined.

All wild strains tested were found to be non-lysogenic upon treatment with mytomycin C and by a shift in the incubation temperature, in contrast to many of the tested industrial strains.

This might indicate that phages are commonly introduced in starter cultures during their presence in the dairy environment. Under these conditions lysogenic strains are naturally selected since they become resistant to the phages they acquired (Jarvis, 1989; Davidson *et al.*, 1990). Lysogenic immunity, conferred by prophages to lysogenic strains, could play a role in the protection of these strains against phage attack (Reyrolle *et al.*, 1982; Séchaud *et al.*, 1990). Strikingly, all tested wild strains were resistant to phages which do affect strains present in a commonly used commercial starter culture. Four industrial strains (B1181, B1182, B1183 and B1184) tested were sensitive to phages present in the phage cocktail (Table 1). The ability of wild strains to withstand a cocktail of phages, which affect strains present in commercial starter cultures, will have practical value for cheese making.

Since acid production and a good proteolysis are required for cheese making (Limsowtin *et al.*, 1995), it is necessary to combine these wild strains with industrial strains to prepare appropriate defined strain starter for practical application in cheese making. This will guarantee sufficient acidification of the milk during cheese making in combination with typical flavour profiles during cheese ripening.

Cheese trials and analysis

Cheeses were made with (DVI) preparations of combinations of wild strains and SK110 (DSS), the latter being responsible for a good acidification of the milk. The control cheeses, made with SK110 alone, achieved pH 5.5 after approximately 6 h, which is normal for Gouda cheese making. The rates of acid production during manufacturing in the cheeses made with DSS B1157+SK110 and B1153+SK110 were similar to the control cheeses. In cheeses made with DSS B1158+SK110 and B1155+SK110 and strain B1152, the rate of acid production was slightly faster than in the control, while in the cheeses prepared with DSS B1156+SK110, B1159+SK110 and B1154+SK110 acid production was significantly slower than in the control situation; e.g., it took up to 12 h to reduce the pH to 5.5 in these cheeses (data not shown).

Since lysis of the starters is thought to be an important parameter for proper cheese flavour development, the stability of the wild starter cultures either individually or in combination with industrial strains was assessed during cheese ripening. The total numbers of viable cell counts for a number of cheeses were determined during 6 months of ripening (Fig. 1). The results show that after 12 weeks of cheese ripening, the numbers of cells of starter culture SK110 in the control cheese was significantly reduced to 1.3×10^2 cfu g^{-1} cheese (panel A) whereas, the numbers of wild starter in a cheese made with DWS B1152 was still 5.9×10^7 cfu g^{-1} (panel B). In cheeses made with DSS B1156+SK110, the colony forming units of SK110 decreased even faster than in the control cheese (panel C). The same was found in cheeses prepared with B1159+SK110 and B1154+SK110 (data not shown). In fact, the growth of

SK110 was even reduced during cheese-making (panel C). These results together with those of the acid production during the first 6 h of cheese manufacturing, could suggest that these non-dairy wild strains have an antagonistic effect against SK110.

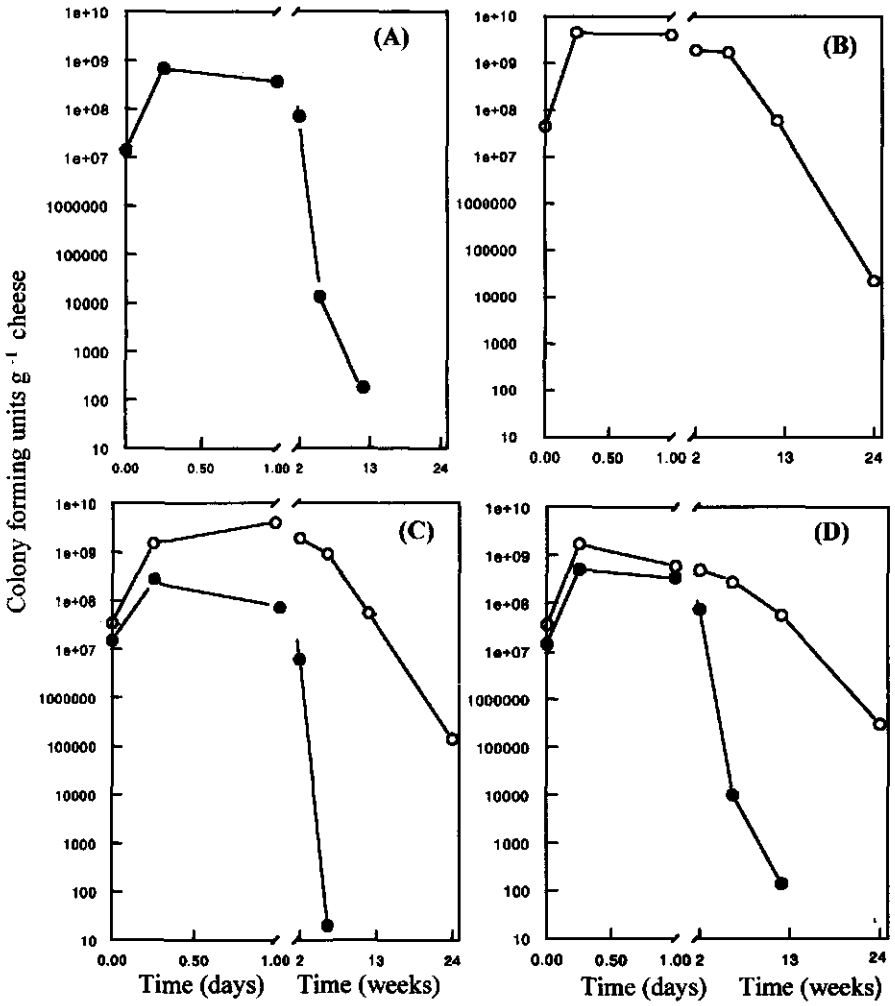

Fig. 1. Population dynamics of starter cultures in cheese prepared with combination of wild strains and SK110 (DSS) (mean of duplicates). Wild starter culture (o), commercial starter SK110 (•). (a): cell counts in cheese made with 1% SK110; (b): cheese made with 1% B1152; (c): cheese made with 2% B1156+1% SK110; (d): cell counts in cheese made with 2% B1158+1% SK110.

In cheeses made with DSS B1158+SK110 (panel D) as well as DSS B1157+SK110 and B1155+SK110 (data not shown), the colony-forming units of SK110 decreased similarly to that in the control situation (panel A), while the wild strains were found to be more stable during ripening.

Table 2. Composition of experimental cheeses two weeks after production and determination of proteolysis during ripening[a].

Cheese sample	Fat %	Moisture %	Salt %	pH	Proteolysis[b]			
					6 weeks		3 months	
					SN	AN	SN	AN
Trial 1:								
1% SK110 (control)	30.0	40.9	2.0	5.18	-[c]	-	-	-
2% B1157 + 1% SK110	31.0	40.8	2.0	5.18	-	-	-	-
2% B1158 + 1% SK110	31.5	40.4	1.9	5.15	-	-	-	-
2% B1156 + 1% SK110	29.5	42.5	2.1	5.18	-	-	-	-
1% B1152	30.5	41.1	2.1	5.15	-	-	-	-
Trial 2:								
1% SK110 (control)	29.0	42.1	2.0	5.19	12.4	2.8	22.1	5.4
2% B1159 + 1% SK110	28.2	43.0	2.0	5.19	10.6	1.4	19.5	3.1
2% B1153 + 1% SK110	29.0	41.7	1.9	5.24	11.4	2.6	21.8	4.7
2% B1155 + 1% SK110	28.5	42.2	2.0	5.16	11.2	2.7	21.7	5.4
2% B1154 + 1% SK110	28.7	42.0	2.0	5.22	10.0	1.1	18.1	2.7

[a] Results are mean of two analyses with standared error ≤ 0.3.

[b] SN, soluble N; AN, amino N. Results expressed as % of TN (Total Nitrogen).

[c] -, not determined.

The values for fat, moisture, salt and pH, after two weeks of ripening are summarized in Table 2. There was no apparent difference in cheese composition between control cheeses and cheeses made with wild strains, as the levels are within margins for normal composition of Gouda-type cheese. Proteolysis after 6 weeks and 3 months of cheese ripening was assessed by chemical analysis of the nitrogen content of the soluble nitrogen fraction (SN) and the amino acids nitrogen fraction (AN) (Table 2). The average results obtained for the two fractions during ripening of cheese made with two DSS (B1153+SK110 and B1155+SK110) were not different from the control cheese, and normal for Gouda-type cheeses. Thus, there were no significant differences in proteolytic breakdown by these DSS as compared to a commercial starter culture during cheese ripening. However, the values of SN and AN were slightly lower in cheeses manufactured with DSS B1159+SK110 and B1154+SK110 than in

the control cheeses. This finding is probably due to the quick decline in numbers for the proteolytic industrial strain SK110 in both cheeses. Some selected wild strains, e.g., B1155 and B1153, which have low proteolytic activity gave almost the same level of AN as the control when used in combination with SK110. This result is in agreement with work of Stadhouders *et al.* (1988), who reported that the flavour development in cheese made with 80% prt- and 20% prt+ was about equal to that made with 100% prt+. Apparently, the presence of a relatively low amount of prt+ starter is sufficient to give a good proteolysis and flavour development.

Population dynamics of defined wild strain starter cultures

DVI systems are used by several cheese industries since this method is easier and more convenient for the cheese producers although more expensive. These systems generally consist of mixtures of strains (DSS) that are designed to give a fast acidification of the cheese milk, a high phage resistance and good taste and texture of the final product. Some DSS used as DVI cultures in cheese making during the present study were not satisfactory, because the acidification rate in a number of cheeses was far too low which might have been caused by an inhibition of SK110 in the mixtures. Therefore, more knowledge is required for understanding of the mutual interaction between the strains in the mixtures.

The behaviour of wild strains in simple defined-strain starter cultures with the industrial strain SK110 was investigated in milk cultures to determine the interactions between the strains. The population dynamics of seven wild strains (B1153, B1154, B1155, B1156, B1157, B1158 and B1159) each one mixed with SK110 was followed in different combinations (2:1 and 1:4). Studies of population dynamics in mixed cultures can only be carried out if a general method is available to distinguish clearly the different strains (Hugenholtz, *et al.*, 1987b). The changes in the population dynamics in our study were followed in milk during 48h on GMA-plates to recognize the individual strains in such mixtures due to the difference in proteolytic activity between wild strains and industrial strains. Fig. 2 shows some examples of the population dynamics of defined strain starter cultures B1158+SK110 and B1156+SK110. The results of the population dynamics of all defined strain starter cultures showed that wild strains B1158, B1157, B1155 and B1153 can grow well with the industrial strain SK110 in a defined-strain starter culture, while other wild strains, e.g., B1156, B1159 and B1154, inhibited the growth of SK110. These results, together with the results of cheese trials prepared with the same defined strain starters, indicated that these wild strains directly affect the growth of SK110 in the defined strain starter cultures. Many different interactions can occur which effect the composition of these cultures such as competition, antibiotics production and bacteriophages (Meers, 1973).

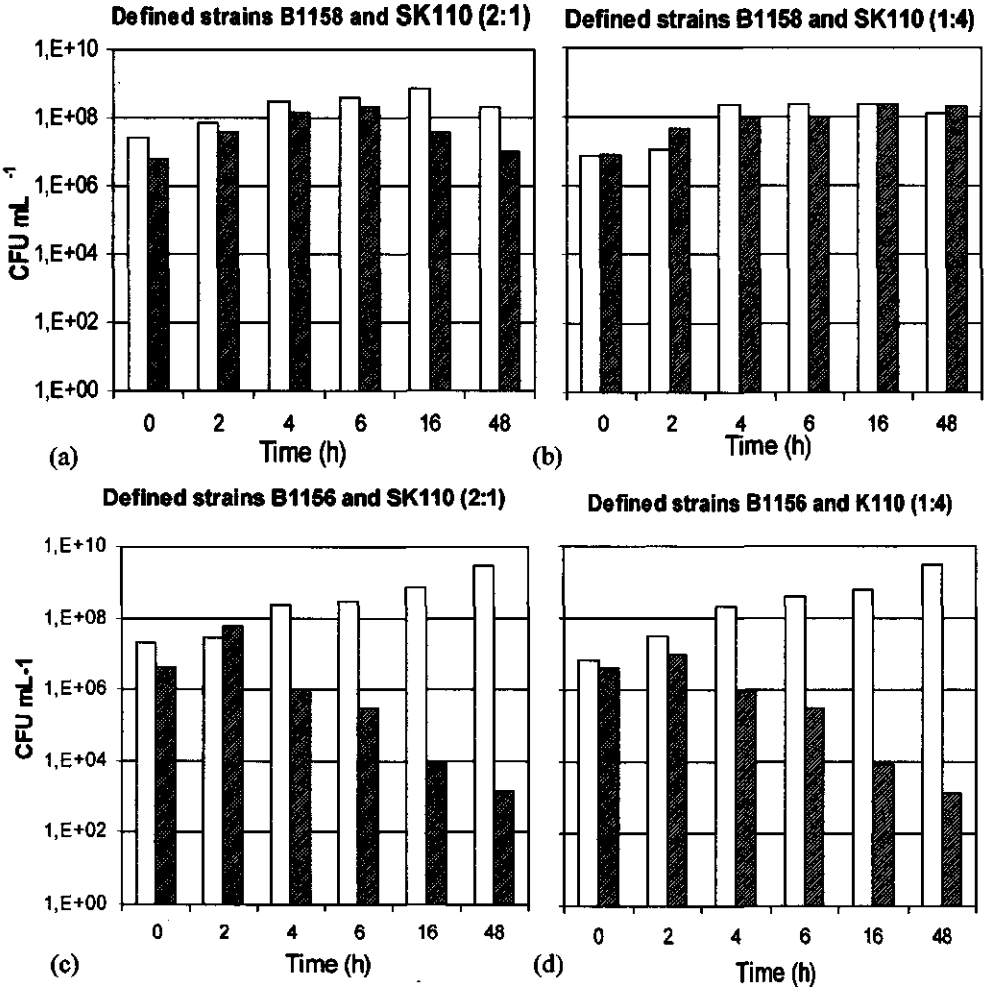

Fig. 2. Population dynamics of defiend strain starter cultures (open bars) with SK110 (filled bars), B1158+SK110 (a and b) and B1156+SK110 (c and d) in different combinations (2:1 and 1:4) during 48h in milk cultures. Results are presented by means of two analyses.

The population dynamics of defined strain starter cultures were followed during three inoculation/growth cycles (Fig. 3). The same results showing no inhibition in B1158+SK110 or inhibition in B1156+SK110 were found during three subcultivations. These results indicated that these phenomena of mutual interaction do not change upon subculturing.

Bacteriocin production

Antimicrobial activity of the eight wild strains tested was investigated using an agar well-diffusion assay against *Lactococcus lactis* subsp. *cremoris* SK110 and *Micrococcus flavus*. One DWS (B1152) and 4 NDWS (B1153, B1154, B1156 and B1159) appeared to have antimicrobial activity against the indicator organisms. Since many lactic acid bacteria are able to produce bacteriocins or bacteriocin-like substances (Jack *et al.*, 1995), these antimicrobial activities are likely to be a consequence of bacteriocin production.

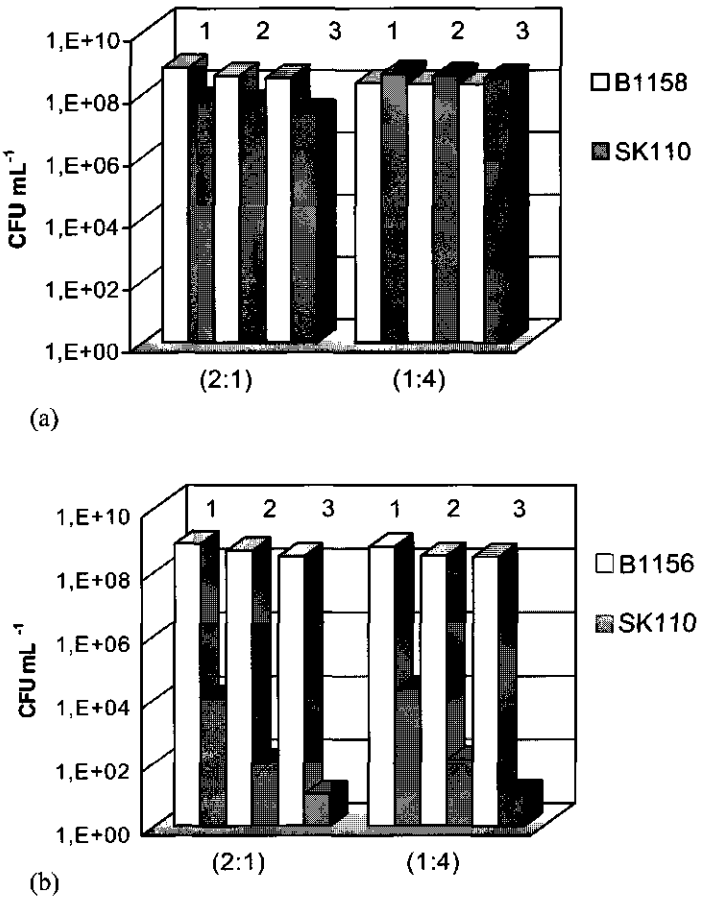

Fig. 3. Population dynamics of defined strain starter cultures with SK110 (mean of duplicates), B1158+SK110 (a) and B1156+SK110 (b) in different combinations (2:1 and 1:4) in milk cultures after 16 h during three subcultivation.

The antibacterial compounds produced by tested wild strains were further characterised on the basis of their susceptibility to proteolytic enzymes (trypsin, proteinase K, α-chemotrypsin and pepsin) and to boiling for 30 min. Bacteriocins produced by the strain B1154, B1156 and B1159 were identified as nisin on the basis of their resistance to heat treatment, inactivation by α-chemotrypsin treatment and on their activity towards both indicator strains; features typical for nisin (Hurst, 1981; Gupta & Prasad, 1989). Moreover, analysis of culture supernatants of these strains showed a clear peak at a retention time identical with that of pure nisin as measured by HPLC analysis (data not shown). Similarly, wild strain B1153 (*Lactococcus lactis* subsp. *cremoris*) most likely produces diplococcin, as the inhibitory substance was inactivated by trypsin and α-chemotrypsin as proposed by Davey and Richardson (1981). B1152 produced an unknown bacteriocin which was characterized by heat sensitivity and its inactiviation by α-chemotrypsin only. Our findings agree with those of several others (Kozak *et al.*, 1978; Scherwitz *et al.*, 1983; Gupta, 1993), who also recorded the production of diverse types of bacteriocins by different lactococci. It is noteworthy that 5 out of 9 wild strains tested showed bacteriocin production, indicating that this seems to be a rather common feature for lactococci in their natural environments. Whether such strains were not selected in the past, or they loose this feature upon subculturing, remaines to be established.

The production of these bacteriocins compounds will obviously have a strong influence on the composition of mixed starter cultures, which will tend to become dominant in these mixtures. These changes in the bacterial population will only occur if the other strains present are sensitive to the bacteriocin. Therefore, these particular strains should be combined with highly acidifying strains which are also resistant to the bacteriocin in order to prepare defined strain starter cultures.

Flavour production in cheese trials

Despite the fact that the acidification rate in some of the cheeses was rather slow, all cheeses were assessed for flavour development during ripening. Cheeses prepared with single, defined strain starter cultures and industrial strain SK110 were assessed sensorically after 3 and 6 months for flavour, consistency and firmness. All cheese samples had good texture characteristics, not noticeably different from the control cheese (results not shown). Table 3 presents the mean grade scores and standard deviations for flavour of cheeses after 3 and 6 months of ripening. The sensory results show that the wild strains produced typical flavours in cheeses which are distinct from that produced by the industrial strain SK110. The typical flavours mentioned by the sensory panel (Table 3) are in agreement with those encountered in a previous study using model systems (Ayad *et al.*, 1999).

Table 3. Sensory evaluation of cheeses prepared with wild *Lactococci* strains and industrial starter SK110 (Mean±SD).

Cheese sample	3 months		6 months	
	Description of flavour (intensity)[a]	Grade[b]	Description of flavour (intensity)	Grade
Trial 1				
1% SK110 (control)	Sour (0.5), creamy (0.5).	5.8±0.4	Sour (2.0), sharp (05), Gouda-like (0.9).	6.5±0.3
2% B1157 + 1% SK110	Malty (3.0), chocolate (0.6), bitter (0.4).	4.4±0.5	Chocolate (3.5), malty (3.0), sour (2.5).	5.0±0.4
2% B1158 + 1% SK110	Acid (0.9), H_2S (0.4), malty (0.4), bitter (1.2).	4.9±0.3	Sour (2.5), H_2S (0.5).	6.0±0.5
2% B1156 + 1% SK110	Fruity (0.3), sweet (0.2), flat (0.2), acid (1.0), sharp (0.4).	6.2±0.4	Sharp (0.5), acid (0.5), farm cheese-like.	7.5±0.3
1% B1152	Malty (2.6), bitter (1.6), salty (0.3).	4.0±0.6	Chocolate (2.0), malty (1.5), sour (2.0), bitter (2.0), scorched.	4.2±0.5
Trial 2				
1% SK110 (control)	Sour (0.9), flat (0.5), sharp (0.4).	5.8±0.6	Sharp (0.5), salt (1.0), Gouda-like (1.6).	6.5±0.4
2% B1159 + 1% SK110	Malty (1.4), chocolate (0.8), acid (0.5), scorched (0.4).	5.8±0.7	Chocolate (0.6), malty (0.9), acid (0.7).	5.9±0.5
2% B1153 + 1% SK110	Sour (1.0), sharp (0.6), Gouda like (0.7).	6.4±0.3	Sharp (1.0), Gouda-like (1.0).	6.5±0.4
2% B1155 + 1% SK110	Sour (1.1), Gouda like (1.0), Kernhem-like (0.4).	6.2±0.4	Fruity (1.0), sweety (0.4), H_2S (1.0), sharp (2.0), thermophilic (1.5), Gouda-like (1.1).	7.5±0.2
2% B1154 + 1% SK110	Sour (0.7), scorched (0.9), malty (1.0), yeasty (1.6).	4.1±0.3	Scorched (0.8), sharp (0.4), malty (0.5).	5.0±0.2

[a] intensity was scored on scale from 0 (absent) to 4 (very strong).

[b] Grade on scale from 3 (very bad) to 8 (very good), results are means with standard deviations.

Cheeses made with DSS B1156+SK110, B1153+SK110 and B1155+SK110 received the highest flavour scores while cheese made with single wild strain B1152 received the lowest score. These results indicate that selected wild strains are able to produce typical/new flavour characteristics in real cheese. The grading of the cheeses was carried out as Gouda-type cheeses with a new flavour, therefore these gradings (Table 3) should not be treated in an absolute manner. It is more important to focus on the flavour attributes and their intensity.

The volatile compounds produced in 3-months old cheeses prepared with individual and mixed-starter cultures were identified using purge-and-trap TDCT GC-MS. Many different compounds were detected and characterized in the cheeses. Each starter culture produced a typical pattern of volatile compounds which matched with the sensory flavour descriptions. Fig. 4 shows some examples of GC-MS aroma profiles of cheeses made with SK110, B1152, DSS B1159+SK110 and DSS B1153+SK110. Cheeses manufactured with B1152 and DSS B1159+SK110 (Fig.4) and DSS B1157+SK110 and B1154+SK110 (data not shown) contained high levels of methylalcohols (2-methylpropanol, 3-methylbutanol and 2-methylbutanol) and corresponding aldehydes (2-methylbutanal, 3-methylbutanal and 2-methylpropanal). Particularly, the aldehydes can be linked to the chocolate/cacao and malty flavours in these cheeses. Methylalcohols and methylaldehydes likely originate from the conversion of the branched-chain amino acids leucine, isoleucine and valine. These compounds have been recognized as off-flavours in raw milk produced by metabolic activity of *Lactococcus lactis* biovar *maltigenes* (Morgan, 1976; Molimard & Spinnler, 1996). However, such aromas are also recognized as key flavour compounds in some cheese types, e.g., some artisanal, Proosdij and Parmesan cheeses (Bosset & Gauch, 1993; Barbieri *et al.*, 1994; Neeter *et al.*, 1996). These branched-chain alcohols and aldehydes are normally not found in Gouda-type cheese in high levels. Cheese prepared with DSS B1158+SK110 was judged as slightly malty after 3 months ripening, however, this flavour was reduced after 6 months. This is most likely due to a further conversion of aldehydes to the corresponding alcohols. Cheese prepared with DSS B1157+SK110 contained, in correspondence with the sensory evaluation, a relative high concentration of methylaldehydes. However, this strain gave only a slightly chocolate aroma in model systems (Ayad *et al.*, 1999), which suggests that certain flavours when present in balance with other volatile compounds may be applied in a positive way in special cheeses depending on the DSS used.

Some cheeses, considered to show a fruity, sweet and yeasty flavours (Table 3), contained different levels of ethylesters (ethylacetate, ethylbutanoate and 3-methylbutylacetate) likely produced by the reaction of fatty acids with ethanol. These compounds are responsible for fruity and sweet (low amount) notes as found in cheese made with DSS B1156+SK110 and yeasty (high amounts) character as in cheese made with DSS B1154+SK110 (data not shown).

Farm cheese-like flavour, Kernhem-cheese-like flavour and H_2S were noticed during

sensory evaluation of some cheeses; these flavours could be attributable to sulphur compounds. Sulphur components were found in cheeses prepared with DSS B1158+SK110, B1155+SK110, B1156+SK110 and B1153+SK110 and with SK110 (control cheese) after 3 months of ripening by using HS-FPD method (Fig. 5).

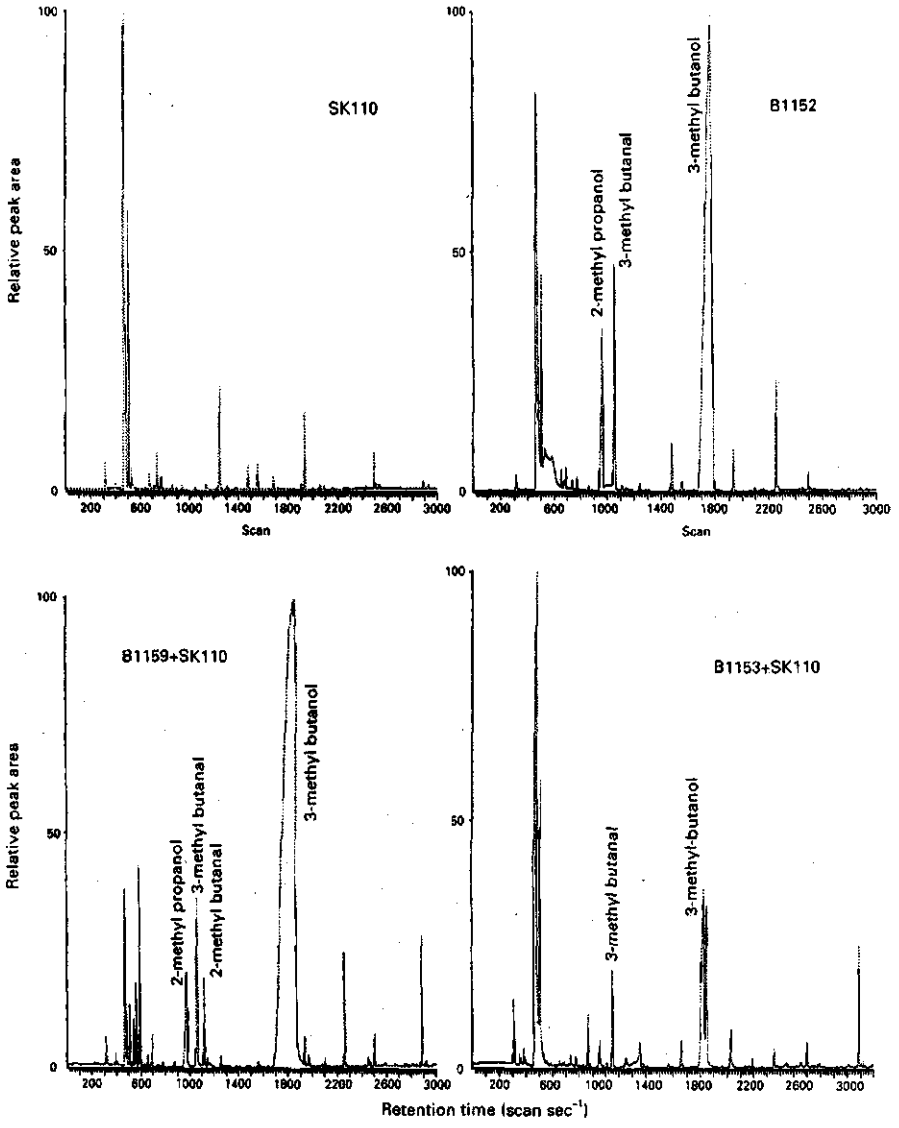

Fig. 4. GC-MS aroma profiles of volatile compounds purged from 3-month-old cheese prepared with industrial strain SK110 and wild starter cultures mixed with SK110.

