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

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Abstract

A number of wild lactococci of 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.

Keywords: Natural starters; Cheese making; Cheese flavour; *Lactococcus lactis*; Wild lactococci; Population dynamics; Bacteriocins

1. Introduction

Starter cultures used in manufacturing cheeses such as Gouda, Edam and Cheddar usually consisting 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 (Cogan et al., 1997; Weerkamp, Klijn, Neeter & Smit, 1996) and other non-dairy sources such as plants, animals and soil (Sandine, Radich & Elliker, 1972; Collins, Farrow, Phillips & Kandler, 1993; Williams & Collins, 1990; Klijn, Weerkamp & De Vos, 1995). In a previous study (Ayad, Verheul, De Jong, Wouters & Smit, 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 contain 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

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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.

2. Materials and methods

2.1. 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).

2.2. Technological characterization 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;

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

Strains	Subspecies	Sources[a]	Acidification activity (units°N)[b]	Proteolytic activity[c]	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.

Hugenholtz, Splint, Konings & Veldkamp, 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 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).

2.3. Population dynamics

Individual strains were pre-grown for 16 h at 30°C in sterilized 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 48 h 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 48 h.

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.

2.4. Cheese trials and analyses

Gouda-type cheeses were made from 200 L portions of pasteurized (10 s, 74°C) milk in a manner characteristics for Gouda cheese (Walstra, Noomen & Geurts, 1987). Two series were made; in each series five starter 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) (Stanley, 1996; Osborne, 1992). 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 cheesemaking and during ripening of the cheeses, the total number of bacterias 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 scales 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 evaluation data with standard deviations were determined.

2.5. Analysis of volatile compounds

Volatile compounds in 3-month-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, Dekker, DeJong, Neeter & Visser, 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, Neeter, Boelrijk and Smit (2000).

2.6. Bacteriocin production

Antimicrobial activity was determined in an 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 into 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.

3. Results and discussion

3.1. 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. 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, Powell & Hillier, 1990). Lysogenic immunity, conferred by prophages to lysogenic strains, could play a role in the protection of these strains against phage attack (Séchaud et al., 1990; Reyrolle, Chopin, Letellier & Novel, 1982). 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.

3.2. 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

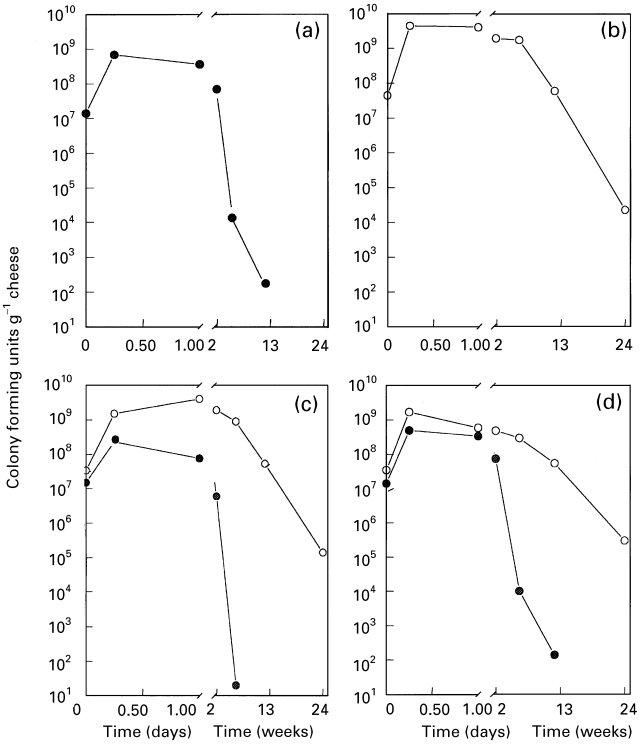

Fig. 1. Population dynamics of starter cultures in cheese prepared with combination of wild strains and SK110 (DSS) (mean of duplicates). Wild starter culture (○), commercial starter SK110 (●). (a): cell counts in cheese made with 1% SK110; (b): cheese made with 1% B1152; (c): cheese made with 2% B1156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

Table 2
Composition of experimental cheeses two weeks after production and determination of proteolysis of 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	—	—	—	—
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 standard error ≤ 0.3.
[b]SN, soluble N; AN, amino N. Results expressed as % of TN (total nitrogen).
—, not determined.

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. 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.

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-acid-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, Toepoel & Wouters (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.

3.3. 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 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, Veldkamp & Konings, 1987b). The changes in the population dynamics in our study were followed in milk during 48 h on GMA-plates to recognize the individual strains in such mixtures due