The results indicated that cheese prepared with DSS B1155+SK110 had the highest level of H_2S and methanethiol followed by cheese prepared with B1156+SK110 for H_2S and B1153+SK110 for methanethiol, while cheese made with DSS B1158+SK110 had the highest amount of dimethylsulphide and CS_2.

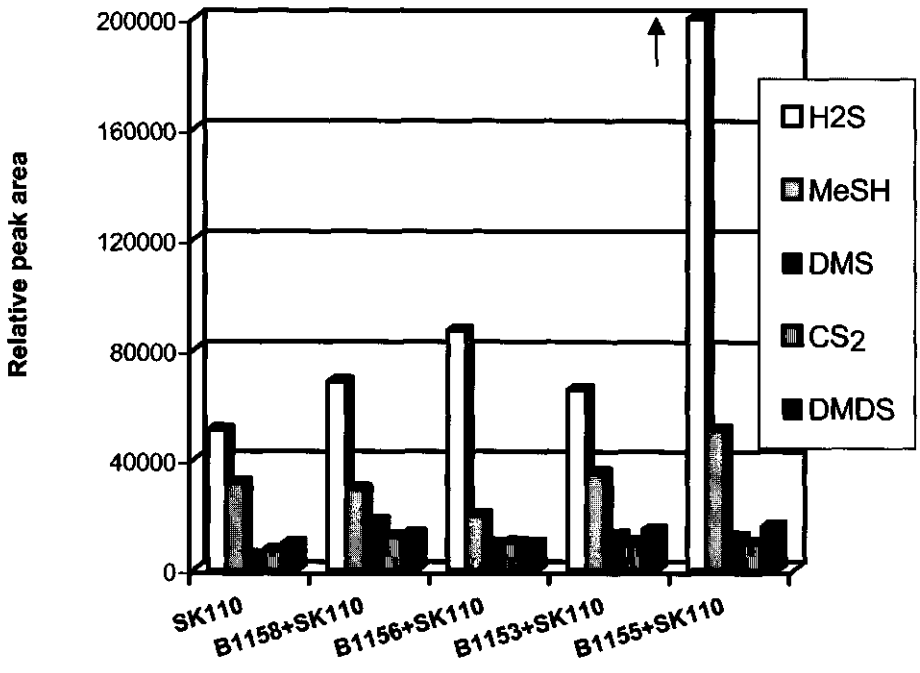

Fig. 5. Relative amounts of sulphur compounds formed during ripening of cheese after 3 months. H_2S, hydrogen sulphide; MeSH, methanethiol; DMS, dimethylsulphide; CS_2, carbon disulphide; DMDS, dimethyldisulphide.

These results are in accordance with the sensory evaluations. Dimethylsulphide, originating from methionine breakdown, has been recognized as a very important flavour compound with a relative low odour threshold in cheeses such as Limburger, Cheddar and Gouda (Parliment *et al.*, 1982; Urbach, 1993) and can be formed by enzymatic degradation of methionine by *L. lactis* (Engels *et al.*, 1997).

CONCLUSIONS

Wild strains generally show a low acidification activity indicating that these strains have to be combined with industrial strains to prepare defined strain starter cultures. However, these wild strains were not lysogenic and were resistant to phages affecting strains present in commercial cultures. Therefore, DSS have to be prepared, composed of wild strains together with industrial strains and tested in cheese making. The chemical composition of cheeses made with different DSS were similar to those of the control cheese prepared with industrial strains. Wild strains, either individual or mixed with an industrial strain, were also able to produce typical/new flavours characteristics in a real cheese environment. The results of GC/MS analysis showed that various volatile compounds were produced by selected wild strains in cheese which was linked to sensory evaluation of these cheeses. This corroborates previous results in model systems (Ayad *et al.*, 1999), indicating that testing in model systems is a useful tool in selecting strains with potentially interesting properties as starter cultures.

The population dynamics of the DSS revealed that a number of wild strains are able to grow well in defined strain starters whereas others produced antimicrobial factors. This activity should be tested before preparing new DSS. In conclusion, the development of DSS including wild strains offers new possibilities.

Further research needs to focus on the possibility to control the flavour development by preparing DSS with the right balance between flavour-producing strains and other characteristics required in Gouda cheese (e.g., eye-formation). Furthermore, the stability of these properties as well as the routes of flavour formation by these strains will have to be determined.

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Antimicrobial producing wild lactococci isolated from artisanal and non-dairy origins

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ABSTRACT

In order to apply wild lactococcal strains with diverse properties, isolated from dairy and non-dairy origins and characterised by their flavour forming abilities, as a new starter culture for dairy products, they were tested for antagonistic activities. A high percentage of the strains tested (40%), inhibited the growth of indicator strains in an agar well diffusion assay. The antibacterial factors/bacteriocins produced by these strains (16 of which produced standard flavours as compared by those produced by industrial strains and the other 16 strains produced specific flavours) were characterised on the basis of their susceptibility to proteolytic enzymes (α-chymotrypsin, trypsin, proteinase K and pepsin) and to boiling for 30 min. The bacteriocins produced by these strains were placed into four groups: nisin, diplococcin, lactococcin and unknown bacteriocin-like compounds. Seventeen strains appeared to produce nisin, which was confirmed by polymerase chain reaction (PCR) and by HPLC. Furthermore, eight of these strains were found to be able to produce nisin A and nine strains produced nisin Z. The technologically important traits in these wild strains have been examined, which allowed the selection of those strains as part of tailor-made starter cultures for the manufacture of dairy products.

INTRODUCTION

Lactococci are the most important group of lactic acid bacteria (LAB) from the point of practical applications, since they are used as starter cultures in the manufacture of several fermented foods e.g., cheese. They are responsible for protein hydrolysis to the sensory attributes and also assist in preventing the rapid spoilage of protein-rich products, mainly through the acidic conditions created during their growth (Lindgren & Dobrogosz, 1990). Beside these characteristics, they can have other relevant properties such as bacteriophage resistance or have the ability to produce bacteriocins. Bacteriocins or bacteriocin-like substances are proteins with bactericidal activity against microorganisms usually related to the producer strain as originally defined by Tagg *et al.* (1976). Production of bacteriocins is widespread among LAB (Klaenhammer, 1988; Jack *et al.*, 1995). Consequently, the topic of LAB bacteriocins have been reviewed by several authors (Daeschel, 1989; Piard & Desmazeaud, 1991; Piard & Desmazeaud, 1992; Klaenhammer, 1993). The production of these compounds by starters will obviously have a strong influence on the composition of mixed of multiple starter cultures (Meer, 1973). The main negative technological consequence of the application of bacteriocin production by a lactococcal starter is the possible dominance of the strain in a mixed starter culture on successive subcultivation. This can also occur during cheese making, as was shown by Gupta and Prasad (1989), Piard *et al.* (1990) and Ayad *et al.*

(2000). The positive aspect of using the bacteriocin producing strains is the possibility of inhibition of undesirable gram-positive bacteria including the genera *Bacillus* and *Clostridium* and bacteria pathogenic to humans (*Listeria monocytogenes* and *Staphylococcus aureus*) (Hurst, 1981; Piard *et al.*, 1990; Ray & Daeschel, 1992; Maisnier-Patin *et al.*, 1992). Over the past decennium, there has been a strong interest to produce more 'natural' foods. Lactic acid bacteria are generally regarded as safe, and some of the bacteriocins produced by them are used to control the growth of spoilage and pathogenic microorganisms in food (Hoover & Steenson, 1993; De Vuyst & Vandamme, 1994). Bacteriocin-producing strains have also been used successfully in starter cultures for cheese making in order to improve safety and quality of the cheese (Lipinska, 1973; Maisnier-Patin *et al.*, 1992; Delves-Broughton *et al.*, 1996; Ryan *et al.*, 1996).

In a previous study, we investigated lactococcal strains isolated from artisanal and non-dairy origins with specific flavour forming abilities as well as strains producing a standard flavour comparable with that produced by industrial strains (Chapter 2; Ayad *et al.*, 1999). The behaviour of strains producing specific flavours in the presence of industrial strains in a defined starter pair was studied. Some strains inhibited the growth of the industrial strains, due to the production of bacteriocins (Chapter 3; Ayad *et al.*, 2000). These wild lactococcal strains could nevertheless be of interest, when their property to produce antimicrobial compounds can be coupled with flavour forming capability in an appropriate starter. Therefore, we focused on the production of antimicrobial compounds by lactococci from artisanal and non-dairy origin in order to exploit this natural diversity in the common practice of cheese making.

MATERIALS AND METHODS

Origin of strains

The strains used in this study were obtained from the culture collection of NIZO food research. Industrial strains were derived from commercial starters. Dairy wild strains (DWS) originated from artisanal production of dairy products and non-dairy wild strains (NDWS) originated from various sources other than milk such as saliva of cow, soil, grass and silage. A total of 79 wild lactococcal strains, 22 producing standard (not specific) flavours and 57 producing specific flavours as compared to industrial strains were examined (Ayad *et al.*, 1999). All strains tested belong to the species *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris*.

Bacteriocin production

Antimicrobial activity was determined in agar well-diffusion assay against two target organisms either *Micrococcus flavus* NIZO B423 or *L. lactis* subsp. *cremoris* SK110 as described before (Ayad *et al.*, 2000). Characterization of the antimicrobial activity was obtained by evaluation of the sensitivity to various heat treatments and the susceptibility for different proteolytic enzymes (see Ayad *et al.*, 2000).

To assess which strains produced nisin, DNA coding for the bacteriocin was identified by the polymerase chain reaction (PCR). Strains *L. lactis* subsp. *lactis* NIZO 22186 and NIZO R5, producing nisin Z and A, respectively, were used as a control. Chromosomal DNA of all wild strains, which produced bacteriocin, was obtained by standard procedures (Kuipers *et al.*, 1991). Oligonucleotide primers that were used for PCR amplifications were R1: 5'-CGCGAGCATAATAAACGGCT-3' and R2: 5'-GATAGTATCCATGTCTGAAC-3'. The amplications were performed by using a Thermocycler 480 (Perkin-Elmer, Gouda, The Netherlands). The reactions were carried out in 0.5 mL tubes which contained 50 μL of the following buffer: 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM NaCl, 2.5 mM (each) deoxynucleosid triphosphate, 1U of Taq-polymerase (GibcoBRL 18038-026) and 250 ng of primer R1 or R2. After being heated to 95°C to eliminate all protease activity, 5 μL of template DNA was added. Amplification was done in 55 cycles of 1 min at 94°C (denaturation), 1 min at 25 °C (annealing), and 8 min at 72°C (extension-polymerization). PCR products were analysed by agarose gel electrophoresis as described by Klijn *et al.* (1991).

Qualitative detection of nisin was performed by analytical reversed-phase HPLC on a Hi-Pore PR-318 column (250 by 4.6 mm; Bio-Rad Laboratories, Richmond, Calif), using a linear gradient of 23 to 28% buffer B (90% aqueous acetonitrile, 0.08% trifluoroacetic acid) for 50 min with a flow rate of 1 mL min^{-1}. Absorbance was monitored at 220 nm. Strains *Lactococcus lactis* subsp. *lactis*, NIZO R5 and NIZO 22186 were used as references for nisin A and Z, respectively.

Quantification of nisin was performed with the agar well diffusion assay as described above using standard nisin solutions in a concentration range from 0.1 to 1000 IU nisin per mL with *Micrococcus flavus* as the indicator strain.

Technological characterisation of strains

The technological characteristics of the strains producing bacteriocin-like compounds was performed as described previously (Ayad *et al.*, 2000). Besides the acidification activity, the ability to hydrolyse casein, the lysogeny and the sensitivity of strains to bacteriophages, also

the ability of the strains to grow at varous temperatures and salt concentrations was tested. The strains were grown at 20, 30, and 40°C in M17 medium (Oxoid, Hampshire, UK) containing 5 g L^{-1} lactose (LM17). Growth was followed by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Ultrospec 3000, Pharmacia Biotech., UK). The sensitivity of the strains to salt was determined by following growth of strains in LM17 medium at 30°C in the presence of 1, 2, and 4% NaCl.

RESULTS AND DISCUSSION

Bacteriocin production

The antimicrobial activity of 79 wild lactococal strains, either producing standard (usual) or specific (unusual) flavours as compared with industrial strains (Ayad *et al.*, 1999), was investigated using the agar well-diffusion assay against *Lactococcus lactis* subsp. *cremoris* SK110 and *Micrococcus flavus* NIZO B423. Thirty two strains (i.e., 40%) exhibited antagonistic activity against the two indicator strains, 16 of those strains produced standard flavours and the other 16 strains produced specific flavours (Table 1). Since various lactic acid bacteria are found to produce bacteriocins (Jack *et al.*, 1995), these antimicrobial activities are likely to be a consequence of bacteriocin production. The 40% of wild strains in our study that showed bacteriocins production is higher than previously reported (Klijn *et al.*, 1995; Cogan *et al.*, 1997; Estepar *et al.*, 1999), however, in one other case also such a high percentage was found (Martinez *et al.*, 1995). It was observed that lactococci in natural niches, either producing specific or standard flavour, more frequently possess the ability to produce antimicrobial compounds. That can possibly be explained by the fact that such abilities enable them to withstand competition against other bacteria. Apparently, this property is found more frequently in strains of a non-dairy origin than in dairy strains (Table 1).

The antibacterial compounds produced by the 32 strains were characterised on the basis of their susceptibility to proteolytic enzymes (trypsin, proteinase K, α-chymotrypsin and pepsin) and to boiling for 30 min. The bacteriocins produced by the strains could be classified on the basis of their characteristics into four groups: nisin, diplococcin, lactococcin and bacteriocin-like compounds (unknown). The bacteriocins produced by 17 wild strains (*Lactococcus lactis* subsp. *lactis*) were distinctly identified as nisin on the basis of their resistance to heat treatment, inactivation by α-chymotrypsin treatment and on their activity towards both indicator strains, features which are typical for nisin as has been reported by Hurst (1981) and Gupta and Prasad (1989). This was confirmed by HPLC analysis of the culture supernatant of these strains, which showed a clear peak with a retention time identical to that of pure nisin.

Table 1. Numbers of wild strains producing bacteriocins.

	Total	Strains producing specific flavours		Strains producing standard flavours[a]	
		Dairy	Non-dairy	Dairy	Non-dairy
Total number of tested strains	79	31	26	16	6
Number of strains with antimicrobial activity	32	6	10	11	5
Nisin	17	2	5	5	5
Diplococcin	2	0	2	-	-
Lactococcin	3	1	0	2	0
Bacteriocin-like compound	10	3	3	4	0

[a] Specific and standard flavours were defined previously as described by Ayad *et al.* (1999).

The preliminary results of three strains have already been described before (Ayad *et al.*, 2000). Moreover, the bacteriocin produced by these strains was also identified as nisin by DNA isolation and polymerase chain reaction (PCR) (data not shown). Seventeen strains tested out of 32, produced nisin indicating that nisin is the most common antimicrobial peptide found to be produced by *L. lactis* subsp. *lactis* (De Vos *et al.*, 1993 & Klijn *et al.*, 1995). The inhibition zone of nisin produced by some of these wild strains were found to be larger than the inhibition zone of two control strains NIZO 22186 (nisin Z-producer) and NIZO R5 (nisin A-producer). This might be due to a stronger expression of nisin biosynthesis or to the prevalence of the nisin Z production trait. Nisin Z exhibits a greater inhibitory effect than nisin A due to its increased solubility (De Vos *et al.*, 1993; Desmazeaud, 1996). The nisin production by the strains studied was qualitatively determined by analytical reversed-phase HPLC. The results indicated that eight strains produced nisin A and nine produced nisin Z. Furthermore, nisin Z was quantitatively determined for these nine strains. Three strains, DWS B12 and NDWS B26 producing standard flavours (Table 1) and NDWS B1174 producing specific flavour (Ayad *et al*,. 2000), were found to produce significantly higher concentrations of nisin Z than the control strain NIZO 22186.

Two of the NDWS, both *Lactococcus lactis* subsp. *cremoris* strains that produce specific flavours B1153 described before (Ayad *et al.*, 2000), most likely produced diplococcin, as the inhibitory substance was inactivated by the trypsin and α-chymotrypsin a specific feature as

proposed by Davey and Richardson (1981). Three *Lactococcus lactis* subsp. *lactis* (DWS) appeared to produce lactococcin based on their resistance to heat treatment, their inactivation by the proteolytic enzymes and on their activity towards both indicator strains as referred by Geis *et al.* (1983) and Gupta (1993). Ten strains (seven *Lactococcus lactis* subsp. *lactis* strains [strain B1152 has been described before: Ayad *et al.*, 2000] and three *Lactococcus lactis* subsp. *cremoris* strains) were shown to produce unknown bacteriocins which were characterized by heat sensitivity and their inactivation by α-chymotrypsin only (Table 1). Lactococci can produce a wide variety of bacteriocins e.g nisin, diplococcin, lactococcin, lactacin 481, lactostrepcins, etc. and many more still remain undiscovered (Davey & Richardson, 1981; Hurst, 1981; Zajdel & Dobrzanski, 1983; Geis *et al.*, 1983; Delves-Broughton, 1990; Gupta & Batish, 1992; Piard *et al.*, 1992).

The production of these bacteriocin compounds by several wild strains will obviously have a strong influence on their usefulness in mixed starter cultures, because they will tend to outgrow the other strains and to become dominant in these mixtures (Ayad *et al.*, 2000). These changes in the bacterial population will only occur if the other strains present are sensitive to the bacteriocin. Therefore, to use such strains in a starter culture, they should be combined with other strains producing or resistant towards the same bacteriocins.

Among the bacteriocins of lactococci, only nisin has been granted the GRAS (generally recognised as safe) status by FAO/WHO and FDA (WHO, 1969 & Anonymous, 1988). It is used as a natural food preservative, because it efficiently inhibits the growth of several Gram-positive bacteria, e.g., *Clostridium*, *Listeria*, *Bacillus*, and *Staphylococcus* spp. Nisin-producing starter cultures of *L. lactis* subsp. *lactis* have been used as natural preservatives in cheese against these undesirable micro-organisms. They have to be combined with nisin-resistant strains to ensure adequate performance of the cheese starter (Lipinska, 1977; Roberts, *et al.*, 1992; Delves-Broughton *et al.*, 1996). The combination of existing nisin-producing starters with strains having the flavour-generating properties described in this paper will offer new avenues for the development of tailor-made nisin-producing starters.

Technological characteristics of strains

Sixteen of the 32 bacteriocin-producing strains (6 DWS and 10 NDWS) responsible for specific flavours after growth in milk have already been characterised previously (Ayad *et al.*, 2000). The technological characteristics of the other 16 bacteriocin-producing strains (11 DWS and 5 NDWS) giving rise to standard flavours comparable with those of industrial strains (Ayad *et al.*, 1999), were determined to complete our knowledge about their potential application in tailor-made multiple strain starter cultures (Table 2).

Table 2. Characteristics of wild *Lactococcus lactis* strains producing standard flavours.

Strains	Subspecies	Sources	Acidification activity (units°N)[a]	Proteolytic activity[b]	Phage Sensitivity[c]	Bacteriocin -like compounds
Dairy strains						
NIZO B7	*lactis*	raw milk	24	-	-	nisin A
NIZO B8	*lactis*	raw milk	26	-	-	nisin A
NIZO B11	*lactis*	raw milk	26	±	-	nisin Z
NIZO B12	*lactis*	raw goat milk	25	±	-	nisin Z
NIZO B19	*lactis*	raw milk	38	+	-	nisin A
NIZO B1176	*cremoris*	raw sheep milk	32	+	-	unknown
NIZO B1231	*lactis*	fermented raw milk	34	+	-	unknown
NIZO B1232	*lactis*	raw sheep milk	39	+	-	lactococcin
NIZO B1233	*lactis*	raw sheep milk	36	+	-	lactococcin
NIZO B1234	*lactis*	raw goat milk	26	-	-	unknown
NIZO B1235	*lactis*	raw goat milk	27	-	-	unknown
Non-dairy strains						
NIZO B26	*lactis*	chinese radish seed	25	-	-	nisin Z
NIZO B1236	*lactis*	soil	24	-	-	nisin Z
NIZO B1238	*lactis*	saliva of cow	23	-	-	nisin A
NIZO B1239	*lactis*	silage	37	+	-	nisin A
NIZO B1240	*lactis*	soil	26	±	-	nisin Z

[a] The acidity is expresed as degree *N* (the number of mL 0.1 N NaOH to neutralise 100 mL of milk).
[b] +, proteolytic; -, not proteolytic; ±, weakly proteolytic.
[c] Phage sensitivity as tested against a cocktail of phages isolated from commercial production sets of Gouda cheese in The Netherlands (Ayad *et al.*, 2000)

Five of 11 DWS and one of 6 NDWS showed acid production in a level from 32 to 38 N° when grown in milk, the others produced rather low amounts of acid in a range of 23-27 N°. Some of both DWS and NDWS were able to hydrolyse milk protein upon culturing on GMA. Although the NDWS were isolated from environments where casein is not the normal substrate, these strains were able to hydrolyse casein. However, the proteolytic activity and also the capacity for acid production were generally found to be higher in DWS than in NDWS.

All tested strains were found to be non-lysogenic upon treatment with mytomycin C and by a shift in the incubation temperature. Moreover, all strains were resistant to phages, which do affect strains in commonly used commercial starter cultures. This may give them a practical value for cheese making, because one of the first requirements in careful selection of starter strains is trying to avoid problems with phages (Heap, 1998).

All wild strains tested, either subsp. *cremoris* or *lactis*, were able to grow at 40°C and in the presence of 4% NaCl (data not shown). This ability might be functional for application in certain cheeses, which are cooked, to high temperatures, e.g. Cheddar, and in others, which contain relatively high salt concentrations.

Acid production and good proteolysis as well as phage resistance are required in starter cultures (Timmons *et al.*, 1988; Limsowtin *et al.*, 1995). These strains could be combined with other wild strains in order to prepare successfully defined strain starters with the required properties for practical application in cheese making and concomitantly, with the desired flavour formation capability.

CONCLUSIONS

The pool of wild lactococci isolated from dairy and non-dairy niches contains several strains, which are interesting for use in cheese making. We have shown before that they are able to produce specific flavours as well as standard flavours comparable with those of industrial strains. In this study, several of this wild lactococci were shown to produce a bacteriocin-like compound, in fact 32 of 79 strains examined had this property. These strains can be useful in defined strain starter cultures for cheese manufacture, but only if applied in combination with other strains-resistant producing the same bacteriocin. For that purpose, the stability of their technological properties and their behaviour in a defined starter culture should be evaluated.

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Chapter 5

Population dynamics of lactococci from industrial, artisanal and non-dairy origins in defined strain starters for Gouda-type cheese

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ABSTRACT

Lactococcal strains from various artisanal and natural niches were studied for the development of defined strain starter (DSS) mesophilic cultures with specific flavour forming characteristics as well as with other properties required for Gouda-type cheese. These 'wild' lactococcal strains were found to be stable up to 50 subcultivations with regard to their morphology, genetic profile, bacteriocin production, proteolytic and acidification activity, bacteriophage resistance, citrate utilizing ability as well as their ability to grow at 40°C and in the presence of 4% NaCl. In addition, the flavour forming abilities of the strains were found to be stable during subcultivation, making them suitable as starter cultures. Different DSS were prepared, each one composed of a limited number of strains for Gouda-type cheese making. These starters included proteolytic, non-proteolytic and citrate utilising strains, as well as a specific flavour-generating wild strain. One of the DSS consisted of nisin-producing together with nisin-resistant strains. The characteristics of the strains in DSS were studied in milk cultures as well as in cheese making experiments. The population dynamics revealed that a number of strains were able to grow well together and thus were suitable to be applied in tailor-made starter cultures, whereas other strains could not be maintained during growth in a mixed DSS. The results of population dynamics in the cheese corroborated the results obtained in a milk culture. These results may open new avenues for the construction of tailor-made starters for new types of cheese.

INTRODUCTION

Mesophilic starter cultures of lactic acid bacteria which are essential in the manufacture of various cheese types are mainly composed of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (Thomas & Mills, 1981; Law & Kolstad, 1983; Grow *et al.*, 1993; Limsowtin *et al.*, 1995; Broome & Limsowtin, 1998). Other types of bacteria are used as starter adjuncts depending on the type of cheese. In Gouda-type cheese, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc* spp. are responsible for diacetyl formation and CO_2, the latter being essential for the eye formation (Daly, 1983; Akkerman *et al.*, 1989; Johnson *et al.*, 1998). Generally, for cheese makers it is important to produce cheeses with a constant high quality. The use of the right starter cultures is essential, and therefore, it must be emphasised that a rigorous characterisation of starter strains is vital to ensure a good practical performance of a culture. Research has been focused on the role of starters in cheese ripening and on the potential exploitation of their antimicrobial properties (Olson, 1990; Visser, 1993; Fox *et al.*, 1996). The most important attributes of starter lactic acid bacteria required for cheese manufacture include phage insensitivity, acid-producing

activity in milk and their effect on flavour development (Marshall, 1991; Heap, 1998). In contrast to the depth of knowledge about the properties of individual starter strains, there is a lack of detailed information on their behaviour within the population of strains, which are present in a starter culture (Grow *et al.*, 1993).

Basically, two types of starter cultures are used in cheese industries: mixed cultures of unknown strains and defined cultures of known strains. Mixed cultures are used especially in Europe (mesophilic mixed starters, originated in northern Europe, particular in the Netherlands, Scandinavia and Germany, as well as thermophilic mixed starters from Italy, Switzerland and France). Limited information is available about the origin and the number of strains present in these complex starter cultures (Timmons *et al.*, 1988). The composition of a bacterial population in a mixed culture can change depending on the incubation temperature, growth medium and frequency of subculturing (Hugenholtz, 1986) and the final culture composition will also vary depending on phages present during the processing in the dairy plants. Defined strain starters (DSS) have been used mostly in countries where Cheddar and similar types of cheese are manufactured (e.g., New Zealand, Australia, UK and Ireland). DSS are usually blends of two or more defined strains, which can be mesophilic and/or thermophilic lactic acid bacteria (Lawrence & Pearce, 1972). However, DSS are vulnerable to phage infection which can cause loss of starter activity. Multiple strain DSS that include a selection of phage-resistant strains are successfully used to avoid this risk (Limsowtin *et al.*, 1977; Limsowtin *et al.*, 1978; Timmons *et al.*, 1988; Heap, 1998).

In a previous study, lactococcal strains isolated from artisanal and non-dairy environments were investigated with regard to their flavour forming abilities (Chapter 2; Ayad *et al.*, 1999). In addition, the behaviour of strains producing specific flavours was investigated in mixing such a culture with an industrial strain (Chapter 3; Ayad *et al.*, 2000). This was initiated in order to test the possibility of using such strains for cheese flavour diversification. These so called 'wild' lactococcal strains could be of interest, whether they produce a standard flavour like industrial strains or a specific flavour, to be combined with other strains for the preparation of tailor-made starter cultures with new properties.

The present work focusses on using wild lactococcal strains for cheese flavour diversification in a defined mixture of strains essential for Gouda-type cheese. Up till now, not many attempts were reported in the literature using defined strain starter cultures for the manufacture of such cheese.

Studies on population dynamics of mixed or defined strain starter cultures can only be performed if appropriate methods are available to distinguish between different strains. A number of techniques has been used including, microbiological techniques such as immuno-fluorescence (Jablon *et al*, 1976; Otto, 1981; Hugenholtz & Veldkamp, 1985; Hugenholtz, *et al.*, 1987b), or molecular biological techniques as random amplified polymorphic DNA (RAPD) and 16S rRNA sequencing based on random amplified polymorphic DNA finger

printing (Cocconcelli *et al.*, 1997).

In order to develop and test such DSS, it is essential to obtain detailed information on the population dynamics within these mixtures of strains. Beforehand, attention must be given to the stability of the wild strains during subculturing, since that is a prerequiste for prolonged use.

MATERIALS AND METHODS

Origin of strains

The strains used in this study were obtained from the culture collection of NIZO food research. Industrial strains were derived from commercial starters. Dairy wild strains (DWS) originated from artisan production of dairy products and non-dairy wild strains (NDWS) originated from various sources other than milk such as soil, grass and silage (Ayad *et al.*, 1999). All strains tested belonged to the species *Lactococcus lactis* subsp. *lactis,* subsp. *cremoris* and subsp. *lactis* biovar *diacetylactis*.

Characteristics of strains used in DSS

Acidification activity of the strains was measured by the change in pH after 6 h of incubation at 30°C in NILAC milk powder (NIZO food research, Ede, The Netherlands), the acidity being expressed as degree N° (the number of mL 0.1 NaOH to neutralize 100 mL of milk; Stadhouders & Hassing, 1981). The ability of the strains to hydrolyse casein was determined on GMA plates as described before (Ayad *et al.*, 2000). The sensitivity of strains to bacteriophages was tested using a phage enrichment technique as described before (Weerkamp *et al.*, 1996). The ability of the strains to grow at 20, 30, and 40°C was examined in M17 medium (Oxoid, Hampshire, UK) containing 5 g L^{-1} lactose (LM17). Growth was followed by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Ultrospec 3000, Pharmacia Biotech., UK). Antimicrobial activity was determined in agar well-diffusion assay against two target organisms *Micrococcus flavus* NIZO B423 or *L. lactis* subsp. *cremoris* SK110.

Stability of lactococcal wild strains

The study of the stability of individual strains during up to 50 subcultivations included eight wild lactococcal strains, B851, B1152, B1154, B1155, B1156, B1157, B1162 and B1173, which are able to produce specific flavours; three wild strains B87, B88 and B926, which produced standard flavours like industrial strains do (Ayad *et al.*, 1999); as well as two

industrial strains, SK110 and B86, were used to. These strains originating from various DWS and NDWS sources, were selected with different characteristics (Ayad *et al.*, 2000; Table 1). Individual strains were grown overnight at 30°C in litmus milk with $CaCO_3$ (Difco Laboratories, Detroit, MI) containing 0.5% yeast extract. Aliquots (1.2 mL) of these cultures was frozen at - 40°C as the zero sample. Subsequently, these cultures served as the primary inoculum (1% v/v) for the series of 50 overnight subcultivations (50 inoculations and growth cycles). Inoculations were carried out in a laminar air-flow unit. Samples (1.2 mL) from each subculture after 5, 10, 15, 20, 25, 30, 40, and 50 cycles were stored in stock at - 40°C until further use.

Subcultures of each strain were examined morphologically and four of them, randomly chosen, were studied genetically using Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) fingerprinting after 0, 15, 30 and 50 subcultures (Williams *et al.*, 1990; Te Giffel *et al.*, 1997).

Subcultures of individual strains were also pre-grown for 16 h at 30°C in sterilised milk containing 0.5% yeast extract. Subsequently, 1% of such culture was added to 100 mL skimmed UHT milk. Sensory evaluation of the milk cultures was carried out after incubation at 30°C for 48 h by 5 to 8 experienced graders. The attributes were recorded and statistically analysed. The flavour intensity was scored on a scale ranging from 0 (non or/ absent) to 4 (very strong).

Phenotypical and technological characteristics of the strains were investigated. The ability of strains to hyrolyse casein, their acidification activity, and the ability to grow at 40°C and in the presence of 4% NaCl were measured as described before (Ayad *et al.*, 2000). The sensitivity of strains to bacteriophages was tested using a phage enrichment technique and the ability to hydrolyse arginine were assessed as described previously (Weerkamp *et al.*, 1996). The ability of strains B86, B87 and B88 to ferment citrate was determined on whey with calcium citrate, calcium lactate and casaminoacids agar (WACCA) plates (Galesloot *et al.*, 1961).

Antimicrobial activities of subcultures of strains B1152, B1154, B1156 and B1162, which are known to be able to produce bacteriocin-like compounds, were determined in agar well-diffusion assay as described previously (Ayad *et al.*, 2000).

Population dynamics of defined strain starter cultures in milk

Four different DSS cultures were prepared, each one consisting of four strains including proteolytic, non-proteolytic and citrate utilising strains. The strains were combined together in equal ratios at a final inoculum level of 1%. The following DSS were chosen: DSS1: B851+B1155+B88+SK110, DSS2: B851+B1155+B86+SK110, DSS3: B1156+B1162+B895 + B1271 (a nisin producing system) and DSS4: B1173+B926+B87+SK110. Individual strains

were pre-grown for 16 h at 30°C in sterilised milk with 0.5% yeast extract (Difco Laboratories, Detroit, MI) for protease-negative (prt^-) and without yeast extract for protease positive (prt^+) strains. Subsequently, 1% as a final inoculum level of each DSS (0.25+0.25+0.25+0.25 % of the four pre-grown cultures) was grown in 100 mL skimmed UHT milk for 48 h at 30°C. The population dynamics of strains in the DSS were followed by measuring colony-forming units. Samples (duplicates) were taken after 0, 2, 4, 6, 24 and 48 h, diluted and spread on GMA plates (Limsowtin & Terzaghi, 1976; Hugenholtz *et al.*, 1987a) and incubated at 30°C and at 40°C under anaerobic conditions for 2-3 days. The cultures were also plated on whey media with calcium lactate and casaminoacids agar WACCA (Galesloot *et al.*, 1961), which is made turbid by means of calcium citrate. Only citric acid fermenting strains produce clear zones around their colonies on this medium. The WACCA plates were incubated at 35°C for 3-5 days. Based on the differences in the ability to hydrolyse casein, to grow at 40°C and to ferment citrate, the strains could be followed individually in each DSS.

The population dynamics of each DSS (each including four strains) were also followed as mentioned above during three subsequent subcultivations. The cultures were taken after 16 h and inoculated (1%) for the next subculture in 100 mL skimmed UHT milk.

The sensory evaluations of the milk cultures incubated with DSS in the first culture as well as in the three subcultures were carried out after 48 h at 30°C (see above).

Cheese making and analysis

Cheese was made using the standard technology for manufacture of Gouda cheese as described by Walstra *et al.* (1987). Gouda-type cheese was made from 200 L of pasteurised (74°C, 10 s) milk, standardized for fat (Gouda 48^+). Two cheese series were made, each from a different batch of milk. In each series two DSS cultures were used. The strains were pre-grown as a pure culture for 16 h at 30°C in low-fat milk with 0.5% yeast extract for protease-negative (prt^-) and without yeast extract for protease positive strains (prt^+) and the cultures were subsequently mixed. The acidifying activity of each DSS was determined prior to the cheese making experiments. The DSS used appeared to have a sufficient activity for acidification of the cheese milk, which is usual for Gouda cheese. The mixed culture was added into processed milk via direct vat inoculation (Stanley, 1996; Osborne, 1992). In the first series, the cheeses were manufactured using the sets DSS1 and DSS2, respectively. The strains were used in equal ratios (each 0.25% inoculum) in the DSS at final total inoculum level of 1%.

In the second series, the cheeses were made with the defined starter sets DSS5 and DSS6, respectively. DSS5 was a combination of a proteolytic *L. lactis* subsp. *cremoris* strain NIZO B894 (nisin-resistant strain) and a citrate-utilising *L. lactis* subsp. *lactis* biovar *diacetylactis* NIZO B895 (nisin-producing strain), mixed together in equal amounts and inoculated at a

final level of 1.6%; this combination was used at NIZO successfully for cheese manufacture. The other cheese was prepared from milk inoculated with DSS6: B894+ B895+B1156 in the ratio 0.8% : 0.8% : 0.5% at final inoculum level of 2.1%. All cheeses were ripened for 6 months at 13°C and analysed at various intervals.

For measuring the population dynamics of strains in DSS during cheese making and ripening of the cheeses, the total numbers of bacteria in each sample were determined in duplicate. Cheese samples were diluted 10 times in 2% trisodium citrate solution (w/v), and subsequently, homogenized for 5 min in a stomacher (Lab-Blender 400, Seward London). Viable counts were enumerated on GMA plates and on WACCA. The cell number of the individual strains could be monitored in the DSS as described above.

The compositions (fat, salt, pH, and moisture) of cheeses were analysed as described by IDF standards (IDF, 1997; 1979; 1989 and 1982, respectively).

The sensory evaluation was carried out by an experienced taste panel after 6 weeks and 3 months of cheese ripening. The cheese flavour intensity was scored on a scale ranging from 0 (none or/absent) to 4 (very strong). The scale for consistency ranged from 3 (very bad) to 8 (very good) and the scale for firmness from 1 (very soft) to 7 (very firm), see for more details Ayad *et al.* (2000).

The concentrations of nisin produced in cheeses prepared with DSS5 and DSS6 were estimated after 6 weeks of cheese ripening by an agar well diffusion bioassay with *Micrococcus flavus* NIZO B423 as the indicator strain. Five gram of cheese were diluted 10 times in 0.02 N HCl at 45°C, and subsequently, homogenized for 5 min in a stomacher (Lab-Blender 400, Seward, London). The pH of the mixture was adjusted to 2.0 using 0.5 N HCl. The mixture was centrifuged (10 min, 16000 *g*) and the supernatant was adjusted to pH 6.5 using 50% NaOH. Twenty μL of the neutralized and filter-sterilized supernatant were dispensed in wells (3mm in diameter) and the plates were incubated overnight at 30°C. After this time the zones of growth inhibition were measured and the content of nisin was estimated from the calibration curve in a concentration range from 10 to 1000 IU nisin per mL.

RESULTS AND DISCUSSION

Characteristics of strains used in DSS

Characteristics which play an important role for cheese making were investigated in the tested strains in order to be able to use them in a defined strain starter for preparation of tailor-made cheese starter cultures (Table 1). Some of these strains (SK110, B1155, B1156, B1162 and B1173) were characterised previously (Ayad *et al.*, 2000). Three of eight wild strains, B1162, B851 and B926, showed medium or high level of acid production (33, 35 and 45 N°,

respectively) when grown in milk; the other strains produced a low amount of acid in a range of 22-24 N°. The same strains were able to hydrolyse milk protein upon culturing on GMA. All strains were resistant to phages which do affect strains in commonly used commercial starter cultures. This rendered the strains useful for cheese making, since problems with phages should be avoided beforehand (Heap, 1998). The wild strains were able to grow at 40°C except the *L. lactis* subsp. *lactis* biovar *diacetylactis* strains B87 and B88.

Table 1. Distinguishing characteristics of lactococcal strains used in defined strain starter cultures

Strains	Subspecies	Sources	Proteolytic activity[a]	Nisin production[b]	Growth at 30°C	Growth at 40°C
Industrial						
SK110	*cremoris*	Commercial starter	+	-	+	-
NIZO B86 (RU4)	*lactis* biovar *diacetylactis*	Commercial starter	-	-	+	-
NIZO B894	*cremoris*	Commercial starter	+	Nis^r	+	-
NIZO B895	*lactis* biovar *diacetylactis*	Commercial starter	-	Nis^r	+	-
NIZO B1271	*cremoris*	Commercial starter	+	Nis^r	+	-
Wild strains						
NIZO B87	*lactis* biovar *diacetylactis*	Swedish cheese	-	-	+	-
NIZO B88	*lactis* biovar *diacetylactis*	Raw milk	-	-	+	-
NIZO B851	*lactis*	Raw sheep milk	+	-	+	+
NIZO B926	*lactis*	Fermented milk	+	-	+	+
NIZO B1155	*lactis*	Fermented raw milk	-	-	+	+
NIZO B1156	*lactis*	Grass	-	Nis^+	+	+
NIZO B1162	*lactis*	Raw goat milk	+	Nis^+	+	+
NIZO B1173	*lactis*	Silage	-	-	+	+

[a] +, proteolytic; -, not proteolytic.

[b] Nis^r: nisin-resistant strain, Nis^+: nisin-producing strain.

Nisin-producing starter cultures of *L. lactis* subsp. *lactis* have been used as natural preservatives in cheese against several undesirable Gram-positive bacteria (Roberts, *et al.*, 1992) but none of the existing nisin-producing starters have the flavour-generating properties and the bacteriophage resistance which are required for the manufacture of most cheese types (Lipinska, 1977; Delves-Broughton *et al.*, 1996). Two wild strains B1156 and B1162 which are able to generate specific flavours (Ayad *et al.*, 2000), were nisin-producing and were selected to be used in a DSS. These strains need to be combined with highly acidifying, nisin-resistant strains in order to apply them in a nisin-producing DSS. Therefore, two industrial

nisin-resistant strains (B1271 and B895) were used to complete the DSS3.

Stability of specific properties of lactococci

In order to be able to use wild lactococci with specific desired properties (Ayad *et al.*, 1999), these strains should maintain such specific properties when grown individually for many generations in a rich environment such as milk. Therefore, the stability of various lactococcal strains from natural niches (DWS and NDWS), producing a specific flavour or a standard flavour like industrial strains, was investigated using up to 50 subcultures in milk. The industrial strains SK110 and B86 were used as a control in these experiments. In total, 13 strains were tested with respect to different properties desired for use as starter cultures. Microscopic examination of the cultures showed no change in morphology during subcultivation, except for strain B1162 which showed a slightly different morphology (some clumping of cells in this subculture).

After subculturing 15, 30 and 50 times, the strains SK110, B1154, B1155, B1156 and B1157, randomly chosen, were studied genetically using RAPD-PCR fingerprint (Fig. 1) and the profiles were compared with those of the original strains. The profiles did not change during subcultivation, which indicates that the overall genetic stability of strains up to 50 subcultivations was high.

Subcultures of the tested strains were sensorically evaluated in milk, in order to determine the changes in their aroma characters during 50 subcultivations. All cultures appeared to produce flavours similar to those produced by the original cultures (data not shown). Only a slight decrease in the flavour intensity was found in case of strain B1162 in the late subcultures; most notably, the viscosity of this culture increased. This may be related to the clumping of cells as mentioned before.

The other phenotypical and technological characteristics of the tested strains did not change during subculturing cycles. The proteolytic activity, acidification activity, the ability to grow at 40°C and in the presence of 4% NaCl, the bacteriophage resistance and the ability to hydrolyse arginine were not altered after 50 subculturing (data not shown). The ability of B1152, B1154, B1156 and B1162 to produce bacteriocins was not altered over the 50 subcultivations. Also the citrate fermenting ability of strains B86, B87 and B88 was stable over the 50 subcultivations. Taken together, no significant changes in flavour production and other characterestics by these wild strains were observed upon subcultivations, thus making these strains suitable for use as starter cultures.

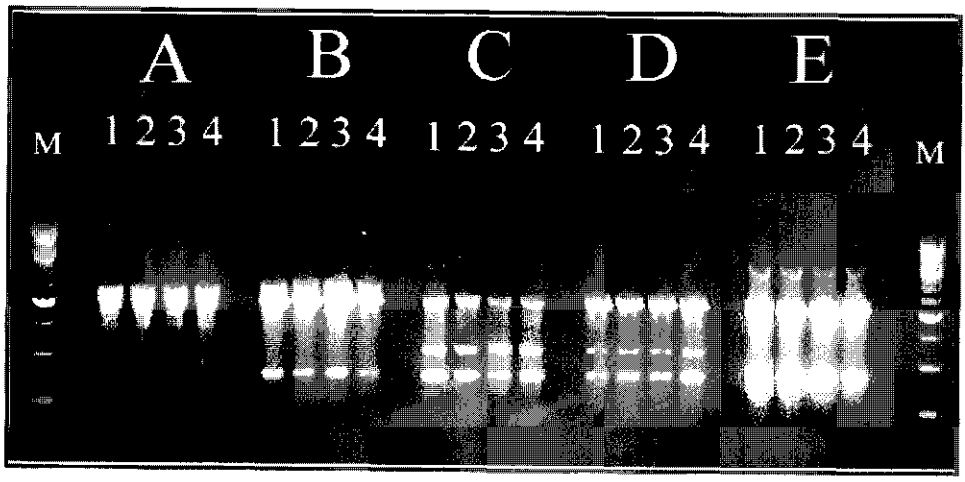

Fig. 1. Random amplified polymorphic DNA-Polymerase chain reaction RAPD-PCR fingerprinting of five strains (A-E) during 50 subculturings. Lanes (1-4) show the RAPD-fingerprint patterns after: (1) zero, (2) 15, (3) 30, and (4) 50 subculturings. Letters are the strains (A) SK110, (B) B1157, (C) B1155, (D) B1156 and (E) B1154. M= marker (Biozym, medium molecular mass standard).

Population dynamics of DSS cultures in milk

In order to prepare tailor-made DSS cultures with typical characteristics required for Gouda-type cheese, it is necessary to understand their population dynamics. For this purpose the behaviour of wild lactococcal strains was studied in milk cultures together with industrial starter strains. Four mixtures of DSS strains were investigated. Each mixture was a blend of strains suitable for Gouda-type cheese. The population dynamics of strains were followed by measuring colony-forming units during 48 h. The strains used could be distinguished individually based on differences in proteolytic activity, maximal growth temperature and the ability to ferment citrate (Table 1). DSS1 was different from DSS2 in the *diacetylactis* strains, DSS3 contained nisin-producing strains (Nis^+) B1156 and B1162 and nisin-resistant strains (Nis^r) B1271 and B895. Fig. 2 shows the population dynamics of the four DSS studied. In DSS1 and DSS3, all strains grew well together and no dominant strain was detected. The initial balance between the strains remained stable during co-cultivation, reflecting that these strains can be used as DSS. In DSS2 and DSS4, the total cell density in the population of strains reached a normal value, however, the *diacetylactis* strains B86 and B87 dramatically decreased in number within 6 h.

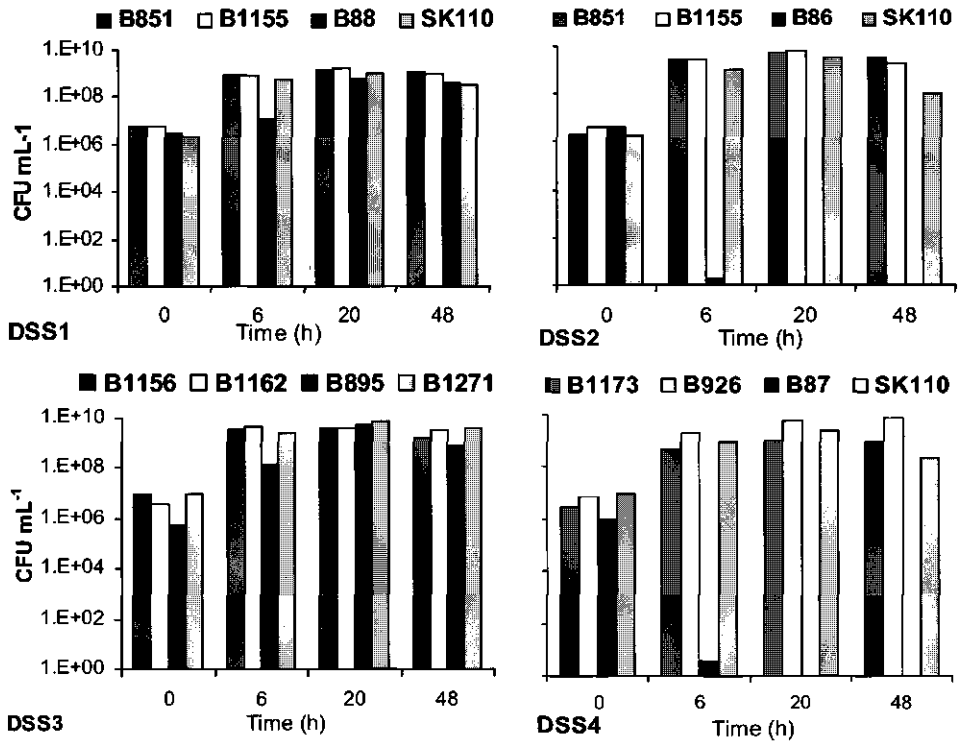

Fig. 2. Population dynamics of defined strain starters (mean of duplicates), DSS1: B851+B1155+B88+SK110; DSS2: B851+B1155+B86+SK110; DSS3: B1156+B1162+B895+B1271 (nisin system) and DSS4: B1173+B926+B87+SK110 in milk cultures during 48 h.

Similar results were found when B87 was used instead of B86 in DSS2 and vice versa (data not shown). On the other hand, when other *diacetylactis* strains, e.g., B88 or B630 (*L. lactis* subsp. *lactis* biovar *diacetylactis* strains) from the NIZO food research collection were used as alternatives in these DSS, they both grew well (data not shown). These results indicate that strains B86 and B87 were inhibited specifically in these DSS. The strains present in DSS2 and DSS4 do not produce nisin (Table 1), indicating that an unknown strain-specific inhibitory effect is present in these DSS. Many interactions can affect the population-dynamics of a mixture of starter cultures such as competition, antibiotic production and the presence of bacteriophages (Meers, 1973). Future work needs to focus on studying the interaction between these particular *diacetylactis* strains and other strains in such DSS.

The population dynamics of the defined strain starter cultures were followed during three inoculation and growth cycles (Fig. 3). The same behaviour of each strain was found during three subcultivations. The strains in DSS1 and DSS3 grew well together whereas in DSS2 and DSS4, the *diacetylactis* strains did not grow. These results indicate that the phenomena of mutual interaction did not change upon subcultivation. Thus stable DSS can be composed after careful selection of strains.

Defined strain starter cultures are not a common practice for Gouda-type cheese manufacture. Usually a mixed starter is used which contains a complex mixture of many different strains that may vary in the activities relevant for cheese manufacture (Gilliland, 1971; Law & Kolstad, 1983; Hugenholtz & Veldkamp, 1985). Changes in composition in these mixed starter cultures during cultivation should be avoided, because they may have strong effects on the acidification rate, flavour development and susceptibility to bacteriophages during the process of cheese making. The results indicate that some wild lactococci strains can be used successfully to develop tailor-made defined starter cultures with specific properties, because they are apparently able to maintain themselves in these starters.

Flavour production by DSS in milk cultures

DSS were grown in milk to determine their flavour forming abilities. Milk incubated with DSS1 was described as creamy, chocolate-like, coarse; acid and yoghurt-like were also mentioned. B851 is responsible for chocolate-like flavour; acid production resulted primarily from SK110 and B851 due to their proteolytic activity; and creamy and yoghurt-like flavours were most likely produced by B1155 and SK110 (Ayad *et al.*, 1999; 2000). DSS2 produced flavours similar to those produced by DSS1. These results indicate that B851, B1155 and SK110 together are responsible for the flavours mentioned, whereas both *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* strains B88 and B86 do not seem to contribute strongly to the flavour. DSS3 produced a specific flavour described variably as yeasty, fruity, sweety, flowery or 'esters'. Most of these typical flavours were found previously (Ayad *et al.*, 2000) and were produced by strain B1156. DSS4 produced a flavour described as creamy and dry grass-like. In general, the flavour forming abilities of the individual DSS were similar to those produced by the same DSS during three subcultivations (data not shown). These results indicate that wild lactococcal strains are able to produce specific flavours in DSS and that the flavour forming abilities are stable during subcultivations.

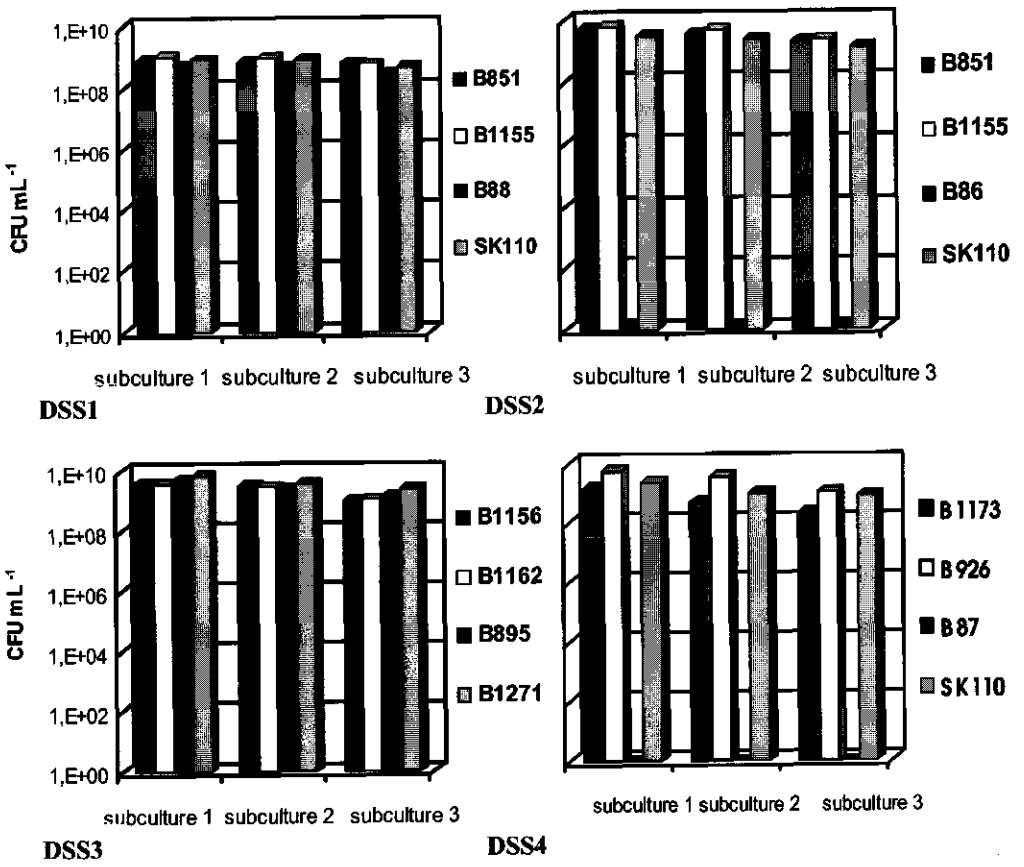

Fig. 3. Population dynamics of defined strain starter cultures (mean of duplicates), DSS1: B851+B1155+B88+SK110; DSS2: B851+B1155+B86+K110; DSS3: B1156+B1162+B895+B1271 (nisin system) and DSS4: B1173+B926+B87+SK110 in milk cultures after 16 h during three subcultivation.

Population dynamics of defined strain starter cultures in cheese

Gouda-type cheeses were manufactured with various DSS in order to investigate the potential application of wild lactococcal strains in the cheese environment and to confirm the behaviour of these DSS in milk cultures. The rates of acid production during cheese making were sufficient in all cheeses; pH 5.5 was achieved after approximately 6 h, which is normal for Gouda cheese making. The values for fat, moisture, salt and pH after 2 weeks of cheese ripening are summarized in Table 2. The levels in all cheeses were within the range for

normal composition of Gouda-type cheese. All cheese samples had good texture characteristics as shown in Table 2.

Table 2. Composition of experimental cheeses 2 weeks after production and texture after 3 months of ripening [a].

Cheese sample	Fat %	Moisture %	Salt %	pH	Texture (Mean±SD)	
					Consistency[b]	Firmness[c]
Series 1						
DSS1: B851+B1155+B88+SK110	31.0	41.5	2.1	5.18	6.7±0.4	3.8±0.4
DSS2: B851+B1155+B86+SK110	30.5	41.3	2.0	5.14	6.5±0.3	4.0±0.4
Series 2						
DSS5: B894+ B895	29.9	41.7	1.8	5.28	5.0±0.5	3.6±0.3
DSS6: B894+ B895+B1156	29.5	41.4	1.9	5.26	5.2±0.4	3.1±0.5

[a] Results of the chemical composition are the mean of two analyses with standard error ≤ 0.3.
[b] Consistency was scored on scale from 3 (very bad) to 8 (very good).
[c] Firmness was scored on scale from 1 (very soft) to 7 (very firm).

The total viable cell counts in the cheeses were determined during 6 months of ripening (Figs 4 & 5). In cheeses made with DSS1, the viability of wild-type lactococci B851, B1155 and B88 was relatively stable until 6 weeks of ripening and then decreased in the following months of ripening. SK110 already started to decrease after the first week of ripening similar to cheese made with SK110 only (Ayad *et al.*, 2000). As a result, after 3 months of ripening the cell numbers of wild strains B851, B1155 and B88 were significantly higher than the numbers of the industrial strain SK110. Taken together, the results clearly show that these wild strains can grow together with SK110 in the DSS during cheese making, which is in accordance with the results obtained in milk cultures prepared with the same DSS. In cheese made with DSS2, the number of wild type lactococcal B851 and B1155 cells was found also to be stable until 6 weeks of ripening and started to decrease thereafter. However, the total counts of the *diacetylactis* strain B86 was significantly reduced already 24 h after cheese making. These results corroborated the results obtained in milk cultures. In the second cheese making series trials were performed with a nisin-producing starter culture and a nisin-immune strain in a DSS suitable for the manufacture of Gouda-type cheese. Again, strains from natural niches were generally more stable than strains from industrial starter cultures. In the cheese made with DSS5, the number of colony forming units of B895 was stable until 3 months while those of B894 decreased. In cheese made with DSS6, the counts of B894 and B895

after 3 months of ripening were apparantly similar to those in the DSS5 situation, while the wild starter B1156, was found to be more stable during ripening than the other strains (Fig. 5). The levels of nisin produced after 6 weeks of ripening were approximately 200-360 IU g^{-1}, an amount which is expected to be functional as a natural biopreservative in cheese manufacture for prevention of butyric acid fermentations (Lipinska, 1973).

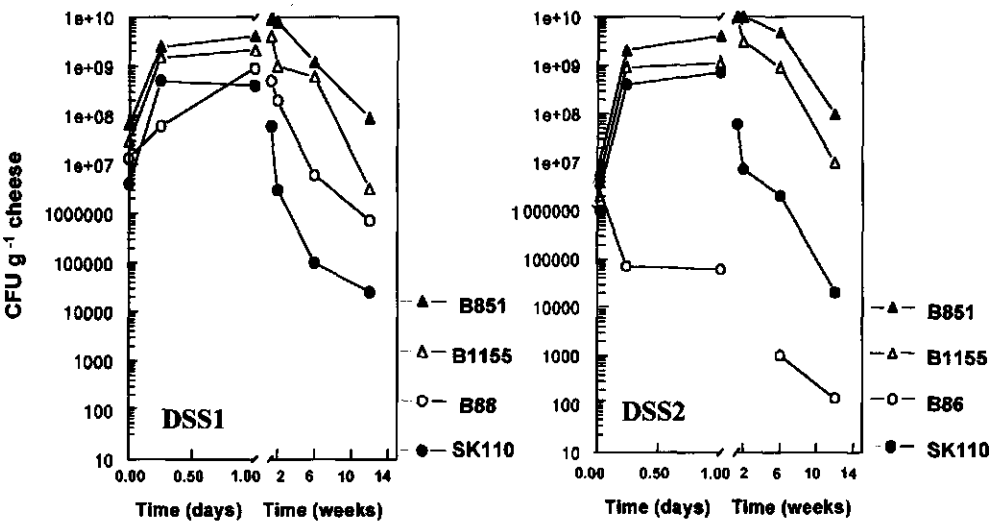

Fig. 4. Population dynamics of defined strain starter cultures (mean of duplicates) in Gouda cheese prepared with DSS1: B851+B1155+B88+SK110 and DSS2: B851+B1155+B86+SK110. The strains presented are B851 (▲), B1155 (Δ), SK110 (●) and B88 and B86 (○).

The results for populations in cheese are in accordance with the results obtained with the growth of the DSS in milk and the present findings open new possibilities for preparing tailor-made starter cultures especially with the focus on generating specific flavour notes.

Flavour production by DSS in cheese

Cheeses prepared with DSS were assessed sensorically after 6 weeks and 3 months of ripening (Table 3). The data for cheese made with DSS1 and DSS2 show that the wild strains in either DSS1 or DSS2 were able to produce typical flavours. These flavours closely resembled the results found in milk cultures prepared with the same DSS. After 6 weeks of ripening, cheeses were rather acid and received a high of bitter and malty flavour score. After 3 months of ripening the bitterness of the cheeses was reduced, wherease the malty flavour seemed to have completely disappeared. Instead, descriptions like sweet, thermophilic and sharp were mentioned during the sensory evaluation. After 3 months of ripening the flavour

became more balanced. This balance was correlated with the observed decline in cell numbers (Fig. 4), which might be an indication of cell lysis. This phenomenon is reported to be essential for the development of flavour components in cheese due to the release of flavour-generating enzymes (Visser, 1993). In a previous study (Weerkamp *et al.*, 1996) it was reported that strain B851 (Sar18) produced a highly malty and bitter flavour in cheese when used as a single starter culture Such a flavour intensity was reduced when mixed cultures were used (Ayad *et al.*, 2001).

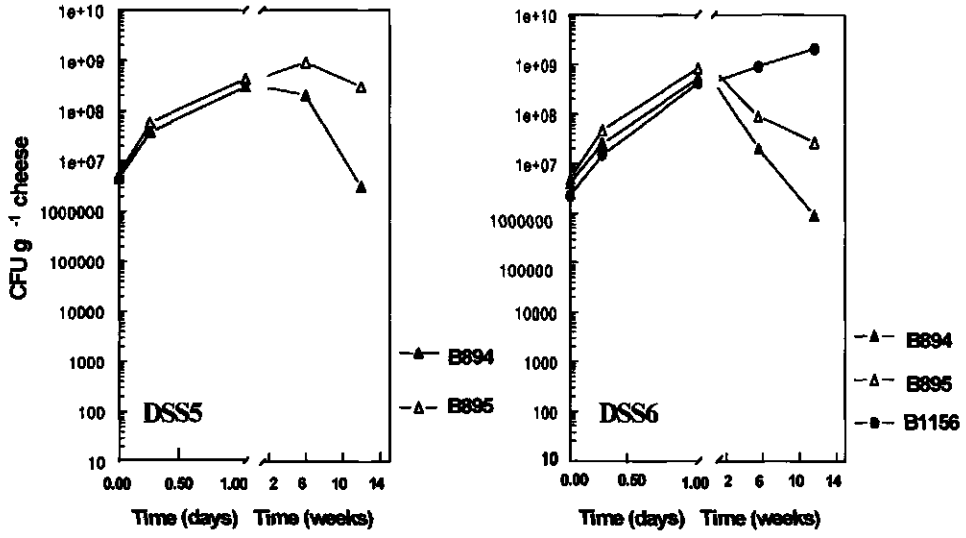

Fig. 5. Population dynamics of defined strain starter cultures (mean of duplicates) in Gouda cheese prepared with DSS5: B894+B895 and DSS6: B894+B895+B1156. The strains presented are B894 (▲), B895 (Δ) and B1156 (●).

These results suggest that choosing the appropriate composition of the starter culture is essential in order to obtain a well-balanced flavour. Gouda cheese made with the nisin-producing strains showed a good flavour intensity during ripening for 6 weeks and 3 months. The cheese manufactured with DSS5, showed some bitterness after 3 months of ripening. The addition of strain B1156 to this combination (DSS6) led to a decrease in bitterness, which is a positive contribution of this strain. The relatively high intensity of bitterness of cheese made with DSS5 could be due to the high stability of strains and thus the delay of lysis during cheese ripening (Fig. 4). A reduction in lysis sensitivity of a starter culture results in bitterness during cheese ripening (Meijer *et al.*, 1998). Despite the stability of strain B1156 in DSS6

during the ripening process, the intensity of bitterness was still reduced. Perhaps this wild strain possesses a debittering activity, which does not require lysis of the cells. Further work needs to focus on the possibility to control the flavour intensity in order to obtain well-balanced but desired flavours by DSS.

Table 3. Sensory evaluation of cheeses prepared with DSS containing wild *Lactococci* strains (summary of comments).

Cheese sample	6 weeks	3 months
	Description of flavour (intensity) [a]	Description of flavour (intensity)
Series 1		
DSS1:		
B851+B1155+B88+SK110 (0.25%+0.25%+0.25%+0.25%)	Sour (1.1), bitter (2.3), malty (2.7), slightly coarse (0.3)	Sour (2.7), bitter (1.1), flat (0.4), sweet (0.2), feta-like, sharp (0.4), thermophilic
DSS2:		
B851+B1155+B86+SK110 (0.25%+0.25%+0.25%+0.25%)	Sour (0.7), bitter (2.0), coarse (0.5), scorched malty (2.6)	Sour (1.9), bitter (1.2), thermophilic, malty, yeasty, coarse (1.0)
Series 2 (nisin system)		
DSS5:		
B894+B895 (0.8%+0.8%)	Sour (0.9), flat (0.2), Gouda-like (1.4), bitter (1.4)	Acid (1.1), bitter (1.2)
DSS6:		
B894+B895 +B1156 (0.8%+0.8%+0.5%)	Sour (0.9), flat (0.5), Gouda-like (1.6), bitter (1.1), fruity (0.3)	Sour (0.6), flat (0.3), sweet (0.2), bitter (0.6), farm cheese-like, sharp (0.4), fruity

[a] Intensity was scored on scale from 0 (absent) to 4 (very strong).

CONCLUSIONS

The morphology and genetic profiles of different wild lactococcal strains were found to be stable up to 50 subcultivations. Moreover, the flavour production and the phenotypical and technological properties including proteolytic activity, acidification activity, the bacteriophage resistance, the ability to ferment citrate as well as the ability to grow at 40°C and in the presence of 4% NaCl, were found to be stable. The frequency of subcultivation of these strains during practical application would not lead to loss of the relevant activity, which ensures a constant quality and a reproducibility in the cheese manufacture. Therefore, the use of these strains as starters for cheese looks promising.

The study on population dynamics of strains in the DSS in milk cultures and in pilot-scale production of Gouda-type cheese revealed that several strains were able to grow well together

and can be applied as tailor-made starter cultures, while others were inhibited. Further work needs to focus on the mechanism of specific inhibition of some strains (e.g., *diacetylactis*) by other strains. The tailor-made DSS cultures may be able to produce specific flavours in milk as well as in cheese as they were found to be stable in their performance.

ACKNOWLEDGMENT

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Chapter 6

An investigation on the specific inhibition of *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* strains in defined strain starter cultures

Eman H.E. Ayad, Annette Verheul, Jan T.M. Wouters and Gerrit Smit

ABSTRACT

Specific inhibition of two *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* strains (B86 and B87) in a defined starter culture (DSS), developed for Gouda cheese, by wild *Lactococcus lactis* strains B1155 and B926 was studied. The results of interaction studies revealed that the inhibition was not caused by competition between the strains for nutrients, nor by aeration, decrease of pH value during growth, production of known bacteriocins or bacteriophage release by the wild strains. The inhibition of the *diacetylactis* strains was also observed during growth in the presence of supernatant derived from exponential phase cultures of certain lactococcal strains. However, the inhibition was stronger if living cells were present. These results suggest that a specific inhibitory factor is produced by certain strains against *diacetylactis* strains. Since not all *diacetylactis* strains were affected by this factor, it appears to be a very strain-specific inhibition. This inhibitory factor was found to be a small molecular weight compound (less than 1 kDa), but heat stable up to 100°C for 30 min and unstable during prolonged incubation times at different temperatures. In addition, it was found to be inactivated by proteinase K, indicating that it might be a proteinaceous compound. These studies offer a first identification and characterisation for a new mechanism of specific inhibition between strains in a starter culture, and show its importance for understanding the underlying population dynamics.

INTRODUCTION

Lactic acid bacteria in mixed starter cultures play an important role in dairy manufacturing (Stadhouders, 1961; Schmidt *et al.*, 1976; Thomas & Mills, 1981; Law & Kolstad, 1983; Broome & Limsowtin, 1998). Different species contribute to flavour and texture and are needed to achieve the characteristics of a typical dairy product (Crow *et al.*, 1993; Limsowtin *et al.*, 1996). In cheese manufacture, the quality of the finished product depends on the starter cultures used including their functional properties, the levels of starters used and the distribution of their enzyme activities for ripening (Olson, 1990; Visser, 1993; Fox *et al.*, 1996). In starter cultures which consist of a complex mixture of strains, the composition of the bacterial population can change, depending on the incubation temperature, growth medium and frequency of subculturing (Hugenholtz, 1986). Consequently, the presence of different species can vary and thereby the activities relevant for cheese manufacture (Collins, 1961; Gilliland, 1971; Exterkate, 1976; Limsowtin *et al.*, 1978). In these starters many different interactions might occur between strains such as competition for nutrients, production of antimicrobials and the presence of bacteriophages from lysogenic strains (Meers, 1973;

Hugenholtz & Veldkamp, 1985; Ayad *et al.*, 2000). These interactions or changes can have drastic effects on the main functions of starters during the cheese making. In order to obtain stable starter cultures, whose use results in fermented products with a consistently high quality, factors affecting the population dynamics in such a starter culture must be known. Based on this understanding a careful selection of starter cultures should be emphasised to avoid problems during cheese making. Cheese starter cultures especially for Gouda cheese often consist of various strains, responsible for rapid acidification, proper flavour development, proteolytic activity and eye-formation. *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* strains in these starters are able to ferment citrate into, among others, the functional products diacetyl (butter flavour) and carbon dioxide (eye formation in cheese) (Starrenburg & Hugenholtz, 1991; Limsowtin *et al.*, 1996). These citrate-utilizing strains are thus very important for the flavour and appearance of the cheese.

In a previous study, defined strain starter cultures (DSS) with specific flavour characteristics and properties required for Gouda-type cheese were studied (Chapter 5; Ayad *et al.*, 2001). Several strains in those DSS were shown to be promising for designing tailor-made starter cultures for cheese manufacture. On the other hand, it was observed that some *diacetylactis* strains were found to be specifically inhibited in these DSS, a phenomenon which might also occur in more complex starter cultures. The present work is a first attempt to elucidate the specific inhibition of these strains by other *L. lactis* strains in order to understand the mechanism behind it. Knowledge in this field will facilitate the selection of strains for tailor-made starter cultures.

MATERIALS AND METHODS

Origin of strains

The strains used in this study, including industrial strains and wild lactococci strains originated from artisanal production of dairy products, all belong to the species *Lactococcus lactis* subsp. *lactis*, subsp. *cremoris* and subsp. *lactis* biovar *diacetylactis* (Ayad *et al.*, 2001). All strains were obtained from the culture collection of NIZO food research.

Growth experiments

Individual strains were pre-grown for 16 h at 30°C in sterilised milk with 0.5% yeast extract for protease-negative (prt^-) and without yeast extract for protease positive (prt^+) strains. The behaviour of two wild lactococcal strains (B1155 and B926) was individually

tested in a culture paired together with the *diacetylactis* strains B86, B87, B88, and B630. Each pair was mixed in ratios, *lactis* : *diacetylactis* being 1:1 and 1:2 in the final inoculum level of 1% in 100 mL skimmed UHT milk and subsequently incubated for 24 h at 30°C. The same study was carried out in M17 medium (Oxoid, Hampshire, UK) containing 5 g L^{-1} lactose (LM17) at different incubation temperatures (16, 20, 30, 35°C) under aerobic or anaerobic conditions. The population dynamics of strains were followed by measuring colony-forming units (CFU mL^{-1}) as described previously (Ayad *et al.*, 2001).

The pH value was measured after 6 h of the incubation of the bacteria in milk cultures at 30°C.

Interaction studies between strains

To evaluate the antagonistic interaction between wild strains and *diacetylactis* strains, the antimicrobial activity in culture filtrates of the former was determined in an agar well-diffusion assay against *diacetylactis* strains B86 and B87. Plates were prepared as described previously (Chapter 3; Ayad *et al.*, 2000) and subsequently, 50 μL of the neutralised and filter-sterilised (0.45 μM) supernatants obtained from overnight cultures of either B1155 or B926, grown in LM17 at 30°C, were dispensed in wells. In addition, the supernatants prepared from the wild strains tested were concentrated 10 times by ultrafiltration (UF, 500 Da). Ultrafiltration was performed at 4°C in a stirred-cell type ultrafiltration module (Amicon Corporation, MA, USA), operating under a nitrogen pressure of 300 kPa using Pall Filtron DISC membrane OMEGA type (100 Da molecular mass cut-off). The concentrated filter-sterilised supernatant was dispensed in wells. Then, the plates were incubated for 2 h at 4°C, and subsequently overnight at 30 °C as described before.

A plaques-assay for counting bacteriophages of *Lactococcus* strains B1155 and B926 against two *diacetylactis* strains (B86 and B87) was performed. Filter-sterilized supernatant (phage filtrate) was prepared from the following cultures grown in LM17 media at 30°C: (a) overnight cultures of strains B1155 and B926, (b) mixed cultures of B1155 and B926 each one paired with B86 and B87, and (c) 10 times concentrated supernatant (by ultrafiltration). Each supernatant was tested against the host cells prepared from an overnight culture of *diacetylactis* B86 or/and B87 in LM17 at 30°C. 0.1 mL of the host cultures was mixed with 50 μL of 1 M calcium-borogluconate and 0.1 mL of each phage filtrate and incubated for 10 min at room temperature. Subsequently, 3 mL of LM17 semi-hard agar medium was added to that mixture; this mixture was poured onto fresh plates prepared from LM17 hard-agar medium containing 1% calcium-borogluconate (1 M). The plates were incubated for 16-20 h at 30°C.

The cells of B1155 and B926 strains taken from overnight cultures, grown at 30°C in LM17 broth medium, were either inactivated by UV light, high temperature (100°C for 15 min), 1% chloroform or by chloramphenicol (2 μg mL^{-1}). The cell free extract (CFE) was prepared from these overnight cultures. The cells were harvested by centrifugation (30 min, 14000 rpm, 4°C) and washed twice with 50 mM potassium phosphate buffer (pH 7.5). The washed cells were resuspended in 5 mL of the same buffer and the cells were disrupted using a French pressure cell (Spectronic Instruments, Inc., Rochester, New York, USA) for two times and kept in ice. The suspension was centrifuged (5 min, 14000 rpm, 4°C) to remove intact bacteria and cell debris, and the supernatant (CFE) was collected and filtered through a 0.45 μM-pore-size filter (Millipore corp., Bedford, Mass.). Fifty μL of either overnight culture (cells), inactivated cells or CFE were dispensed in individual wells in agar plates, against two *diacetylactis* strains, the plates were incubated overnight at 30°C.

The growth of *diacetylactis* strains was followed by measuring CFU mL^{-1} in neutralised and filter-sterilised supernatants from cultures of the wild *Lactococcus* strains B1155 and B926. The supernatant was prepared by centrifugation (30 min, 14000 rpm, 4°) of the cultures obtained either from exponential growth phase, growth of 1% lactococcal strain in LM17 medium at 30°C for 4-5 h (OD_{600} 0.55, pH 6.8), or from overnight cultures (stationary phase).

Dialysis

Dialysis experiments were performed in a 100 mL cylinder divided in two equal volumes of 50 mL, each contaning LM17 medium. The inside of the dialysis tube (cut-off value 10,000-15,000 D) was inoculated with 1% lactococcal strain (B1155 or B926) and the outside part with the *diacetylactis* strain B86 or B87. The growth of each strain was followed by measuring CFU mL^{-1}.

Effect of temperature

The effect of temperature on the inhibitory factor present in the supernatant prepared from the exponential phase culture of wild strains was investigated. The supernatant was heat-treated at 60 and 100°C for 15, 20 and 30 min, then the growth of *diacetylactis* strains in the supernatant was followed by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Manual spectronic instruments 100-240, Vitatron, Holland).

Stability testing

The stability of the inhibitory factor was checked under different conditions. The supernatant was stored overnight at different temperatures: -20, 4 and 20°C. In addition, it

was stored at 4°C under anaerobic conditions. The presence of the inhibitory factor was tested by following the growth of B86 in the stored supernatant and in the fresh supernatant as the control.

Protease treatments

The effect of protease on the activity of the inhibitory factor was investigated by treating a sample with proteinase K and trypsin, 10 mg mL^{-1}, for 2 h at 30°C. To inactivate the enzymes, the samples were heated for 10 min at 100°C.

Ultrafiltration

Ultrafiltration was performed at 4°C in a stirred-cell type ultrafiltration module (Amicon Corporation, MA, USA), using different filter membranes sizes (cut-off values 1-10, 20, 30, and 50 kDa). The retentate was diluted with LM17 to its origin volume and subsequently, the filtrate and retentate were filter sterilised before used in the assay.

Silica cartridge separation

Silica cartridges (Isolute solid Phase Extraction column C18, International sorbent technology, Ltd., Mid-Glamorgan, UK), were used to test binding and elution conditions. Ten-mL samples were loaded on a silica cartridge column and eluted using different concentrations of ethanol (10, 30, 50 and 90%). Eluted material was collected and ethanol was evaporated under N_2 and subsequently the concentrated material was diluted to its original volume. The solution obtained was filter sterilised before used in the assay.

RESULTS AND DISCUSSION

Growth behaviour of *diacetylactis* strains in presence of wild lactococci

Previously, it was observed that some *diacetylactis* strains (B86 and B87) were found to be inhibited in defined strain starter cultures (Ayad *et al.*, 2001). This effect was very significant and the use of such DSS resulted in cheeses without eye formation and with poor diacetyl production. That raised the question about the underlying mechanism for this inhibition. The DSS used consisted of the strains B851+B1155+B86+SK110 (DSS1) and B1173+B926+B87+SK110 (DSS2), respectively. To obtain insight in the interactions

between strains in these DSS, the effect of the individual strains present in each DSS on the growth of the *diacetylactis* strains B86 and B87 was studied. The population dynamics in the cultures were followed by measuring the growth of individual strains during 24 h (Fig. 1 & 2). When strain B86 was tested against each strain of DSS1 individually (Fig.1), it appeared that this strain could grow well with strains B851 and SK110, but not in the presence of strain B1155.

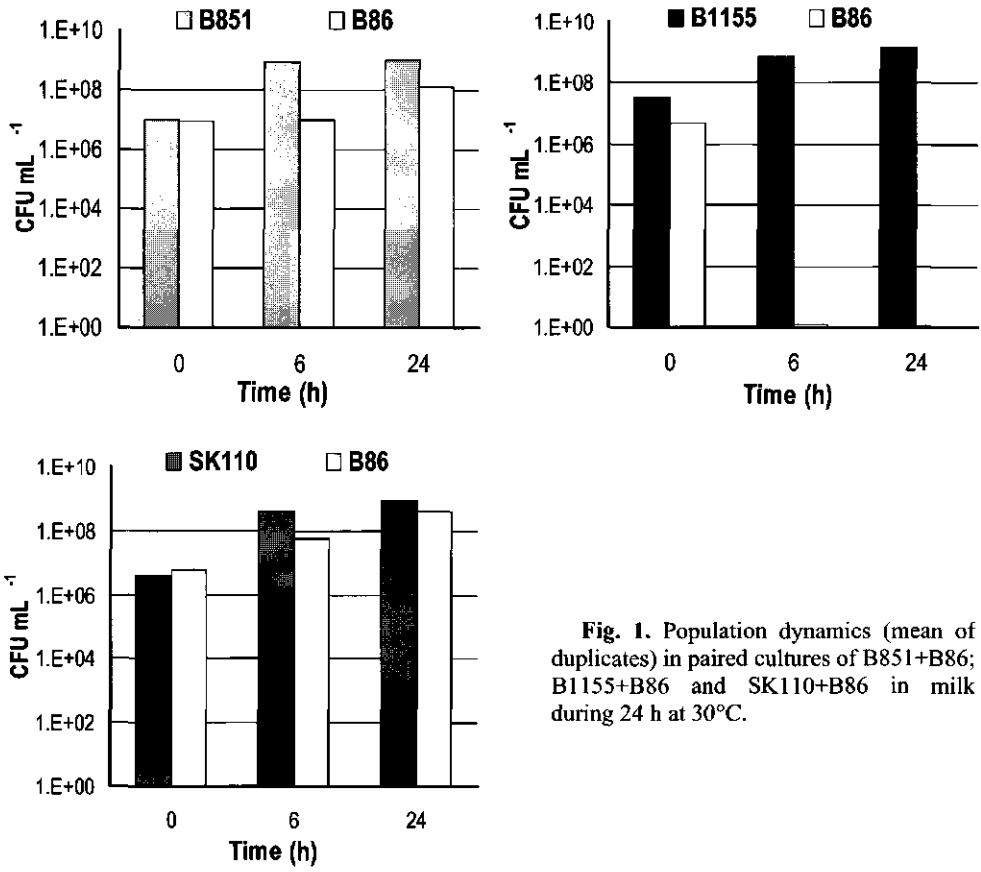

Fig. 1. Population dynamics (mean of duplicates) in paired cultures of B851+B86; B1155+B86 and SK110+B86 in milk during 24 h at 30°C.

The same results were found when the other *diacetylactis* strain (B87) was tested with the individual strains SK110, B851 and B1155 indicating that strain B87 also did not grow in the presence of strain B1155 (data not shown). Strain B87 was also tested with the individual strains of DSS2 (Fig. 2) and could grow well with strain B1173 and SK110, but not with strain B926. Similar results were found with strain B86 (data not shown). The results of these population dynamic studies in milk clearly revealed that both *diacetylactis* strains B86 and

B87 are not able to grow in the presence of the wild strains B1155 or B926. Many different interaction mechanisms can occur in mixed cultures as mentioned earlier by Meers (1973). In order to elucidate the mechanism behind these cases of growth inhibition, a number of growth studies were performed.

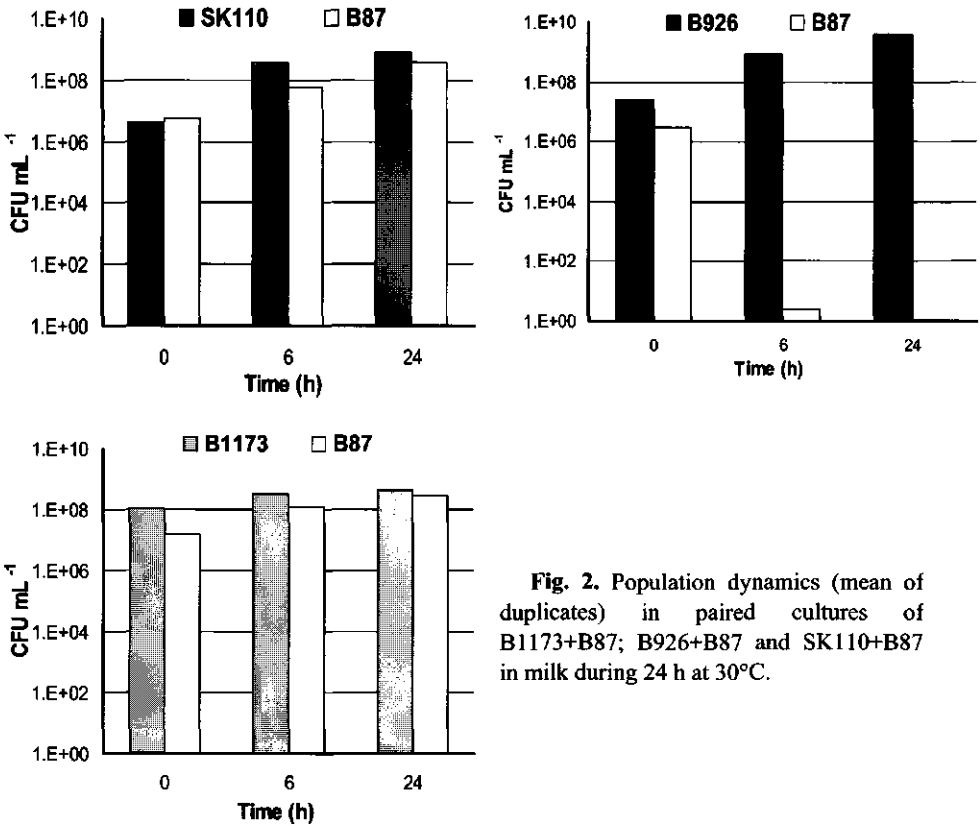

Fig. 2. Population dynamics (mean of duplicates) in paired cultures of B1173+B87; B926+B87 and SK110+B87 in milk during 24 h at 30°C.

The interaction of the wild strains with the individual *diacetylactis* strains B86, B87, B88 and B630 was followed in paired cultures in LM17 medium. The pairs were inoculated in two ratios (1:1 and 1:2, *lactis* and *diacetylactis*, respectively). Strains B88 and B630 appeared to grow well in the presence of strain B926 (Fig. 3) and strain B1155 (not shown). On the contrary, the strains B86 and B87 were also in LM17 medium not able to grow in the presence of the wild strains, under different conditions of growth temperatures (16, 20, 30 and 35°C) as well as under aerobic or anaerobic conditions (Fig. 3 and other data not shown). These results indicated that the interaction between the strains appeared to be specific, not all *diacetylactis* strains were found to be sensitive for this inhibition by strains B926 and B1155.

Moreover, the interaction was not specific for the milk medium, but also occurred in another rich medium like LM17.

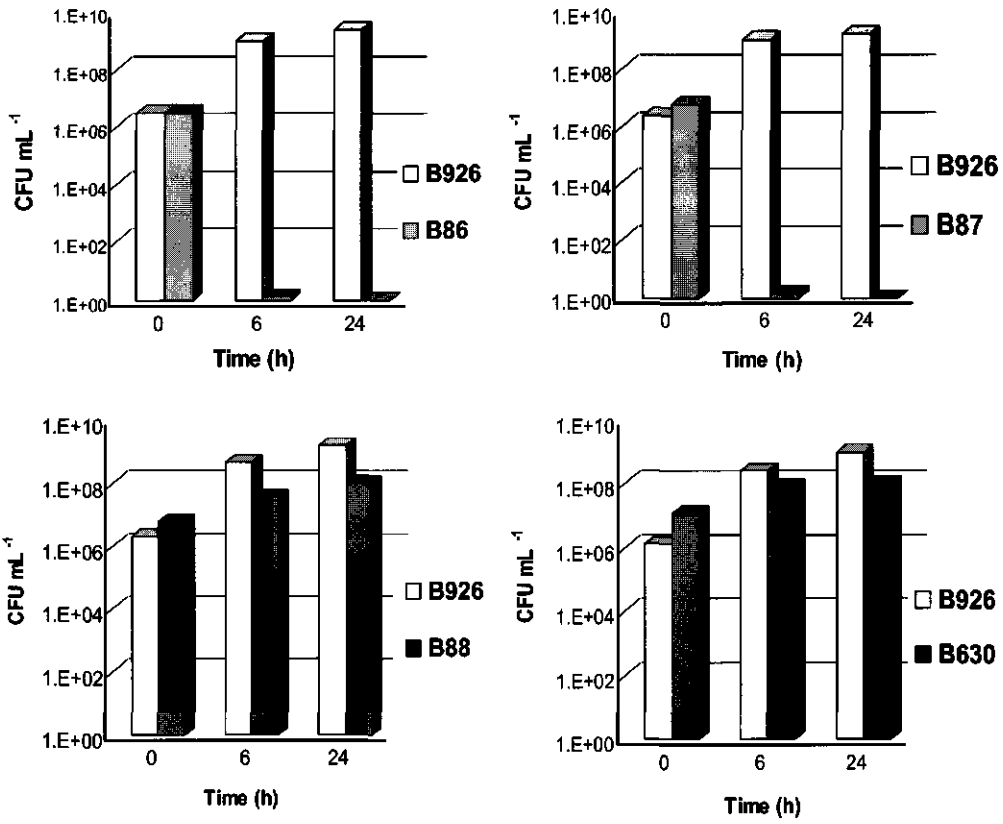

Fig. 3. Enumeration of viable counts during cultivation of strain B926 (open bars) together with the *diacetylactis* strains B86, B87, B88 and B630 (filled bars) in LM17 medium for 24 h at 30°C (mean of duplicates).

The pH values after 6 h of growth of the DSS1 and DSS2 cultures were almost the same, ranging from 5.5 to 5.7 (Ayad *et al.*, 2001). This is in the range of the pH required for the growth of *diacetylactis* strains; their citrate utilization has been shown to be pH dependent, with a maximum rate observed also between pH 5.5 and 6.0 (Starrenburg & Hugenholtz, 1991). The *diacetylactis* strains B86 and B87 grew well in cultures paired with the proteolytic strain B851 (Fig. 1 and other data not shown), but not well with the proteolytic strain B926 (data not shown and Fig. 2). In all these cases, the pH value after 6 h was 5.5. In cultures with the non-proteolytic strain B1155, which also caused growth inhibition of the *diacetylactis*

strains, the pH value was 6.0 after 6 h. These results indicated that there is no relationship between the pH value of the cultures and the observed inhibition of growth.

Possible mechanisms of inhibition

Lactic acid bacteria (LAB) can inhibit or eliminate the growth of microorganisms, including bacteria, by the production of a variety of antimicrobial compounds, such as organic acid, diacetyl, hydrogen peroxid, enzymes, phages, lytic agents and bacteriocin compounds (Lindgren & Dobrogosz, 1990; Blom & Morvedt, 1991; Ray & Daeschel, 1992). Several inhibiting factors produced by these bacteria have been characterised and identified (Piard & Desmazeaud, 1992; Desmazeaud, 1996). In order to understand the interaction mechanism described above, different possibilities were examined.

Although, we reported previously that strains B1155 and B926 did not produce bacteriocin-like compounds against two target microorganisms, *Lactococcus lactis* subsp. *cremoris* SK110 and *Micrococcus flavus* (Ayad *et al.*, 2001), these strains may produce compounds with a very narrow host range and specific for the *diacetylactis* strains B86 and B87. Such narrow spectrum bacteriocins should then be active against strains closely related to the producer strain (Ray & Daeschel, 1992; Klaenhammer, 1993; Stiles, 1996). In order to test this, the antimicrobial activity in the supernatant of an overnight culture of the strains B1155 and B926 was tested against the two *diacetylactis* strains B86 and B87 using an agar well-diffusion assay. No inhibition zones were observed, indicating that both strains B1155 and B926 did not produce a bacteriocin-like antimicrobial activity against B86 and B87 under these conditions. After concentration of the neutralised and filter-sterilised supernatants, the results were not different and again no inhibition was found. Thus, these results do not support the production of specific narrow spectrum bacteriocins, since such compounds are commonly identified in this manner. Also the fact that *Micrococcus flavus* is not inhibited corroborated this. However, the mode of action of the inhibition between the wild strains and the *diacetylactis* strains seemed to be as rapid as the mode of action of bacteriocin-like compounds, since the viable population of the sensitive strains decreased during the first few hours, when grown together. Thus it could be still possible that the inhibition was due to an unknown bacteriocin-like compound that is not stable and yet undefined.

Initially, it was observed that the inhibition could only be demonstrated in a co-culture of an inhibitory and a sensitive strain. Therefore, the question was raised whether living cells (of the inhibitory strain) are a prerequisite for the inhibition activity.

The cells (overnight cultures) of B1155 and B926 and their CFE were tested against *diacetylactis* strains in an agar well-diffusion assay. Inhibition zones were found only with the living cells. When these cells of the inhibitory strains were inactivated by different treatments, i.e., UV radiation, high temperature, 1% chloroform or chloroamphenicol (2 μg mL^{-1}), and

tested against *diacetylactis* strains, no inhibition zones were found. These results clearly support that living cells are needed for a strong and specific inhibition.

It can be envisaged that the inhibition could be caused by bacteriophages produced or carried by wild strains, since these may attack closely related strains (Neve, 1996). In order to test this, the phage titres in the cultures of strains B1155 and B926 against *diacetylactis* strains B86 and B87 were determined in a plaque assay. However, no plaques were found, even when concentrated supernatants (phage filtrates) prepared from single cultures of each strain or from paired cultures were tested. Thus the presence of phages as an explanation seems to be unlikely.

It can also be speculated that the inhibitory strains synthesise a compound, which is partly secreted into the culture medium and exerts its action only when the producing strain is actively growing. To evaluate this, the growth of *diacetylactis* strain B86 was followed in LM17 medium containing either 10% CFE or 10% concentrated supernatant from an actively growing culture of B1155. The results of this experiment showed that the CFE prepared from these B1155 cells had no effect on strain B86, but in the presence of a concentrated supernatant an effect was observed (Fig. 4). This led to the conclusion that actively growing cells of B1155 were found to synthesise an inhibitory factor which is secreted into the medium.

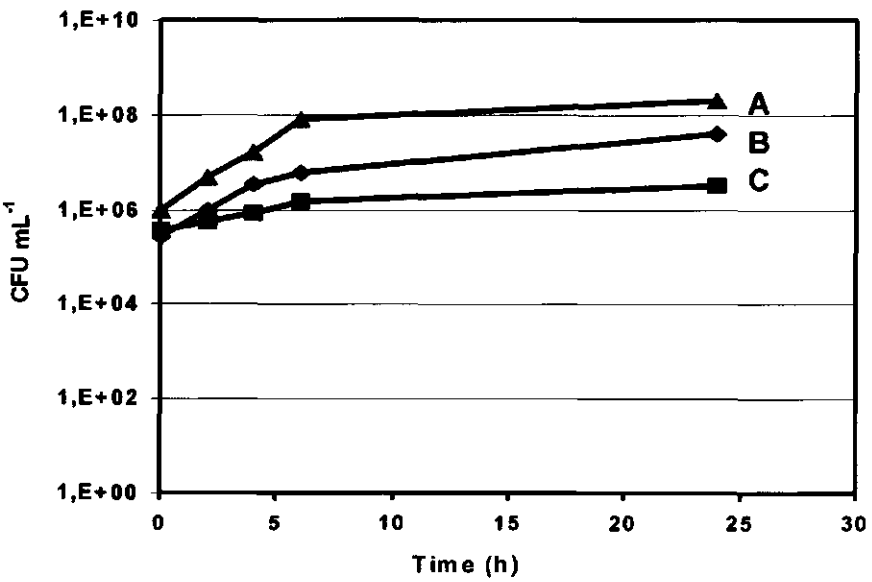

Fig. 4. Growth of the *diacetylactis* strain B86 (mean of duplicates) in, (A): LM17 medium (control), (B): LM17 medium containing 10% cell free extract prepared from strain B1155 and (C): LM17 medium containing 10% concentrated supernatant prepared from B1155 strain.

The fact that this factor could not be found in the supernatant of an overnight culture (see above), could be explained by assuming that this factor is rather unstable.

To confirm this further, the growth of B86 was followed in supernatants prepared from exponential and from stationary phase cultures in LM17 medium of B1155 (Fig. 5). The results showed that B86 experienced no inhibition when cultivated in the growth supernatant of B1155 cells harvested in the stationary phase of growth and similar results were found for strain B926. This means that on the one hand the medium was still rich enough for the growth of the *diacetylactis* strain and that competition for nutrients was not the explanation for the observed inhibitory effects. On the other hand, the B1155 supernatant obtained from stationary phase culture was not able to inhibit the growth of *diacetylactis* B86. However, a significant inhibition of the growth of B86 was observed during growth in a supernatant obtained from an exponential phase culture of B1155. This indicates that the inhibitory factor is produced during the exponential phase of growth.

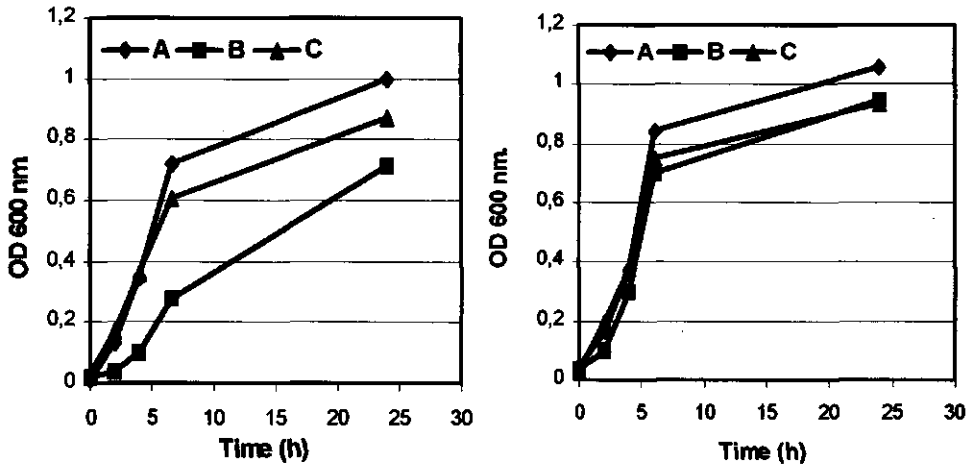

Fig. 5. Growth of *diacetylactis* strains B86 (left) and B88 (right) in (A): LM17 medium (control); (B): supernatant prepared from exponential phase culture of strain B1155; (C): supernatant prepared from stationary phase culture of strain B1155 for 24 h at 30°C (mean of duplicates).

The inhibitory factor is presumably unstable, which might explain why no inhibition was found with supernatants of overnight cultures tested in agar well assays (see above). For comparison reasons, the same experiments were carried out with the other *diacetylactis* strain B88, which is able to grow in the presence of strain B1155. No inhibition activity against this strain was found in all culture supernatants tested (Fig. 5).

For further evaluation of the characteristics of the inhibitory factor, an experiment was set up in which cultures were co-cultivated with a dialysis membrane as sole separation between the cultures. The wild strain was inoculated at the inner side of the dialysis tube, whereas the *diacetylactis* strain was present at the outside of the dialysis tube. The growth of the strains in this system is shown in Fig. 6. The results showed that the *diacetylactis* strain B86 is inhibited in its growth under these conditions, but the cells were not killed, and confirmed that living cells are needed for the inhibitory effect (Fig. 6). This result indicated that the inhibitory factor should be a relatively small compound, since the cut-off value of the dialysis tube used was 10,000-15,000 Da. On the other hand, the inhibition was not as severe as in co-cultures without culture separation.

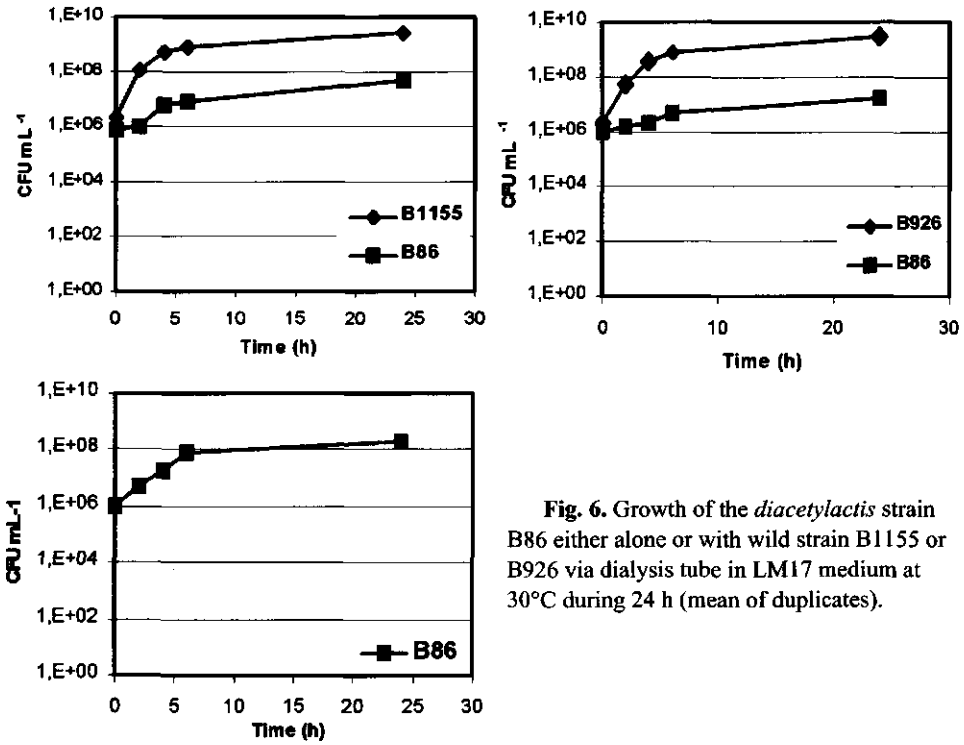

Fig. 6. Growth of the *diacetylactis* strain B86 either alone or with wild strain B1155 or B926 via dialysis tube in LM17 medium at 30°C during 24 h (mean of duplicates).

Preliminary characterisation of inhibitory factor

The characteristics of the inhibitory factor present in the supernatant prepared from the exponential phase culture of wild strains were studied in further detail. For this, the effect of the inhibitory factor on the growth of B86 (positive control) was compared with that on B88

strain (negative control). The inhibitory factor was found to be heat stable up to 100°C for 30 min. Remarkably, the inhibitory factor was shown to be unstable upon overnight storage, irrespective of the temperature (- 20, 4 and 20°C). The presence or absence of oxygen did not influence this storage effect. This observation implied that all testing should be performed as quickly as possible after isolation of the inhibitory factor from a given culture. Therefore, further studies were carried out using fresh supernatants.

The results of these studies showed that the inhibitory factor was inactivated/inhibited by proteinase K, since growth of the *diacetylactis* strain B86 was not inhibited after treatment of the supernatant with this enzyme (data not shown). Although the inactivation by trypsin was found to be less pronounced, these results indicated that the inhibitory factor could be proteinaceous.

The estimated molecular mass of the inhibitory factor present in the supernatant of wild strain cultures was found to be less than 1 kDa (data not shown), as determined by ultra filtration using an 1kDa molecular weight cut-off filter. This result was in line with the results from the experiment with the dialysis culture proving that the inhibitory factor is a small size molecule.

Moreover, the inhibitory factor was able to bind to a silica cartridge C18 and could be eluted from this material by ethanol, indicating that the nature of the inhibitory factor appears to be hydrophobic. Since it can be eluted without loss of its activity, this means that further purification would be possible.

Further work should concentrate on the isolation of this compound by HPLC and on its characterisation. A better understanding of this factor and its mode of action will be very valuable in the selection of stable starter cultures.

CONCLUSIONS

Wild lactococci strains B1155 and B926 inhibited the growth of *diacetylactis* strains B86 and B87 in DSS indicating that a specific interaction exists between these strains. The studies on the possible mechanism of this interaction indicated that the inhibitory behaviour is not due to competition between the strains for nutrients. In addition, the inhibitory action was not affected by aeration, pH value during the growth of strains, or release of bacteriophages by the wild lactococcal strains. However, it was found that the presence of living cells of the inhibitory strains is a prerequisite for a strong and lasting inhibition of the growth of *diacetylactis* strains. This phenomenon was believed to be due to the accumulation of an inhibitory factor in the supernatant of cultures of wild lactococcal strains, only in the exponential phase of growth. The preliminary apparent characteristics of the inhibitory factor were: (*a*) a small compound less than 1 kDa, (*b*) heat stable up to 100°C for 30 min, (*c*)

unstable when stored for 24 h at different temperatures (from - 20° to 20°C), (*d*) sensitive for proteinase K (proteinaceous compound), and (*e*) binding to silica cartridge C18 material. The characteristics did not match common characteristics of already known antimicrobial compounds, for instance, there was no activity against *M. flavus*.

Further research work needs to focus on the exact mechanisms of this interaction and the identification and characterisation of the inhibitory factor.

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Chapter 7

Enhanced flavour formation by combination of selected lactococci from industrial and artisanal origin with focus on completion of a metabolic pathway

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ABSTRACT

Combinations of lactococcal strains from various origins with diverse properties were developed as new starters for new dairy products. Flavour formation by such tailor-made cultures was studied. In some cases, a strongly enhanced flavour was observed. For instance, the combination of B1157 and SK110 strains in milk resulted in a very strong chocolate-like flavour. B1157 produces only a moderate chocolate-like flavour, whereas SK110 alone fails to produce this flavour. Headspace gas chromatography results corroborate the organoleptic evaluations. High levels of branched-chain aldehydes were found when B1157 and SK110 were grown together. The enzyme activities involved in this pathway were studied, both strains contain transaminase activity. Although B1157 had a very high amino acid decarboxylating activity, its release of amino acids from milk protein was limited. SK110 was strongly limited in decarboxylating activity, although this strain is very active in proteolysis. By combining these strains, the substrates released by SK110 can directly be used by the other strain, resulting in completion of the whole flavour-formation pathway. This opens new avenues for the preparation of tailor-made cultures.

INTRODUCTION

Flavour development in dairy products is essentially an enzymatic process mainly performed by the starter microorganisms. During cheese ripening, proteolytic enzymes of the starter culture play a significant role in protein breakdown (Law *et al.*, 1974; Bie & Sjöström, 1975). This breakdown of proteins is important for the formation of a desirable flavour and texture, and therefore, proteolysis has been investigated extensively (Pritchard & Coolbear, 1993; Visser 1993; Exterkate & Alting, 1995; Law & Mulholland, 1995). It has been demonstrated that proteinases and peptidases of starter bacteria release peptides and free amino acids from casein (Olson, 1990; Visser, 1993; Engels & Visser, 1994).

The relationship between release of amino acids and flavour formation in cheese has been assumed for a long time (Mulder, 1952; Solms, 1969). Amino acids may contribute to flavour either directly or indirectly by serving as precursors of volatile aroma compounds such as aldehydes, acids, alcohols, esters and sulphur compounds (Engels & Visser, 1996). In recent years, it has become clear that the conversion of amino acids into volatile (flavour) compounds plays an important role in flavour formation during the ripening process. A number of enzymes involved in amino acid conversion have been identified in starter cultures (Schmidt & Lenoir, 1974; Nakazawa *et al.*, 1977; Lee *et al.*, 1985; Alting *et al.*, 1995; Yvon *et al.*, 1997; Yvon *et al.*, 1998). Generally, these enzymes are involved in various reactions,

including deamination, transamination, decarboxylation and cleavage of the amino acid side chains.

Lactic acid bacteria (LAB), which are present in all types of cheeses, play a major role in generating flavour compounds from amino acids. In lactococci, transamination is a first step in the conversion of aromatic and branched-chain amino acid (Thirouin *et al.*, 1995; Engels, 1997; Gao *et al.*, 1997; Yvon *et al.*, 1997; Engels *et al.*, 2000). Recently, a number of transaminases have been identified and characterized in LAB (Engels, 1997; Yvon *et al.*, 1997; Gao & Steele, 1998; Roudot-Algaron & Yvon, 1998). The keto acids produced by transamination of the amino acids can either undergo spontaneous degradation (Gao *et al.*, 1997), or are degraded enzymatically into the corresponding aldehydes or carboxylic acids (Thirouin *et al.*, 1995; Smit *et al.*, 2000). The transamination reaction is catalysed by aminotransferases, which transfer the α-amino group of amino acids to a keto acid acceptor.

In the manufacture of cheeses such as of Gouda and Cheddar, mixed or defined cultures of LAB are used as starter cultures. In these mixtures, many different interactions between the strains may occur, which not only affect the composition of such mixtures, but may also have an impact on flavour formation. Until now, very little was known about this process. Depending on the enzymes present in the cultures, different flavours can develop due to the contribution of many enzymes, which lead to various flavour compounds. It is important to study the role of starter cultures in flavour formation, the enzymes involved in the conversion of amino acids and the regulation of enzymatic conversions, in order to control flavour formation during cheese ripening. In the present work, flavour formation by the complementary action of defined starter cultures was studied in detail, and the results show that interactions exist which can be applied to develop tailor-made cultures.

MATERIALS AND METHODS

Chemicals

Amino acids (leucine, isoleucine and valine), α-keto acids (α-ketoisocaproic acid (KICA), α-keto-β-methyl-n-valeric acid and α-ketoisovaleric acid) and thiamine pyrophosphate chloride (TPP) were obtained from Sigma Chemicals, α-ketoglutaric acid was purchased from Janssen Chimica, ethylenediaminetetra-acetic acid (EDTA) from BDH Limited, and pyridoxal-5'-phosphate (PLP) from Boehringer Mannheim GmbH. All other chemicals used were of analytical grade.

Micro-organisms and growth conditions

The strains used in this study were: (i) strain *Lactococcus lactis* subsp. *cremoris* SK110 (NIZO B697), which is derived from a commercial starter culture, (ii) the strains *L. lactis* subsp. *cremoris* NIZO B1157, *L. lactis* subsp. *lactis* NIZO B851, *L. lactis* subsp. *lactis* NIZO B850 and *L. lactis* subsp. *lactis* NIZO B1173, which originate from natural niches (Chapter 2; Ayad *et al.*, 1999). Strains were routinely stored in litmus milk with $CaCO_3$ and 0.5% yeast extract and kept at - 40°C. Strains B1157 and B1173 are non-proteolytic strains which were grown in milk with 0.5% yeast extract, whereas SK110, B850 and B851 are proteolytic strains which were cultured in milk without yeast extract.

Flavour production and population dynamics

Individual strains, SK110, B1157, B851 were pre-cultured for 16 h at 30°C in sterilised milk with 0.5% yeast extract for non-proteolytic strains, and without yeast extract for proteolytic strains. Cultures consisting of a strain isolated from natural niches were combined with cultures of the industrial strain (SK110) in different ratios (2:1 and 1:2) at a final total inoculum level of 1% (v/v), and grown together in 500 mL skimmed UHT milk for 48 h at 30°C. The strains were also inoculated individually at 1% and grown under the same conditions.

The total number of cells (colony-forming units, cfu) in each milk culture was determined by plating cells on GMA agar containing 10% skimmed milk, 1.9% β-glycerophosphate (pH 6.9), 0.001% bromocresol purple and 1.3 % agar as described previously (Limsowtin & Terzaghi, 1976; Hugenholtz *et al.*, 1987). Based on the differences in the ability to hydrolyse casein and the ability to grow at 40°C between wild-type strains and the industrial strain (Chapter 3; Ayad *et al.*, 2000), the cell number of the individual strains could be monitored in a mixed population.

The milk cultures were sensorically evaluated by five to eight experienced cheese graders. The attributes were recorded and statistically analysed. The flavour intensity scale ranged from 0 [none] to 4 [very strong].

Analysis of volatile compounds

Branched aldehydes formed by the cultures used were identifed and quantified using headspace gas chromatography (HS-GC). The analytical system used consisted of a headspace autosampler HS800 mounted on a Mega series gas chromatograph (CE instruments, Thermo Quest, Milan, Italy) fitted with a splitless injector, a flame ionisation detector and a fused silica capillary column (25 m x 0.22 mm i.d., $d_f = 1$ μM CP-Sil5 CB-LB,

Chrompack, The Netherlands). After an equilibration time of 20 min at 60°C, headspace samples (1.0 mL) were injected directly (splitless) onto a capillary pre-column (25 cm x 0.53 mm). The column was mounted in a cryotrap model 515 (Thermo Quest, Milan, Italy) inside the oven. During injection the volatile compounds are condensed (-150°C) and adsorbed in this capillary pre-column and afterwards re-injected onto the chromatographic column by flash heating (150°C). Gas chromatographical separation was performed under isothermal conditions (70°C) at a carrier gas flow rate of 1.2 mL min^{-1} hydrogen. Identification of aldehydes was achieved using retention times of standard compounds.

Enzymatic conversion of branched-chain amino acids by strains of *L.lactis*

Cultures were pre-grown in sterilised milk (containing 0.5% yeast extract for non-proteolytic strains) overnight at 30°C, and subsequently, individual and mixed cultures (B1175+SK110 2:1) were grown in 50 mL UHT milk after inoculation at a final inoculum level of 1% (v/v). The following additions were made: (i) no additions; (ii) 10 mM leucine; (iii) 10 mM isoleucine; (iv) 10 mM valine; (v) 10 mM α-ketoisocaproic acid; (vi) 10 mM α-keto-β-methyl-n-valeric acid and (vii) 10 mM α-ketoisovaleric acid. The volatile components formed enzymatically by the strains were detected using direct static headspace injection in combination with gas chromatography and flame ionisation detection. Column and chromatographic conditions were the same as those described above.

Free amino acid analysis

Free amino acids were determined on a 4151 Alpha Plus amino acid analyser (Pharmacia LKB, Uppsala, Sweden). The soluble nitrogen fractions (Noomen, 1977) were prepared from skimmed UHT milk incubated with the individual strains SK110 and B1157 and their mixtures in different ratios at final inoculum level of 1% for 48 h at 30°C.

Preparation of cell-free extract (CFE)

The strains were cultured overnight at 30°C in sterilised milk with 0.5% yeast extract only for non-proteolytic strains. After addition of 1% (w/v) sodium tricitrate, the cells were harvested by centrifugation (5 min, 10000 *g*, 4°C) and washed twice in 50 mM potassium phosphate buffer (pH 7.5). The washed cells were resuspended to an OD_{600nm} of approximately 20 (Ultrospec 3000, Pharmacia Biotech., UK) in the same buffer, added to a plastic tube (Sarstedt 72694, Greiner, Alphen a/d Rijn, NL) with 1 g glass beads (Zirconium beads Ø = 0.1mm, Biospec, Bartlesville, USA) and kept on ice (0°C). The cells were

disrupted by using a Bead beater (multipurpose Orbital mixer, Merlin, Rotterdam, NL) for 3x3 min, and cooled on ice for 2 min after every 3 min of shaking. The treated suspension was centrifuged (30 min, 24000 *g*, 4°C) to remove intact bacteria and cell debris, the supernatant fluid (CFE) was collected and filtered through 0.45-μm-pore-size filter (Millipore Corp., Bedford, Mass.). The CFE was stored at -30°C until further use.

Determination of aminotransferase and decarboxylase activity

The aminotransferase activity in CFE of wild strains and the industrial strain SK110 was measured as follows. A 100 μL volume of CFE (either active or inactive by heat treatment) was incubated in 20 mM potassium phosphate buffer (pH 7.5) containing 1mM EDTA and 20 μM PLP, with leucine (final concentration 20 mM) and co-substrate α-ketoglutaric acid (final concentration 10 mM). The final volume of the incubation mixture was 200 μL. The incubations were performed at 30°C for 1 h in the dark. The reaction was stopped by lowering the pH of the mixture to 2.5 via addition of 0.2 M HCl. The formation of α-ketoisocaproic acid (KICA) during incubation was quantified by measuring its peak area using high-performance liquid chromatography (HPLC). The HPLC equipment consisted of an ISS-100 sample injector (Perkin Elmer, Uberlingen, Germany), two M6000A pumps, an AGC 680 gradient controller (Waters, Milford, MA, USA) and a Kratos 783 UV detector (Kratos Analytical, Ramsey, NJ, USA) operating at 220 nm. Samples were chromatographed at 30 °C on a Bio-Rad HiPore RP-318 reversed-phase column (4.6 x 250 mm) preceded by a Bio-Rad C_{18} cartridge guard column. The elution buffers were 5 % acetonitrile, 0.1 % trifluoroacetic acid (TFA) in water (solvent A) and 90 % acetonitrile, 0.08 % TFA in water (solvent B). The components in the reaction mixture were separated isocratically at 0 % solvent B for 5 min followed by a linear gradient from 0 to 70 % solvent B over 2 min and isocratic elution at 70 % solvent B for 5 min. The flow rate was 0.8 mL min^{-1}. KICA eluted at 22.5 min. The relative amounts of KICA were determined from their peak area. Perkin Elmer Nelson Turbochrom 4.0 software (Cupertino, CA.) was used for processing raw HPLC data.

The conversion of KICA to 3-methylbutanal (3MeA4) by CFE was monitored by determining 3MeA4 using headspace gas chromatography with flame-ionisation detection (see above). CFE (100 μL) either active or inactive by heat treatment, was incubated in 50 mM potassium phosphate buffer (pH 6.0) containing 1mM EDTA, 50 μM TPP and KICA (final concentration 5 mM) at 35 °C for 4 h. The reaction was stopped by adding 50 μL of 6 M (HCl) to reduce the pH to 2.

RESULTS AND DISCUSSION

Growth and flavour production by defined strain starter cultures

Strains B1157 and B851 were grown as described in the Material and Methods section in milk, either individually or in combination with the industrial strain SK110. The growth of strains B1157 and B851, when cultured together with SK110 in two combinations (1:2 and 2:1) was followed by measuring the cell counts of the individual strains. Strains were distinguished individually based on proteolytic activity and the differences between growth temperature characteristics of lactococcal isolates from artisanal, non-dairy origins and industrial strains (Ayad *et al.*, 2000). The growth of individual and mixed cultures are shown in Fig. 1.

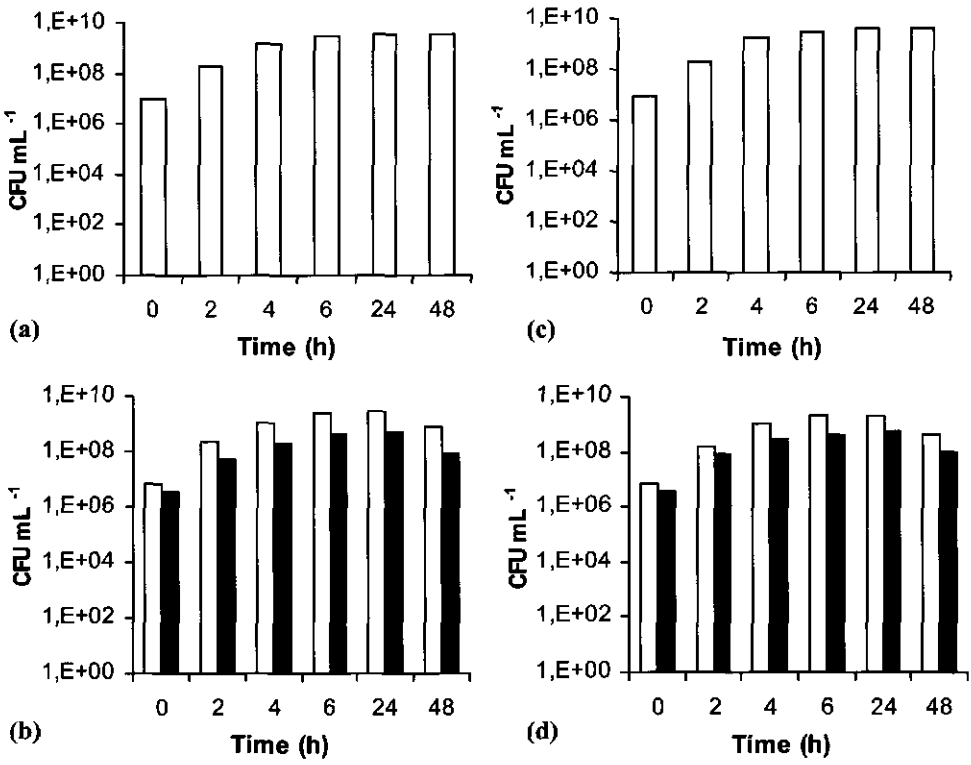

Fig. 1. Changes in starter populations in milk cultures prepared with strains B1157 and B851 (open bars) and strain SK110 (filled bars). (a) strain B1157, (b) strain B1157: strain SK110 (2:1), (c) strain B851 and (d) strain B851: strain SK110 (2:1). Data represent viable counts (mean of duplicates).

Each strain could grow well, both in a mixture as on its own. The initial balance between the strains remained stable during co-cultivation. After growth, the cultures were also evaluated organoleptically (Table 1). Strain B1157 produced a slight chocolate-like flavour in milk, when grown as a pure culture. Surprisingly, this flavour formation was significantly increased when co-cultured with industrial strain SK110. This finding suggests that each culture had a direct effect on the metabolism of the other. Such interactions are highly relevant for practical application. Mixing at a ratio of 2:1 resulted in a higher intensity of the chocolate-like flavour than at 1:2. Strain B851 produced a moderate chocolate-like flavour in milk when cultivated alone, whereas this flavour intensity was decreased when B851 was mixed with SK110 (Table 1). This reduction in chocolate-like flavour production is most likely due to the reduced number of B851 cells present in the mixed cultures compared with the situation in the individual cultures (Fig. 1).

Table 1. Chocolate-like flavour score of milk cultures incubated with wild strains B1157 and B851 and industrial strain SK110 (mean ± SD).

Strain	Chocolate-like flavour[a]
SK110	0 ± 0
B1157	1.3 ± 0.5
B851	1.9 ± 0.4
B1157+SK110 (2:1)[b]	2.9 ± 0.4
B1157+SK110 (1:2)	1.8 ± 0.3
B851+SK110 (2:1)	0.9 ± 0.6
B851+SK110 (1:2)	0.7 ± 0.5

[a] Scale from 0 (none) to 4 (very strong); results are means with standard deviations.
[b] Inoculation ratio.

In view of the chocolate-like flavour that was perceived during the organoleptic evaluation, and the knowledge that branched-chain aldehydes derived from branched-chain amino acids can be responsible for the development of a 'malty or chocolate' flavour in milk and cheese (Morgan, 1976; Dunn & Lindsay, 1985; McDonald, 1992; Urbach, 1993; Barbieri *et al.*, 1994), the milk culture samples were subjected to headspace gas chromatography (HS-GC).

The conversion of leucine, isoleucine and valine proceeds via transamination of the amino acid to the corresponding α-keto acids, and subsequently, via a chemical or enzymatic decarboxylation step to 3-methylbutanal (3MeA4), 2-methylbutanal (2MeA4) and 2-methylpropanal (2MeA3), respectively (Engels, 1997; Yvon *et al.*, 1998; Christensen *et al.*, 1999). The relative amounts of branched-chain aldehydes, formed during incubation of individual and defined strains in milk cultures are presented in Fig. 2. Relatively high levels of particularly 3MeA4 in particular, but also 2MeA3 and 2MeA4 were found in the milk cultures incubated with B1157 and SK110 in the ratio 2:1. In case of the 1:2 ratio, lower levels were found. A much lower amount of these aldehydes were detected in the milk culture incubated with B1157 alone, whereas these compounds were hardly present in the milk culture prepared with SK110 alone.

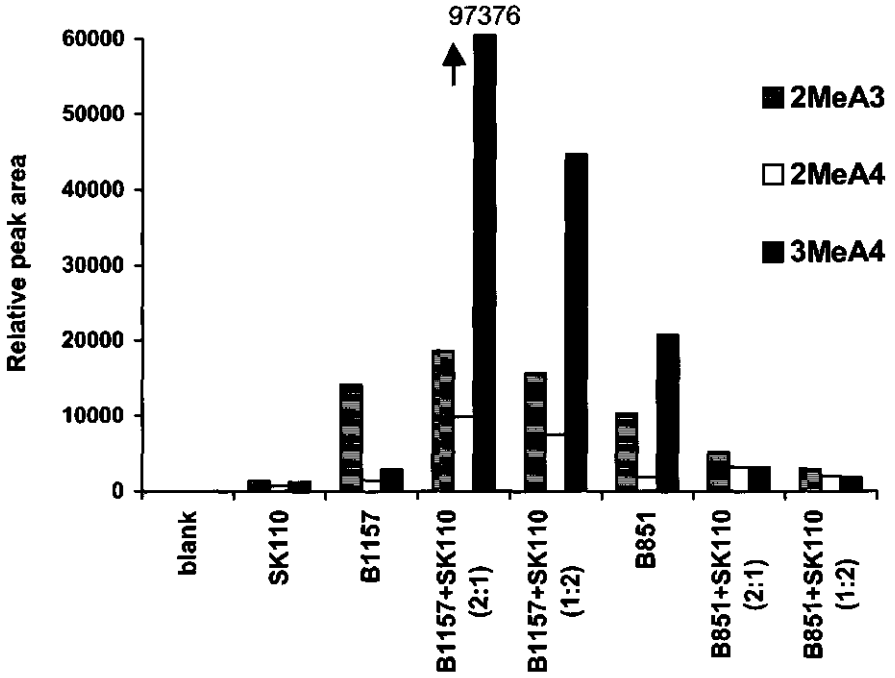

Fig. 2. Relative amounts of branched-chain aldehydes, 2-methylpropanal (2MeA3), 2-methylbutanal (2MeA4) and, 3-methylbutanal (3MeA4) formed during incubation of individual and combined strains in milk culture.

These results corroborate the data from cheese prepared with a defined strain starter culture (B1157 and SK110) that contained a relative high concentration of both branched-chain aldehydes and that their corresponding alcohols (Ayad *et al.*, 2000). The amounts of aldehydes found in milk cultures incubated with mixtures of B851 and SK110 were lower than those encountered in milk incubated with B851 alone. The differences noticed in the amount of aldehydes, correspond with the organoleptic data. The results indicate that in the combination of SK110 and B1157, a complete pathway for the formation of branched-chain aldehydes is, most likely, actively present. Since the individual strains do not produce these aldehydes in high amounts, it is likely that this flavour formation is limited in each strain individually.

Conversion of branched-chain amino acids by lactococcal enzymes

In order to gain further insight in the regulation of flavour formation in mixed cultures, the conversion routes of branched-chain amino acids into the corresponding aldehydes by SK110, B1157 and mixtures thereof were studied. The strains were incubated in milk, either alone or together in a 2:1 ratio (SK110: B1157), in the absence or presence of leucine (Leu), isoleucine (Ile), or valine (Val), or their corresponding α-keto acids (α-ketoisocaproic acid (KICA), α-keto-β-methyl-n-valeric acid or α-ketoisovaleric acid, respectively). The volatile compounds (aldehydes) which were formed by enzymatic conversion were quantified using HS-GC (Fig. 3). Strain B1157 grown in milk contained a higher level of 2MeA3 and 3MeA4 than a culture of SK110, whereas the level of 2MeA4 was apparently similar to those in the culture of SK110. However, a milk culture prepared with a mixture of these strains contained significantly higher levels of 2MeA3 and 3MeA4. These results indicate that strain B1157 is able to convert the branched-amino acids to aldehydes, and that this conversion is likely to be due to a transamination reaction followed by a decarboxylation step (Engels, 1997; Yvon *et al.*, 1997; Yvon *et al.*, 1998; Christensen *et al.*, 1999; Engels *et al.*, 2000).

Addition of leucine to the milk cultures prepared with B1157, and to mixtures of B1157 and SK110 resulted in an increase in the level of 3MeA4, whereas no effect was recorded for the culture of SK110 alone. Addition of isoleucine to a cultures containing B1157 resulted in an increase in the production of 2MeA4, and addition of valine led to an increase of 2MeA3. Addition of α-ketoisocaproic acid, α-keto-β-methyl-n-valeric acid and α-ketoisovaleric acid to pure and mixed cultures containing B1157 led to an increase in the corresponding aldehydes from each α-keto acid (Fig. 3). These results indicate that in the presence of the right substrates, B1157 is able to convert the branched-chain amino acids and corresponding α-keto acids into corresponding aldehydes very efficiently. This strongly indicates that the formation of amino acids is the rate limiting step in aldehyde flavour production by this strain.

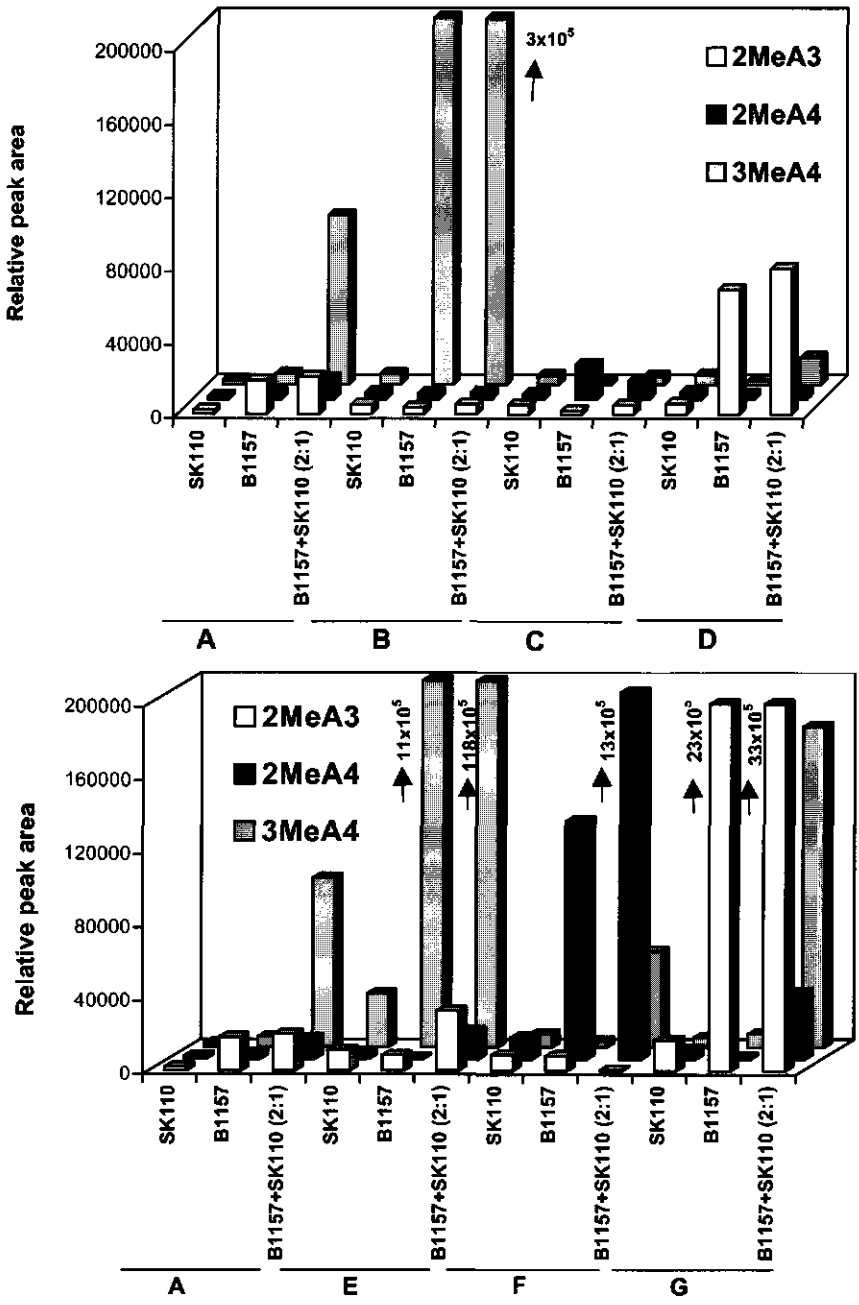

Fig. 3. Relative amounts of branched-chain aldehydes, 2-methylpropanal (2MeA3), 2-methylbutanal (2MeA4) and 3-methylbutanal (3MeA4) formed by B1157, SK110 and (B1157+SK110 2:1) strains in milk cultures without additives (A); with: leucine (B); isoleucine (C); valine (D); α-ketoisocaproic acid (KICA) (E); α-keto-β-methyl-n-valeric acid (F) and α-ketoisovaleric acid (G).

Free amino acid analysis

Free amino acid profiles of milk cultures incubated with SK110 and B1157 and their mixtures revealed that the amino acid patterns were different with SK110 to those with B1157 cultures due to the action of proteolytic enzymes (data not shown).

SK110 is able to release the branched-chain amino acids (Leu, Ile and Val), whereas these amino acids were not liberated by B1157 cells (Fig. 4). Although SK110 is able to produce these amino acids, only low amounts of Val, and neither Leu nor Ileu, were detected in the mixture of B1157 and SK110 at a ratio 2:1. This could be due to the direct conversion of these amino acids to branched-chain aldehydes by B1157. In the case of the mixtures 1:1 and 1:2 (B1157:SK110), branched-chain amino acids were detected (Fig. 4). These findings can most likely be explained by assuming that, when SK110 is present in equal or higher dose than B1157, amino acids converting enzymes become limiting. This corroborates the difference in the organoleptic scores in chocolate intensity between the different mixtures of these cultures (Table 1).

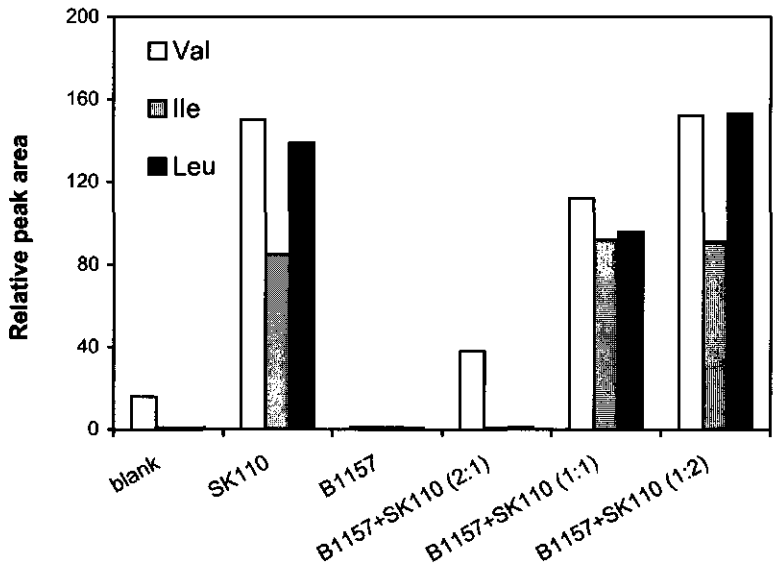

Fig. 4. Relative amount of branched-chain amino acids Leu, Ile and Val in milk cultures. Milk (blank), and milk cultures incubated with SK110, B1157, (B1157+SK110 2:1), (B1157+SK110 1:1) and (B1157+SK110 1:2).

Aminotransferase and decarboxylase activities

The aminotransferase activities towards leucine were determined in CFEs of B1157, B851, SK110 and the other *L. lactis* strains (B1173 and B850) from natural niches (for comparison). All strains showed aminotransferase activity by the formation of KICA although some differences were observed (Table 2). CFE fractions inactivated by heat treatment showed no KICA formation (data not shown). These results indicate that all tested strains contain transaminase activity.

Decarboxylating activity towards KICA was measured in CFE of the strains. The amount of 3MeA4 formed during incubation is indicative of decarboxylating activity present in the CFE (Table 2). The amount of 3MeA4 formed from KICA in the presence of CFE from B1157 was the highest for all strains tested, indicating a strong decarboxylating activity in this strain. Heat-inactivated CFE fractions showed no 3MeA4 formation (data not shown), which indicates that this conversion is enzymatic. No activity was detected in CFE from SK110, suggesting the absence of decarboxylase activity in this strain.

Table 2. Relative amounts of α-ketoisocaprioc acid (KICA) and 3-methylbutanal (3MeA4) formed by cell free extract (CFE) of *L. lactis* strains.

CFE fraction	Peak area	
	KICA[a]	3MeA4[b]
Blank	0.0	0.3
SK110	84.5	0.4
B1157	60.0	400.0
B1173	36.0	93.3
B850	119.0	54.9
B851	136.0	48.0

[a] Relative amounts of KICA as determined by reversed-phase of HPLC after incubation of CFE with leucin.

[b] Relative amounts of 3MeA4 determined by HS-GC after incubation of CFE with KICA (area expressed in arbitary units).

Taken together, the interaction between strains in the tested mixtures is schematically illustrated in Fig. 5. In SK110, the complete pathway from casein to 3-methyl butanal cannot proceed because of the lack of a decarboxylative enzyme in this strain (Fig. 5B). B1157 is a non-proteolytic strain and therefore, is unable to produce enough free amino acids to serve as substrate for the subsequent transamination and decarboxylation steps (Fig. 5C). However, when B1157 and SK110 are incubated together, the strains complement each other with regard to their enzyme activities, resulting in a high production of the chocolate flavour component 3-methyl butanal (Fig. 5D). On the other hand, strain B851 is able to carry out the whole degradation (Fig. 5E), although its decarboxylase activity is lower than that of B1157. As a result, only a moderate chocolate-like flavour is found (Fig. 5F and Table 1). When B851 is mixed with SK110, the chocolate-like flavour intensity experienced is lower (Table 1). This might be due to a further 'dilution' of enzyme activity in the mixture compared with the pure culture of B851 (Fig. 1).

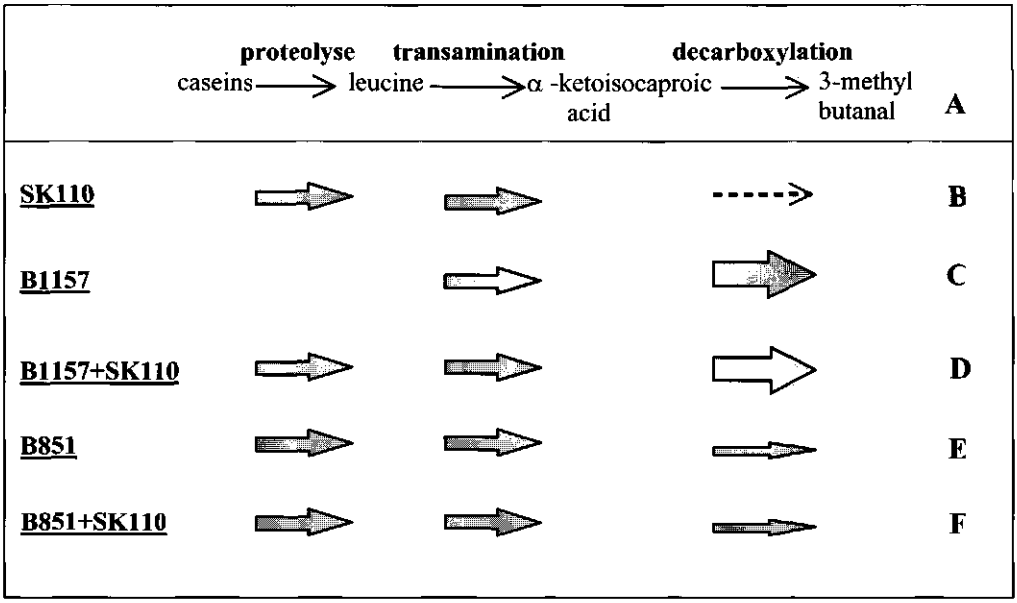

Fig. 5. Proposed pathway of leucine by enzymes from individual and combined lactococcal starter cultures B1157, B851 and SK110. (A) General pathway for the breakdown of casein; (B) SK110; (C) B1157; (D) defined culture (B1157+SK110); (E) B851; and (F) defined culture (B851+SK110). In the decarboxylation step, the narrow arrow represents low decarboxylase activity while the thick arrrow represents high decarboxylase activity.

Despite the fact that the formation of high amounts of these aldehydes has been reported as off-flavours in raw milk (Morgan, 1976; Molimard & Spinnler, 1996), these compounds are also recognized as key flavours compounds in a number of cheeses (Bosset & Gauch, 1993; Barbieri *et al.*, 1994; Neeter *et al.*, 1996), suggesting that good control of flavour production by the starter culture is essential for a well balanced flavour.

In conclusion, the amino acid-converting enzymes of LAB can play an essential role in flavour development. In defined strain starter cultures, many different interactions can occur (Meers, 1973) which not only affect the composition of these mixtures, but, as described in this study, might also have an important impact on flavour production. The combination of a knowledge of flavour formation pathways and functional characteristics of LAB cultures opens new avenues for industrial applications. It can be used to develop tailor-made defined starter cultures, as well as to produce flavour blocks.

Further work will focus on the possibility of applying this knowledge in cheese-making experiments, as well as on the purification of the enzymes involved in the pathways described.

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Chapter 8

Improvement of a starter culture for tailoring the flavour of Proosdij-type cheese

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This chapter has been submitted for publication and for a patent application.

ABSTRACT

To tailor the flavour development of a Proosdij-type cheese made with a combination of an acidifying mesophilic and an adjunct thermophilic culture, the use of the additional mesophilic strain B851 with specific flavour forming abilities was tested. This strain was selected with regard to its ability to produce the branched chain aldehyde 3-methyl-butanal, which is a key flavour compound in Proosdij cheese. In order to control the flavour intensity, the selected strain was first tested in different doses in a defined strain starter (DSS) culture as well as in combination with a mixed strain starter (MSS) culture. The latter is generally used for Gouda and Proosdij-type cheese productions. The results of population dynamics, sensory evaluation and analysis of volatile compounds indicated the possibility to control both the cell numbers of strain B851 as well as the flavour intensity resulting from this strain in cheese. Based on this, B851 was used to enhance the flavour development of a Proosdij-type cheese made with a new thermophilic culture S1138. This culture was previously developed to prevent crack formation in Proosdij cheese. In this cheese, the addition of culture B851 led to an increase in the overall flavour intensity, indicating that it is possible to tailor the flavour of cheese using specifically selected cultures, even in combination with complex starter cultures.

INTRODUCTION

Flavour of cheese is one of the most important attributes for the consumer besides the consistency in the quality of the final product. Cheese flavour is believed to result from a balance between a number of components released by enzymic reactions rather than by chemical ones (Delahunty & Piggott, 1995). The characteristics of the flavour profile of ripened cheeses are mainly effected by proteolysis of caseins and in some types also by lipolysis (Crow *et al.*, 1993). The typical cheese flavour results from further degradation of amino acids due to the pathways for conversion of amino acids by starter bacteria (Broome & Limsowtin, 1998). Indeed, the flavour of finished cheese depends mainly on the starter cultures used (Heap, 1998). Various compounds have been identified and characterized as key flavours in different types of cheese (Bosset & Gauch, 1993; Neeter *et al.*, 1996; Engels & Visser, 1994; Engels *et al.*, 1997; Urbach, 1997).

Dutch-type cheese varieties constitute one of the most important types of cheese produced in the world. Traditionally, two main types of cheeses are made in the Netherlands: Gouda cheese and Edam cheese. Both are made by using mixed-strain mesophilic starter cultures containing *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* as acid-forming bacteria and the citrate using *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris* (Walstra *et al.*, 1987; Johnson *et al.*, 1998).

Another Dutch cheese variety that was developed more recently is Proosdij cheese, which is essentially produced similar to Gouda cheese with the addition of an extra mixed-strain thermophilic culture (e.g. APS culture) containing several strains of the species *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Lactobacillus acidophilus*. The APS starter makes Proosdij cheese different in flavour characteristics than Gouda cheese. The difference in the flavour profile between Gouda and Proosdij cheeses has been unraveled by Neeter *et al.*, (1996). They showed that in Proosdij cheese, almost the same aroma compounds are present as in Gouda cheese of the same age, but the concentrations are different. In particular, the content of 3-methylbutanal (3MeA4) was found to be higher in Proosdij cheese than in Gouda. This compound as well as some ketones were found to be key flavour components for this type of cheese.

The eyes in Gouda-cheese originate from the accumulation of carbon dioxide gas (CO_2) produced from the metabolism of citric acid by *Leuconostoc mesenteroides* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* (Akkerman *et al.*, 1989). Production of gas (CO_2) may lead to desirable eye formation, however it may also cause undesirable crack formation, depending on the facture properties of the cheese mass at the time of gas release. In the case of Proosdij cheese it was found that CO_2 formation also results from the decarboxylation of glutamic acid to γ-amino butyric acid (Zoon & Allersma, 1996). This process occurs later on during the ripening of the cheese, and due to the consistency of the cheese, crack formation may occur. Cracks are an undesired characteristic and therefore a new starter, NIZO S1138, was developed, based on strains isolated from the complex culture APS (G. Smit, unpublished results). However, it appeared that the overall flavour intensity of cheese made with this new starter was not as high as in cheese made with the original culture. Moreover, a decline in the production of key flavour compounds such as 3MeA4 (Neeter *et al.*, 1996) was observed (G. Smit, unpublished results).

3-Methylbutanal was found to be a flavour compound which is produced by a number of wild lactococcal strains isolated from dairy and non-dairy sources (Chapter 2; Ayad *et al.*, 1999). The present work focuses on the possibility to control flavour intensity of the new Proosdij-type cheese, by preparing defined strain starter cultures with different dosages of a 3MeA4-producing strain in order to obtain a well-balanced flavour in the cheese. The combination of knowledge of flavour formation and other functional characteristics of these wild lactococci is applied to enhance the formation of specific key flavour components in a directive manner.

MATERIALS AND METHODS

Origin of strains and growth conditions

The strains used in this study were obtained from the culture collection of NIZO food research, Ede, The Netherlands. *Lactococcus lactis* subsp. *cremoris* SK110 was derived from a commercial starter culture. Dairy wild lactococcal strains originated from artisanal production of dairy products and included *L. lactis* subsp. *lactis* B851, *L. lactis* subsp. *lactis* B1155 and *L. lactis* subsp. *lactis* biovar *diacetylactis* B88 (Chapter 5; Ayad *et al.*, 2001b). The mixed-strain mesophilic starter Bos, often used for Gouda cheese making, consisting of a mixture of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *Leuconostoc* species and *L. lactis* subsp. *lactis* biovar *diacetylactis* was also used. In addition the culture NIZOSTAR APS, a mixed-strain thermophilic starter culture containing strains of *Streptococcus thermophilus*, *Lactobacillus acidophilus* and *Lactobacillus helveticus*, was applied. Culture S1138 (T149) is composed of all strains from NIZOSTAR APS, except the glutamic acid decarboxylase-positive strains. For cheese making, strains were precultivated for 16 h at 30° C in milk for the proteolytic strains SK110 and B851 and in milk with 0.5% yeast-extract (Difco Laboratories, Detroit, MI) for the non-proteolytic strains B88 and B1155. Bos was precultivated for 18 h at 20° C in milk and APS and S1138 were precultivated for 40 h at 37° C in milk.

Cheese making

Three individual series of cheese making trials were performed using standard Gouda cheese making technology. The first series of cheese trials were made from 200 L portions of pasteurised (10 s, 74°C) milk, standarized on fat according to the protocol for Gouda 48^{+} cheese. The second and the third series of cheese trials were performed on a larger scale (2000 L) under the same protocol. The first series of cheese trials consisted of four cheese vats, using four defined strain starter (DSS) culture sets, consisting of SK110 (*L. lactis* subsp. *cremoris*), B851 and B1155 (*L. lactis* subsp. *lactis*), and B88 (*L. lactis* subsp. *lactis* biovar *diacetylactis*). Strain B851 was inoculated from the preculture in different doses (0.00, 0.025, 0.10 and 0.25% v/v), the other three strains were applied in equal dosages of 0.25% v/v each in all cheese vats (Table 1). Cheese prepared without B851 (a) was used as a control. The second series was performed using four mixed-strain starter (MSS) cultures sets, containing the mesophilic Bos-culture as the acidifying cheese starter culture combined with B851 as the adjunct starter. The dosage of the Bos-starter was adjusted in such a way that an acidifying activity was obtained which is usual in Gouda cheese manufacture.

Table 1. Starter cultures used in cheese making

Strain	Series 1				Series 2				Series 3					
	a	b	c	d	a	b	c	d	a	b	c	d	e	f
B1155	0.25[a]	0.25	0.25	0.25	-	-	-	-	-	-	-	-	-	-
B88	0.25	0.25	0.25	0.25	-	-	-	-	-	-	-	-	-	-
SK110	0.25	0.25	0.25	0.25	-	-	-	-	-	-	-	-	-	-
B851	-	0.025	0.10	0.25	-	0.025	0.10	0.25	-	0.025	0.10	0.25	-	0.10
Bos	-	-	-		0.60	0.60	0.60	0.40	0.60	0.60	0.60	0.60	0.60	0.60
APS	-	-	-		-	-	-	-	2.0	2.0	2.0	2.0	-	-
S1138	-	-			-	-	-	-	-	-	-	-	2.0	2.0

[a] % (v/v) of starter preculture added to the cheese vat.

Strain B851 was used in different doses (0.00, 0.025, 0.10 and 0.25% v/v), as shown in Table 1. Cheese prepared with only 0.6% Bos (trial a) was used as a control in this series. The third series of cheeses, consisting of the Proosdij-types, were manufactured using six MSS sets, including mesophilic starter cultures (Bos, 0.6%) and thermophilic starter cultures (2.0%) combined with B851 as the adjunct starter in different doses (Table 1). The thermophilic starter cultures APS was used in four sets (a, b, c and d) of these series. The cheese made with Bos and APS only (0.6%+2.0%, trial a) was used as a control. Cheeses (e) and (f) in these series were made using 0.6% of Bos starter cultures combined with 2.0% of the S1138 culture which is isolated from APS and B851 was added to cheese (f) in the ratio of 0.1%. The cheeses were ripened at 13 °C for 9 months for the first series and for 6 months for the second and third series of cheese trials and analysed at various intervals.

Cheese analysis

The population dynamics of the strains in the first series of cheese trials made with DSS were followed by estimating plate counts during making and ripening of the cheeses. The total cell count of individual strains in each cheese sample was determined in duplicate. Cheese samples were diluted 10 times in 2% trisodium citrate solution (w/v) and homogenized for 5 min in a stomacher (Lab-Blender 400, Seward London). Viable counts were enumerated on GMA plates containing 10% skimmed milk, 1.9% ß-glycerophosphate (pH 6.9), 0.001% bromocresolpurple and 1.3 % agar (Limsowtin & Terzaghi, 1976; Hugenholtz *et al.*, 1987). As well as on a based whey medium with calcium lactate, casaminoacids and agar (WACCA, Galesloot *et al.*, 1961), which is made turbid by calcium citrate. Only citric acid fermenting strains produce clear zones around their colonies on this medium. Based on the differences in the ability of strains to hydrolyse casein, to grow at 40°C and to ferment citrate, their growth and survival could be followed individually in each DSS as described previously (Ayad *et al.*, 2001b).

Compositional analyses for fat, salt, pH and moisture on the cheeses after brining (with an age of one day for cheese made on small scale and of 4 days for cheese made on large scale) were determined according to IDF standards method (1979, 1982, 1989 and 1997). Proteolysis, total nitrogen (TN) soluble nitrogen (SN) and amino acid nitrogen (AN) were performed by the method of Noomen (1977).

Free amino acids were analysed on a 4151 Alpha Plus amino acid analyser (Pharmacia LKB, Uppsala, Sweden) directly in the soluble nitrogen fractions of the cheese slurry, prepared as described previously (Engels & Visser, 1994).

Cheeses were sensorially evaluated by experienced cheese graders after 6 weeks, 3 and 6 months of ripening and after 9 months (only the first cheese series) as described previously

(Ayad *et al.*, 2000). In short, assessment of flavour and consistency on scales from 8 (very good) to 3 (very bad) was used. The cheese flavour intensity was scored on a scale from 0 (absent) to 4 (very strong). During the sensorial evaluation of the third cheese series (Proosdij-type cheeses) the following features were also taken into account: intensity of Proosdij flavour, intensity of chocolate-like flavour and the presence of cracks in the cheeses. The average of sensory evaluations was determined.

Analysis of volatile compounds

Volatile compounds in 6 weeks, 3 months, 6 months and after 9 months old cheeses were identifed and quantified using headspace gas chromatography (HS-GC) essentially as described previously (Ayad *et al.*, 2001a).

RESULTS AND DISCUSSION

Cheese making and analysis

In the three series of cheese trials a total of fourteen different starter cultures were applied. In order to know whether the strains used are stably maintained in the cheese during ripening, the use of a defined strain starter is the only way to test this easily. Such an approach was chosen in the first series, where DSS cultures were used. The ability of strain B851 to produce its specific flavour when combined with the complex mixed strain starter Bos was tested in the cheese trials of second series. The experiences from the first two series of trials were used in the ultimate experiences for the preparation of Proosdij cheese in the final series. In the third series, B851 was combined with Bos and a complex adjunct thermophilic culture APS. Gouda-type cheese was manufactured in the first two series with four different DSS cultures in the series 1, and four undefined MSS culture sets in the series 2. Proosdij-type cheese was made in the third series using six MSS sets (Table 1). The acid production during cheese making was sufficient in all cheeses. After approximately 6 h, all cheeses achieved a pH of 5.5, which is normal for Gouda and Proosdij cheese making.

All the cheeses were prepared with different doses of the selected strain B851 in order to study the possibility to control its specific flavour intensity in cheese. B851 was selected because of its ability to produce the desired aldehyde 3-methylbutanal (3MeA4) (Ayad *et al.*, 2001a), which is responsible for a chocolate-like flavour (Morgan, 1976; Urbach, 1993). This component is also recognized as a key flavour compound in cheeses prepared with the

thermophilic starter APS, which is used in Parmesan and Proosdij type of cheeses (Neeter *et al.*, 1996; Engels & Visser, 1994).

The data on the composition of the cheeses included the values for moisture, fat in dry matter, salt in dry matter and pH after brining (Table 2). The composition of the cheeses in the trials of each series was within the compositional range prescribed for regular Gouda and Proosdij cheeses, respectively.

Series 1: Population dynamics and flavour development in cheese made with DSS

Gouda-type cheeses were manufactured with four DSS sets using different doses of the selected strain B851. In order to be able to follow the population dynamics of the strains and their influence on the flavour development in further detail, the total viable cell counts in cheeses were determined during 6 months of ripening (Fig. 1). Cheese made with DSSa without addition of B851 was prepared as a control. In all cheeses, the strains grew well together and no dominant strain was detected. These results are in agreement with previous work (Ayad *et al.*, 2001b), reflecting that these strains can be used as a stable DSS. The viability of B851, B1155 and B88 was quite high during ripening until 6 weeks and subsequently decreased after 3 months of ripening. The number of SK110 started to decrease after the first week of ripening similar to cheese made with SK110 only (Ayad *et al.*, 2000). These results indicated again that the wild strains used were more stable during ripening than the industrial strain. There was a direct proportional relationship between the numbers of B851 in the cheese and the inoculation dosage (Fig. 1). These results showed that not only a stable defined strain starter culture can be designed, but also that its composition can be affected by choosing the inoculum dosage of one of the strains.

Proteolysis was assessed after 6 weeks and 3 months of cheese ripening by chemical analysis of the nitrogen content of the soluble nitrogen fraction (SN) and the amino-acid nitrogen fraction (AN) (Table 2). The proteolysis increased in the cheeses during the ripening process. The values of AN were slightly higher in cheeses made with DSS containing the additional strain B851 which is protease positive (prt $^{+}$). Especially at 6 weeks this effect was clearly visible. The presence of an extra prt $^{+}$ starter stimulated proteolysis in cheese as has been shown before (Stadhouders *et al.*, 1988).

The cheeses were assessed organoleptically after 6 weeks, 3, 6, and 9 months for flavour, consistency and firmness. The grading of the cheeses was carried out as Gouda-type cheeses with a specific flavour.

Table 2. Composition of the cheeses after brining and proteolysis during ripening

Cheese sample	Moisture %	Fat in dry matter %	Salt in dry matter %	pH	6 weeks		3 months	
					SN	AN	SN	A N
Series 1:								
(a) B1155+B88+SK110 (control) (0.25%+0.25%+0.25%)	48.0	54.3	2.8	5.18	11.30	2.72	17.00	5.35
(b) B1155+B88+SK110+B851 (0.25%+0.25%+0.25%+0.025%)	47.9	53.7	2.6	5.27	11.50	2.97	17.20	5.42
(c) B1155+B88+SK110+B851 (0.25%+0.25%+0.25%+0.1%)	47.4	53.2	2.7	5.17	11.50	3.15	17.30	5.60
(d) B1155+B88+SK110+B851 (0.25%+0.25%+0.25%+0.25%)	46.7	54.0	2.6	5.12	11.60	3.34	17.40	5.75
Series 2:								
(a) Bos (control) (0.6%)	37.5	54.2	3.20	5.19	-	-	-	-
(b) Bos+B851 (0.6%+0.025%)	43.0	51.7	3.63	5.23	-	-	-	-
(c) Bos+B851 (0.6%+0.1%)	43.8	51.2	3.86	5.21	-	-	-	-
(d) Bos+B851(0.6%+0.25%)	37.8	53.8	3.36	5.12	-	-	-	-
Series 3:								
(a) Bos+APS (0.60%+2.0%)	44.1	51.9	2.2	5.28	12.7	4.5	19.5	7.9
(b) Bos+APS+B851 (0.60%+2.0%+0.025%)	43.9	51.7	2.4	5.26	12.6	4.5	19.1	7.6
(c) Bos+APS+B851 (0.60%+2.0%+0.1%)	42.7	51.0	2.8	5.25	12.8	4.7	19.5	7.6
(d) Bos+APS+B851 (0.60%+2.0%+0.25%)	44.2	50.9	2.6	5.23	13.1	5.0	19.7	8.0
(e) Bos+ S1138 (0.60%+2.0%)	44.7	50.3	3.0	5.29	12.2	4.3	17.8	7.5
(f) Bos+S1138 +B851 (0.60%+2.0%+0.1%)	42.8	50.7	2.2	5.26	13.0	5.4	19.9	9.7

Results of the chemical composition are mean of two analysis with standard error 0.3.
-: not determined.

All cheeses had good texture characteristics comparable with those of regular Gouda-type cheese. The cheeses were also evaluated for overall flavours with the focus on the chocolate-like flavour attribute and its intensity. The control cheese made with DSSa without addition of the selected strain B851 as well as the cheese made with 0.025 % of B851 (DSSb) did not receive any score of chocolate-like flavour (data not shown). The intensity of chocolate-like flavour was found to be dependent on the dosage of B851 in the starter. The cheese prepared with DSSc, 0.1% of B851, received a high grading score, while increasing the B851 dosage in the cheese (DSSd) lead to an increase of the chocolate-like flavour intensity and consequently the cheese received a low grading score (data not shown). The cheeses received the relatively highest scores of chocolate-like flavour after 6 weeks of cheese ripening and this score was lower after 3 months of cheese ripening. It appeared that the typical flavour decreased in time, but whether this correlated with the observation that the cell numbers also declined (Fig. 1) is not known. One might speculate that cell lysis played a role in the breakdown of the flavour compound 3MeA4.

The cheeses made with each DSS were analysed for volatile flavour components, with the focus on 3MeA4. Each DSS culture produced a typical pattern of volatile compounds, which matched with the sensory flavour description. The amount of 3MeA4 found corraborated with the organoleptical evaluation. There was also a clear correlation between the amount of 3MeA4 detected in the cheese and the initial inoculum level of B851 in the DSS (for further detail, see series 2 and 3). During cheese ripening the amount of this flavour compound was reduced, most likely due to a further conversion of the aldehydes to the corresponding alcohols (see above). It is also possible that other flavour compounds masked the typical aldehyde flavour. In a previous study, it has been reported that strain B851 produced a certain amount of malty or chocolate-like flavour if grown as a single strain and that the intensity of this flavour was reduced if it grew in mixed cultures (Chapter 7; Ayad *et al.*, 2001a). The findings of these cheese trials series indicate that B851 can have a big impact on the specific flavour of cheese and that the flavour intensity is not only dosage dependent but also dependent on the composition of the starter used.

Series 2: Flavour production in cheese made with MSS

The Gouda-type cheese is often produced by using the regular Bos starter. We focused on the possibility of using the selected strain B851 in different dosage together with the Bos starter in the normal Gouda cheese recipe, in order to be able to add extra flavour tone to the Gouda cheese made with a complex starter culture like Bos. Four cheese trials were made with mixed strain starter (MSS) sets, composed of Bos starter mixed with different doses of B851 (Table 1). Sensory panellists noted that cheeses made with the highest dose of B851 (MSSd, 0.25% B851) received a low score, due to the presence of a high intensity of

chocolate-like flavour (Fig. 2). This indicated that B851 could indeed survive in the cheese with Bos and form its typical flavour.

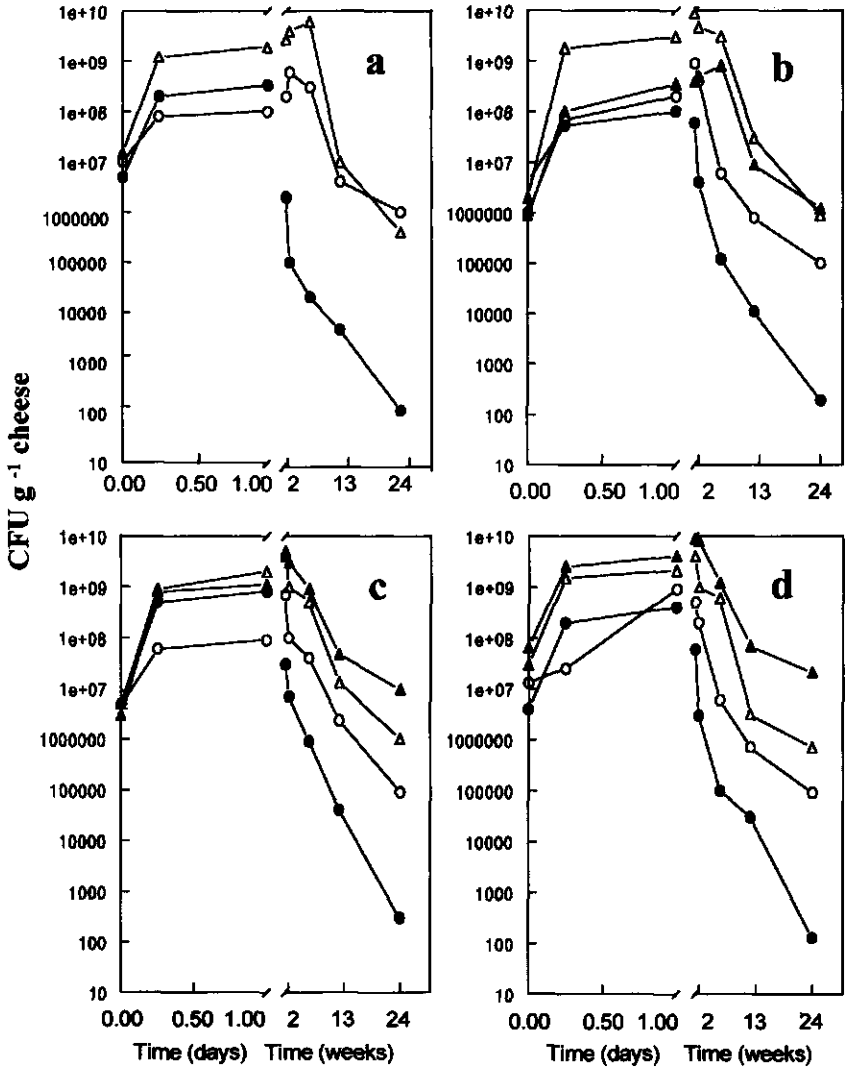

Fig. 1. Population dynamics of defined strain starter cultures (DSS) in Gouda-type cheese (series 1, mean of duplicates). Cheeses prepared with B1155 (Δ), B88 (○), SK110 (●) and different doses of B851 (▲); 025% in DSSb, 0.10 in DSSc, 0.25 % in DSSd and without B851 in DSSa (control).

The volatile flavour compounds in the 4, 8 and 13 weeks old cheeses of these series were quantified using HS-GC (Fig. 3). In general, the amount of 3MeA4 detected was dependent on the dosage of strain B851. A low level of 3MeA4, which slightly increased during ripening was found in the control cheese made with Bos starter (MSSa), indicating that this starter produces a small amount of this flavour compound. In the cheese containing the lowest dosage (0.025%) of B851, the level of 3MeA4 produced was hardly higher than in the control (Fig. 3). As a consequence, the grading score of the cheese made with MSSb was hardly found to give a specific flavour (Fig. 2). The highest level of 3MeA4 was produced after the first weeks of ripening in the cheese made with MSSd set (0.25% dosage of B851) and then decreased by 30% during ripening up to 3 months. The flavour intensity decreased throughout ripening and the Bos-flavour seemed to mask the specific flavour produced by B851 or 3MeA4 could be converted to the corresponding alcohol. Anyway, the results clearly showed that the specific flavour of B851 can be introduced into the Gouda cheese purposely.

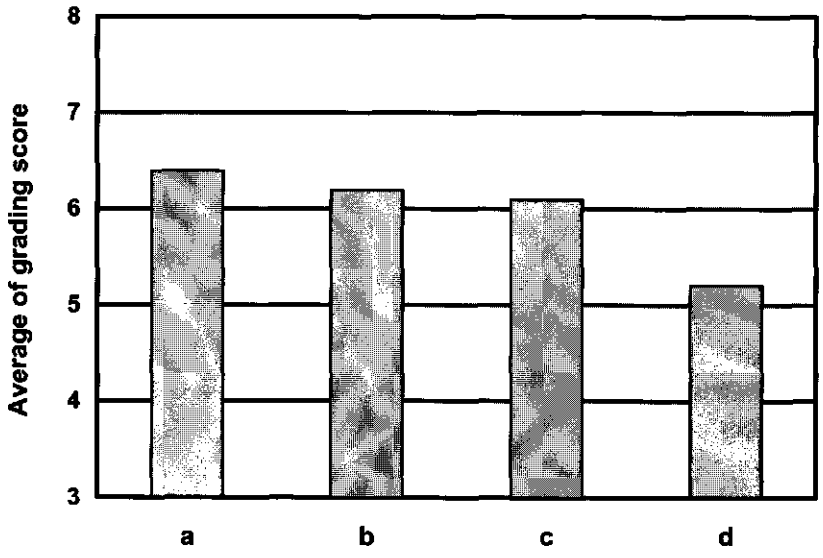

Fig. 2. Average of grading scores for Gouda-type cheese made with MSS (series 2) after 3 months of ripening. The grade on scale ranged from 3 (very bad) to 8 (very good) (for definition of the scale see Methods section). Cheese made with (a) Bos (control); (b) Bos+0.025%B851; (c) Bos+0.1%B851; (d) Bos+0.25%B851.

Series 3: Flavour enhancement in Proosdij cheese

The cheese trials in the series 1 and 2 showed that it is possible to dose the flavour generating properties by adjusting the starter culture. The following question was, whether it is possible to improve the flavour of Proosdij cheese, made with culture S1138 as thermophilic adjunct culture, with the focus on the key flavour component 3MeA4. Previous work (G. Smit, unpublished results) showed that Proosdij cheese made with adjunct culture S1138 scored a lower typical Proosdij flavour than the cheese made with APS. Since, the former was preferred due to the absence of crack formation in the cheeses, the typical flavour should be enhanced and for this the use of a 3MeA4-producing starter might be the solution. Cheeses were manufactured with B851 as an extra culture mixed with the culture Bos as acidifying culture and APS or S1138 as thermophilic adjunct culture (series 3; Table 1).

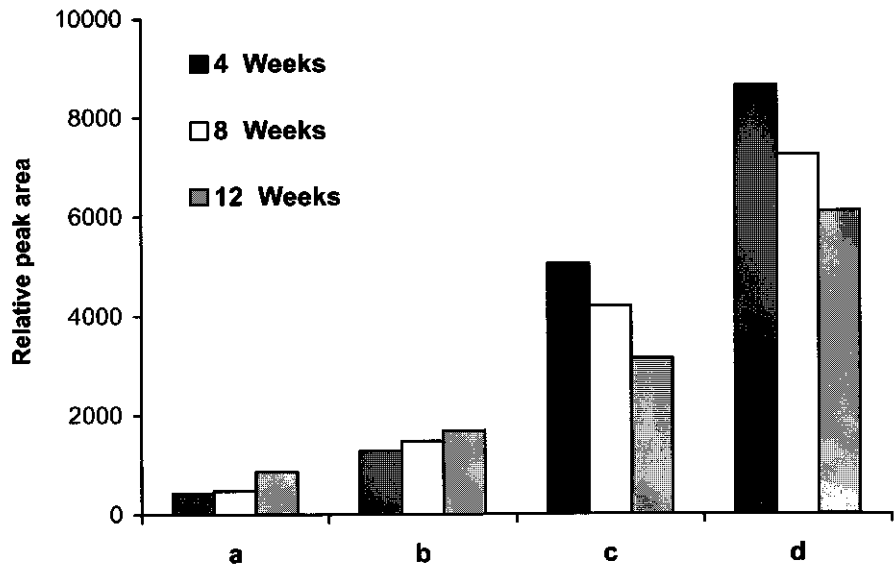

Fig. 3. Relative amount of 3MeA4 formed during ripening of Gouda-type cheese made with MSS (series 2). Cheese made with (a) Bos (control); (b) Bos+0.025%B851; (c) Bos+0.1%B851; (d) Bos+0.25%B851.

The average degree of proteolysis during ripening of these third series of cheeses, derived from the SN and AN values, was higher as compared to Gouda cheese, which is largely due to

the proteolytic activity of the thermophilic APS culture (Smit *et al.*, 2000). The value of AN were slightly lower in the cheeses manufactured with S1138 in MSSe. All the values of AN were further increased when B851 was included in the starter (see b, c, d and f; Table 2). This phenomenon is comparable with that observed in the series 1.

Again the cheeses prepared with APS were found to form cracks, whereas those made with S1138 did not. Analysis of free amino acids and in particular GABA confirmed that GABA was only formed in significant amounts in cheese prepared with APS (Fig. 4). Addition of the proteolytic strain B851 led to an overall increase in amino acid levels, which might be positive for follow-up reactions for flavour formation. GABA levels did, however, not increase in these cheeses. Since, the formation of the key flavour 3MeA4 is also dependent on a decarboxylase activity, there was a minor risk that this could also cause extra CO_2 production. However, the level of conversion into 3MeA4 is relatively low, in the μM level, whereas GABA is produced in the mM level in cheeses made with APS (Fig. 4).

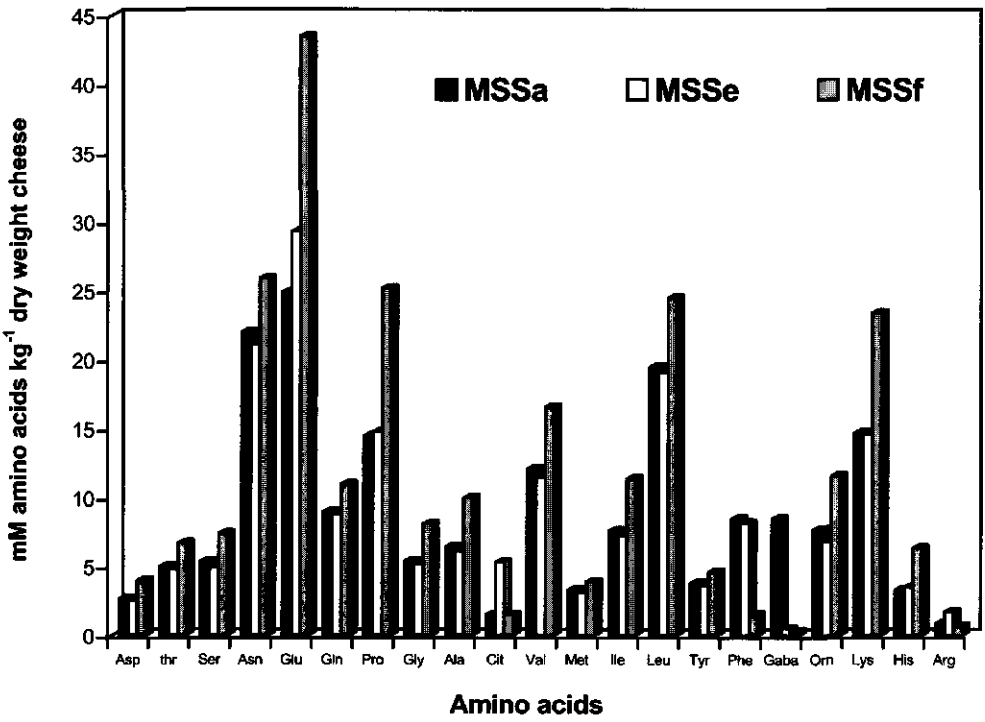

Fig. 4. Free amino acids in extracts of Proosdij cheeses after 3 months of ripening. Cheese made with MSSa: Bos+APS (control), MSSe: Bos+S1138, MSSf: Bos+S1138+0.1%B851. Amino acids are presented in mM kg^{-1} dry mass of cheese.

Only CO_2 production in the mM level might lead to crack formation (Zoon & Allersma, 1996). This observation is important, since the potential improvement of the Proosdij flavour should obviously not be linked to other pathway leading to crack formation.

Cheeses made with APS and low dosages of B851 (MSSb and MSSc) were found to give a similar overall flavour score as the regular Proosdij cheese made with APS alone (MSSa). The grading score for overall flavour of cheeses decreased upon increasing the dosage of B851 up to 0.25%, which resulted in enhancing chocolate-like flavour intensity (Fig. 5).

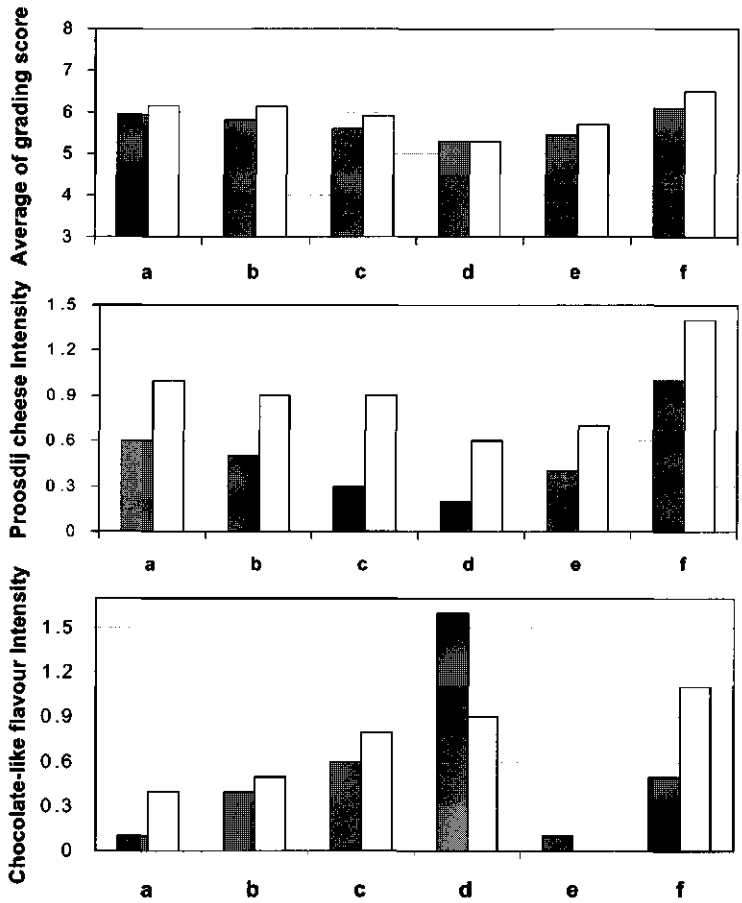

Fig. 5. Sensory evaluation of Proosdij cheese made with MSS (series 3) during ripening after six weeks (filled bars) and after three months (open bars). (1) Average of grading scores for overall flavour ranged from 3 (very bad) to 8 (very good) (for definition of the scale see Methods). (2) Proosdij cheese flavour intensity scale from 0 (absent) to 4 (very strong). (3) Chocolate-like flavour intensity scale from 0 (absent) to 4 (very strong). Cheese made with (a) Bos+APS (control); (b) Bos+APS+0.025%B851; (c) Bos+APS+0.1%B851; (d) Bos+APS+0.25%B851; (e) Bos+S1138; (f) Bos+S1138+0.1%B851.

Apparently, this caused the flavour to be out of the balance, which is characteristic for Proosdij cheese. Cheeses made with S1138 (MSSe) received a lower score for overall flavour as compared with the control cheese made with APS (MSSa). As expected, cheeses made with traditional APS starter developed cracks during ripening, whereas cheeses manufactured with S1138 were not found to have any cracks. Addition of 0.1% of B851 to the latter cheeses (MSSf) resulted in a higher organoleptic score. Both the overall perception as well as the intensity of Proosdij flavour were higher (Fig. 5), suggesting that strain B851 is able to specifically improve this type of cheese.

The relative amounts of branched chain aldehyde 3MeA4 formed during ripening of cheeses are presented in Fig. 6. 3MeA4 were detected in all cheeses as expected for this key flavour compound. Relative high levels of 3MeA4 were found to be dependent of the dose of B851 (MSSd) (Fig. 6) and this result corroborates the results in Gouda cheese (Fig. 3).

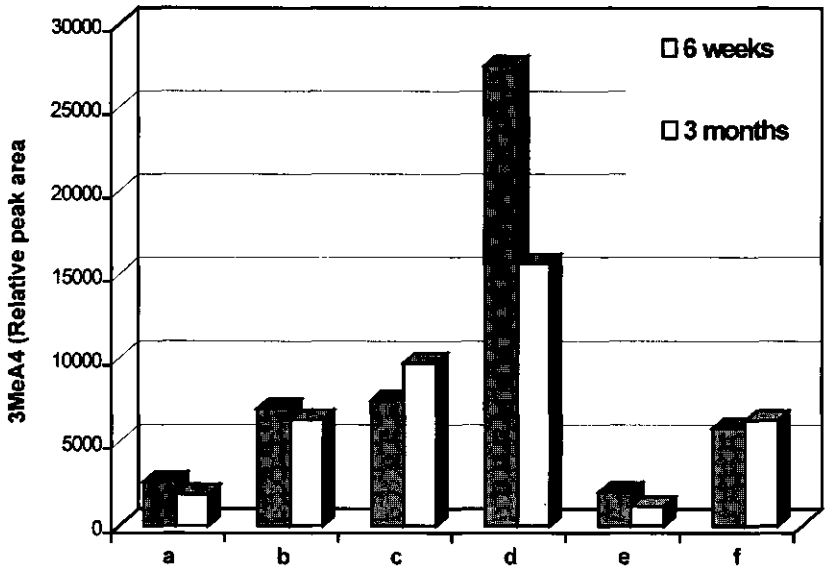

Fig. 6. Relative amount of branched-chain aldehyde 3MeA4 formed during Proosdij cheese ripening (series 3) after six weeks (filled bars) and 3 months (open bars). Cheese made with (a) Bos+APS (control); (b) Bos+APS+0.025%B851; (c) Bos+APS+0.1%B851; (d) Bos+APS+0.25%B851; (e) Bos+S1138; (f) Bos+S1138+0.1%B851.

The key aroma compound (3MeA4) was found in a low amount in cheese prepared with S1138 (MSSe) while this compound was detected in a higher amount in the cheese prepared with S1138 combined with 0.1% B851 (MSSf, Fig. 5). During cheese ripening the amount of

3MeA4 decreased in the cheese made with MSSd after 6 weeks, possibily due to its conversion into the corresponding alcohol 3MeO4 (data not shown). This might also explain the reduction in intensity of the chocolate flavour. The results indicate that B851 is able to enhance the Proosdij flavour in the cheese with the thermophilic starter S1138. The flavour analysis, especially the amounts of 3MeA4 corroborate the organoleptic data (Figs 5 & 6).

CONCLUSIONS

The *Lactococcus lactis* strain B851 was selected for its ability to produce the flavour compound 3MeA4, which is a key flavour compound in Proosdij cheese. The impact of strain B851 on cheese flavour development was tested in defined strain starter (DSS) cultures and in mixed strain starter (MSS) cultures in Gouda and Proosdij cheese. The results showed that DSS can be prepared which are stable on the one hand and result in a selective increase of flavour compounds in cheese depending on the dosage of B851on the other hand. This makes it possible to tailor a desirable flavour by using a selected flavour forming strain. Proosdij cheese made with a new thermophilic strain S1138, that was previously developed to avoid crack formation in this type of cheese, received a low score for overall flavour compared with cheese produced with traditional APS starter. The use of selected strain B851 as an adjunct starter in Proosdij cheese resulted in a cheese with a higher score of Proosdij flavour. Flavour analysis using gas chromatography confirmed that the amount of the key flavour compound 3MeA4 correlated with the addition of B851 in a dose-dependent manner. These results indicate that, strain B851 is able to enhance the flavour development of S1138 for Proosdij cheese flavour without the risk of crack formation. In conclusion, tailoring cheese flavour development by selected cultures opens possibilities for cheese innovations in a directive manner.

ACKNOWLEDMENTS

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Summary and concluding remarks

Summary and concluding remarks

Lactic acid bacteria play an important and economically significant role in the fermentation and preservation of foods, thereby generating desirable flavour and texture attributes. *Lactococcus lactis* strains have been used for millennia in the manufacture of a variety of dairy products such as cheese. Recently, increasing attention has been focused on the need of the dairy industry for 'new' strains to be exploited in product differentiation. For the development of new starter cultures, wild *Lactococcus* strains could be useful since these strains potentially harbour the ability to produce unusual (new) flavours and also other relevant characteristics. To identify *Lactococcus* spp. from natural ecosystems at the species and subspecies level, and to discriminate them from the established strains, several modern molecular microbiological methods are available (Klijn, 1996; Weerkamp *et al.*, 1996).

The work described in this thesis is centred on the study of the characteristics and the behaviour of lactococcal strains isolated from various natural niches and their potential application in cheese manufacture for the formation of new flavours.

Chapter 2 describes the flavour formation ability of a large number of wild lactococcal strains, originating from dairy and non-dairy environments, in milk and in a cheese paste model (Smit *et al.*, 1995). Organoleptic evaluation revealed that several wild strains have the ability to produce specific (unusual) flavours distinct from those produced by conventional industrial strains. GC/MS analysis showed that the major volatile compounds produced by wild strains were most likely originating from amino acid degradation.

Further characterisation of the wild strains indicated that they generally have a low hydrolytic activity towards casein. All wild strains (subsp. *cremoris* and *lactis*) were able to hydrolyse arginine, able to grow at 40°C and in the presence of 4% NaCl in contrast to the industrial strains. These properties might have important implications when these strains are applied in cheese making.

By using the single omission technique, the wild strains were found to requir 1 to 4 amino acids for their growth. The industrial strains, on the other hand, were found to be auxotrophic for up to 10 amino acids. This indicated that these wild strains are more dependent on their own synthesis of amino acids, which could explain their ability to produce unusual flavours. Since, it is hypothesised that amino acid converting enzymes which are involved in flavour formation in cheese (Engels *et al.*, 1996), are in fact involved in the biosynthesis of amino acids.

In Chapter 3 the potential application of wild *Lactococcus* strains in cheese was studied. The strains were characterised on various aspects which play an important role for cheese

making. All strains were found to be resistant to phages which affect strains present in commercial cultures. Moreover, all strains tested were non-lysogenic. The wild strains generally showed a low acidification activity indicating that these strains should be combined with other (fast acidifying) strains in defined strain starter (DSS) cultures. Accordingly, a number of strains were tested in pilot-plant Gouda-type cheese manufacture, either individually or in a simple DSS together with an industrial strain. The chemical composition of cheeses made with different DSS cultures were similar to that of control cheese prepared with industrial strains. Sensory evaluation revealed that wild strains produced their typical flavours in the cheese environment, which corroborated the results in the model systems used in Chapter 2 (Ayad *et al.*, 1999). GC/MS analysis showed that various volatile compounds were produced in cheese by these strains, which confirmed the results of the sensory evaluations.

The behaviour of these strains in mixed cultures was studied by following the population dynamics of the DSS during the cheese making process. Various interactions between the wild lactococcal strains and industrial strains were observed. Some wild strains were able to grow well together with industrial strains, and other strains appeared to inhibit the growth of industrial strains due to the production of bacteriocins. In many cases the bacteriocin was found to be nisin.

From the results obtained in chapters 2 and 3, it can be concluded that some wild lactococcal strains are able to generate specific flavours in milk as well as in cheese. This property makes them interesting for further use in the development of starter cultures.

In order to be able to apply the wild lactococcal strains, described in Chapter 2, the production of antimicrobial compounds by these strains was further evaluated in Chapter 4. The bacteriocins produced by several of these strains were classified into four groups; nisin, diplococcin, lactococcin and bacteriocin-like compounds (unknown). The ability of *Lactococcus* strains to produce antimicrobial compounds was a trait found more frequently in strains of a non-dairy origin than in dairy strains. This ability may enable them to withstand competition from other microorganisms which may be more severe in the hostile natural environment than in the shielded dairy setting. These bacteriocin-producing strains may be used in defined strain starter cultures for cheese manufacture, but only if applied in combination with other bacteriocin-resistant strains.

To assist the development of defined strain starters with specific flavour characteristics and typical properties required for the manufacture of Gouda-type cheese, the behaviour of wild lactococci strains in a complex defined mixture of strains essential for Gouda cheese making was studied in Chapter 5. Before that, it was important to know whether these strains maintain their specific properties when grown individually for many generations. Therefore, the stability of the technological traits of the wild lactococci strains was investigated. The

morphology and genetic profiles of these strains were found to be stable up to 50 subcultivations. Their flavour forming abilities, proteolytic activity, acidification activity and bacteriophage resistance were also found to be stably maintained. The same was true for their ability to ferment citrate as well as their ability to grow at 40°C and in the presence of 4% NaCl.

Different DSS cultures were prepared, each one composed of proteolytic, non-proteolytic and citrate utilising strains. One of these DSS consisted of nisin-producing together with nisin-resistant strains. The population dynamics within the DSS cultures during cultivation in milk and in pilot-scale Gouda-type cheese manufacture showed that several strains were able to grow well together and the mixture was found to be stable in its performance. This opened the possibility to apply these mixtures as tailor-made starter cultures for the production of specific flavours. In some cases however, strains were found to be specifically inhibited in a DSS, a phenomenon that required attention in order to use such strain in DSS.

In Chapter 6, studies on the specific inhibition of *diacetylactis* strains in DSS were carried out to understand the mechanism behind it. Some wild strains used appeared to cause this inhibition. The results of interaction studies showed that the inhibition was not due to competition between the strains for nutrients and not affected by aeration, pH or release of bacteriophages by the wild strains. Growing wild cells were found to be much more inhibitory active than their culture supernatants. In fact, the inhibition of the *diacetylactis* strains was only observed when the supernatant was derived from exponentially phase cultures of the inhibiting strains. The supernatant contained apparently a factor, which acted specifically against some and not all, *diacetylactis* strains. The preliminary characterisation of this inhibitory factor revealed that it was a small compound (less than 1 kDa), heat stable up to 100°C for 30 min, unstable when stored for 24 h at different temperatures. In addition, it was shown to be inactivated by proteinase K, indicating that it might be a proteinaceous compound. However, the general characteristics did not match those of already known antimicrobial compounds. Therefore, further research work needs to focus on the further identification and characterisation of the inhibitory factor.

In Chapter 7, an enhanced flavour production by cocultivation of lactococci from industrial (SK110) and artisanal origin (B1157) was studied. Interestingly, it was found that the strains not only affected their respective growth, but that they also completed each other's metabolism which led to flavour compounds. The combination of both strains resulted in a strong increased chocolate-like flavour. B1157 produced only a moderate chocolate-like flavour whereas SK110 alone failed to produce this flavour. To obtain insight in the underlying mechanism of this enhanced flavour formation by the mixture of cultures, the enzyme activities involved in the pathway leading to these flavour compounds were studied.

The results showed that by combining the strains, the substrates released by one of the strain could directly be used by the other strain, resulting in the completion of the whole flavour-formation pathway.

Although many starter cultures for dairy products are used and combined for several reasons such as preventing or reducing sensitivity for phage attack and formation of eyes in cheese, these cultures have not been selected so far for enhancing the total metabolic activity for the formation of volatile flavour components. The outcome of the present study is providing a new way to enhance or to tailor flavour formation by the cultivation of selected strains.

The combination of knowledge of flavour formation and other functional characteristics of wild *Lactococcus* strains can be applied to enhance or/ improve the formation of specific flavour components of cheese in a directive manner. In Chapter 8, the *Lactococcus lactis* strain B851 was selected for its ability to produce the flavour compound 3-methyl butanal (3MeA4), which is a key flavour compound in Proosdij-type cheese (Neeter *et al.*, 1996). In order to control the flavour intensity and to test the impact of the selected strain B851 on cheese flavour development, this strain was first tested in different doses in a DSS culture as well as in combination with a mixed strain starter (MSS) culture. The latter is generally used for Gouda and Proosdij-type cheese productions. The results showed that DSS cultures can be prepared which are stable on the one hand and result in a selective increase of flavour compounds in cheese depending on the dosage of B851 on the other hand. This showed that it is possible to tailor a desired flavour by using a selected strain with specific flavour-forming abilities. Proosdij cheese made with a new thermophilic strain S1138, that was previously developed to avoid crack formation in this type of cheese (G. Smit, personal communication), received a low score for overall flavour compared with cheese produced with traditional APS starter. In this cheese, the addition of B851 as an adjunct starter resulted in a high score of Proosdij flavour. Analysis of volatile flavour compounds by using gas chromatography confirmed that the amount of the key flavour compound 3MeA4 correlated with the addition of B851 in a dose-dependent manner. Thus, strain B851 is able to assist S1138 in the development of Proosdij cheese flavour without the risk of crack formation. This way of directing cheese flavour development by selected cultures opens possibilities for cheese innovations.

Taken together, the results described in this thesis offer new challenges for the development of tailor-made starter cultures, based on the knowledge of both population dynamics of the strains and their flavour-forming abilities.

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Samenvatting en conclusies

Samenvatting en conclusies

Melkzuurbacteriën spelen een belangrijke en economisch significante rol bij de fermentatie en conservering van voedingsmiddelen. Bovendien zorgen zij voor de gewenste smaak en textuur. Al duizenden jaren worden *Lactococcus lactis*-stammen gebruikt bij de bereiding van diverse zuivelproducten zoals kaas. De laatste tijd is er toenemende aandacht voor de behoefte van de zuivelindustrie aan 'nieuwe' stammen om deze in te zetten bij de productdifferentiatie. Voor de ontwikkeling van nieuwe startculturen zouden wilde *Lactococcus*-stammen van nut kunnen zijn omdat deze mogelijk het vermogen bezitten nieuwe smaken en andere relevante eigenschappen voort te brengen. Om *Lactococcus*-soorten op soort- en ondersoortniveau uit natuurlijke ecosystemen te identificeren en om deze te onderscheiden van de gebruikelijke soorten staan verschillende moderne moleculair-microbiologische methoden ter beschikking.

Dit proefschrift beschrijft het onderzoek naar de eigenschappen en het gedrag van lactococcenstammen geïsoleerd uit diverse natuurlijke bronnen, en hun mogelijke toepassing bij de kaasbereiding om nieuwe smaken te vormen.

Hoofdstuk 2 beschrijft het vermogen van een groot aantal wilde lactococcenstammen afkomstig uit zuivel- en niet-zuivelmilieus om smaak te vormen in melk en in een kaaspastamodel. Uit smaaktesten bleek dat verschillende wilde stammen het vermogen hebben om, naast de smaken gevormd door de gebruikelijke industriële stammen, specifieke nieuwe smaken te vormen. GC/MS-analyse wees uit dat de belangrijkste vluchtige verbindingen gevormd door wilde stammen hoogstwaarschijnlijk afkomstig waren van aminozuurafbraak.

Uit verdere karakterisering van de wilde stammen bleek dat zij in het algemeen een geringe hydrolytische activiteit ten opzichte van caseïne vertonen. Alle wilde stammen (subsp. *cremoris* en *lactis*) konden, in tegenstelling tot de industriële stammen, arginine hydrolyseren en in aanwezigheid van 4% NaCl groeien bij 40 °C. Deze eigenschappen zouden van groot belang kunnen zijn bij toepassing van deze stammen bij de kaasbereiding.

Toepassing van de 'single omission'-techniek toonde aan dat de wilde stammen slechts afhankelijk zijn van een of enkele aminozuren voor hun groei. De industriële stammen bleken daarentegen behoefte te hebben aan soms wel 10 aminozuren. Dit wijst erop dat de wilde stammen een volledigere set enzymen hebben voor hun eigen aminozuursynthese, wat een verklaring zou kunnen zijn voor hun vermogen om nieuwe smaken te vormen; immers de hypothese is dat aminozuuromzettende enzymen die bij de smaakvorming in kaas betrokken zijn, in feite nodig zijn voor de biosynthese van aminozuren.

In hoofdstuk 3 is de mogelijke toepassing van wilde *Lactococcus*-stammen in kaas onderzocht. De stammen zijn gekarakteriseerd naar verschillende aspecten die bij de kaasbereiding een belangrijke rol spelen. Alle stammen bleken resistent te zijn tegen fagen die stammen in commerciële culturen aantasten. Bovendien bleken alle geteste stammen niet-lysogeen te zijn. De wilde stammen vertoonden in het algemeen een geringe verzuringsactiviteit, wat aangaf dat deze stammen met andere (snel verzurende) stammen gecombineerd dienen te worden in samengestelde zuursels, de zogenaamde DSS-culturen (defined strain starter). Een aantal stammen werd dan ook op proefschaal getest bij de bereiding van Goudse kaas, ofwel afzonderlijk ofwel samen met een industriële stam in een eenvoudige DSS-cultuur. De chemische samenstelling van kazen gemaakt met verschillende DSS-culturen kwam overeen met die van controlekaas bereid met industriële stammen. Uit smaaktesten bleek dat wilde stammen hun typische smaak ook in het kaasmilieu vormen, wat de resultaten van de modelsystemen gebruikt in hoofdstuk 2 ondersteunde. GC/MS-analyse liet zien dat deze stammen in kaas diverse vluchtige verbindingen vormen, wat de resultaten van de smaaktests bevestigde.

Uit de resultaten verkregen in hoofdstuk 2 en 3 kan worden geconcludeerd dat enkele wilde lactococcenstammen zowel in melk als in kaas specifieke smaken kunnen vormen. Deze eigenschap maakt hen interessant voor verder gebruik bij de ontwikkeling van starterculturen.

Om de wilde lactococcenstammen beschreven in hoofdstuk 2 te kunnen toepassen is de productie van anti-microbiële verbindingen door deze stammen nader beoordeeld in hoofdstuk 4. De bacteriocines die door een aantal van deze stammen worden geproduceerd, zijn ingedeeld in vier groepen: nisine-, diplococcine-, lactococcine- en bacteriocineachtige verbindingen. Het vermogen van *Lactococcus*-stammen om anti-microbiële verbindingen te vormen was een kenmerk dat bij stammen van niet-zuiveloorsprong vaker werd aangetroffen dan bij zuivelstammen. Dit vermogen kan hen in staat stellen de concurrentie van andere micro-organismen, die in de vijandige natuurlijke omgeving wellicht heviger is dan in de zuivel fermentaties, te weerstaan. Deze bacteriocinevormende stammen kunnen wel in DSS-culturen voor de kaasbereiding worden gebruikt, maar alleen in combinatie met andere bacteriocine-resistente stammen.

Om de ontwikkeling van DSS-culturen met specifieke smaakkarakteristieken en typische eigenschappen vereist voor de bereiding van Goudse kaas te bevorderen, werd het gedrag van wilde lactococcenstammen in een complex gedefinieerd mengsel van stammen die essentieel zijn voor de bereiding van Goudse kaas, bestudeerd in hoofdstuk 5. Het was van belang van tevoren te weten of deze stammen hun specifieke eigenschappen behouden als zij vele generaties afzonderlijk worden voortgekweekt. Daartoe werd de stabiliteit van de technologische kenmerken van de wilde lactococcenstammen onderzocht. De morfologie en

de genetische profielen van deze stammen bleken tot 50 overentingen stabiel te zijn. Hun smaakvormend vermogen, proteolytische activiteit, verzuringsactiviteit en bacteriofaagresistentie bleken eveneens stabiel gehandhaafd te zijn. Hetzelfde gold voor het vermogen om citraat te vergisten evenals het vermogen om te groeien bij 40 °C en in de aanwezigheid van 4% NaCl.

Er werden verschillende DSS-culturen bereid, elk samengesteld uit proteolytische, niet-proteolytische en citraatverbruikende stammen. Eén hiervan bestond uit nisinevormende stammen tezamen met nisine-resistente. De populatiedynamiek binnen de DSS-culturen tijdens het kweken in melk en de experimentele bereiding van Goudse kaas toonden aan dat verschillende stammen goed samen konden groeien en de werking van het mengsel bleek stabiel. Dit opende de mogelijkheid deze mengsels toe te passen als op maat gemaakte starterculturen voor de vorming van specifieke smaken. Aan de andere kant bleken in sommige gevallen stammen in een DSS-cultuur specifiek te worden geremd, een verschijnsel dat nadere aandacht behoeft om dergelijke stammen in DSS-culturen te kunnen gebruiken.

Hoofdstuk 6 beschrijft het onderzoek naar de specifieke remming van *diacetylactis*-stammen in DSS-culturen dat werd uitgevoerd om het mechanisme erachter te begrijpen. Deze remming bleek te worden veroorzaakt door enkele van de gebruikte wilde stammen. De resultaten van interactiestudies lieten zien dat de remming niet was toe te schrijven aan concurrentie om nutriënten tussen de stammen en dat deze niet afhing van beluchting, pH of afgifte van bacteriofagen door de wilde stammen. Groeiende wilde cellen vertoonden veel meer remmende activiteit dan de supernatanten van de groei media waar in ze gekweekt waren. Remming door *diacetylactis*-stammen werd zelfs alleen waargenomen als een dergelijk supernatant afkomstig was van culturen van remmende stammen in hun exponentiële groeifase. De supernatanten bevatten kennelijk een factor die specifiek ageerde tegen sommige maar niet alle *diacetylactis*-stammen. De voorlopige karakterisering van deze remmende factor liet zien dat het een verbinding was met een laag molecuul gewicht (minder dan 1 kDa), hitte-stabiel, maar instabiel bij opslag gedurende 24 h bij verschillende temperaturen. Bovendien werd deze factor geïnactiveerd door proteïnase K, wat erop wijst dat het een eiwitachtige verbinding zou kunnen zijn. De algemene kenmerken kwamen echter niet overeen met die van reeds bekende anti-microbiële verbindingen. Verder onderzoek dient zich daarom te richten op de verdere identificatie en karakterisering van de remmende factor.

Hoofdstuk 7 gaat over het onderzoek naar de versterkte smaakvorming door het co-cultiveren van lactococcen. Belangwekkend was de vaststelling dat de stammen niet alleen elkaars groei beïnvloedden, maar dat zij ook elkaars stofwisseling aanvulden, wat leidde tot versterkte vorming van smaakstoffen. B1157 vormde slechts een matige chocoladeachtige smaak, terwijl SK110 op zichzelf deze smaak niet voortbracht. De combinatie van de twee

stammen leverde een sterk toegenomen chocoladeachtige smaak op. Om inzicht te krijgen in het onderliggende mechanisme van deze versterkte smaakvorming door het mengsel van de culturen werden de enzymactiviteiten onderzocht die betrokken zijn bij de omzettingsroute leidend tot deze smaakstoffen. De resultaten lieten zien dat door combinatie van de stammen de door de ene stam afgescheiden substraten direct door de andere stam konden worden gebruikt, waardoor de hele smaakvormingsroute werd gecompleteerd.

Weliswaar worden reeds veel starterculturen voor zuivelproducten om verschillende redenen in combinatie gebruikt, zoals ter voorkoming van faagbesmetting of vermindering van de gevoeligheid ervoor, of voor de ogenvorming in kaas, maar tot nu toe zijn deze culturen niet geselecteerd om de totale metabolische activiteit voor de vorming van smaakstoffen te versterken. De uitkomst van dit onderzoek verschaft een nieuwe manier om smaakvorming te versterken of aan te passen door de kweek van geselecteerde stammen.

De combinatie van kennis van de smaakvorming en andere functionele eigenschappen van wilde *Lactococcus*-stammen kan worden toegepast om de vorming van specifieke smaakstoffen in kaas gericht te versterken of te verbeteren. In hoofdstuk 8 werd de *Lactococcus lactis*-stam B851 geselecteerd om zijn vermogen de smaakstof 3-methylbutanal (3MeA4) te vormen, een sleutelsmaakstof in Proosdijkaas. Om de smaakintensiteit te beheersen en de invloed van de geselecteerde stam B851 op de ontwikkeling van de kaassmaak na te gaan werd deze stam eerst in verschillende doseringen beproefd, zowel in een DSS-cultuur als in combinatie met een mengzuursel, een zogenaamde MSS-cultuur (mixed strain starter). Dit mengzuursel wordt algemeen toegepast bij de bereiding van Goudse en Proosdijkaas. Uit de resultaten bleek dat DSS-culturen kunnen worden bereid die aan de ene kant stabiel zijn en aan de andere kant een selectieve toename van smaakstoffen in kaas opleveren, afhankelijk van de dosering van B851. Dit toont de mogelijkheid aan om een gewenste smaak te realiseren door een geselecteerde stam met specifieke smaakvormende vermogens te gebruiken. Proosdijkaas gemaakt met een nieuwe thermofiele culture, S1138, die was ontwikkeld om scheurvorming in deze kaassoort tegen te gaan, kreeg een lagere beoordeling voor totale smaak vergeleken met kaas die bereid was met het traditionele APS-zuursel. Door toevoeging van B851 als hulpzuursel kreeg deze kaas een hoge waardering voor Proosdijsmaak. Gaschromatografische analyses van de vluchtige smaakstoffen bevestigden dat de hoeveelheid van de sleutelcomponent 3MeA4 evenredig toenam met de dosering van stam B851. Hieruit volgt dat stam B851 het zuursel S1138 kan helpen bij de vorming van Proosdijsmaak zonder het gevaar van scheurvorming. Deze methode om de kaassmaakvorming met behulp van geselecteerde culturen te sturen toont nieuwe mogelijkheden tot kaasinnovaties op basis van kennis en beheersing van smaakvormingsprocessen.

Kort samengevat, de resultaten beschreven in dit proefschrift stellen nieuwe uitdagingen tot de ontwikkeling van op maat gemaakte starterculturen, gebaseerd op de kennis van zowel de populatiedynamiek van de stammen als hun smaakvormend vermogen.

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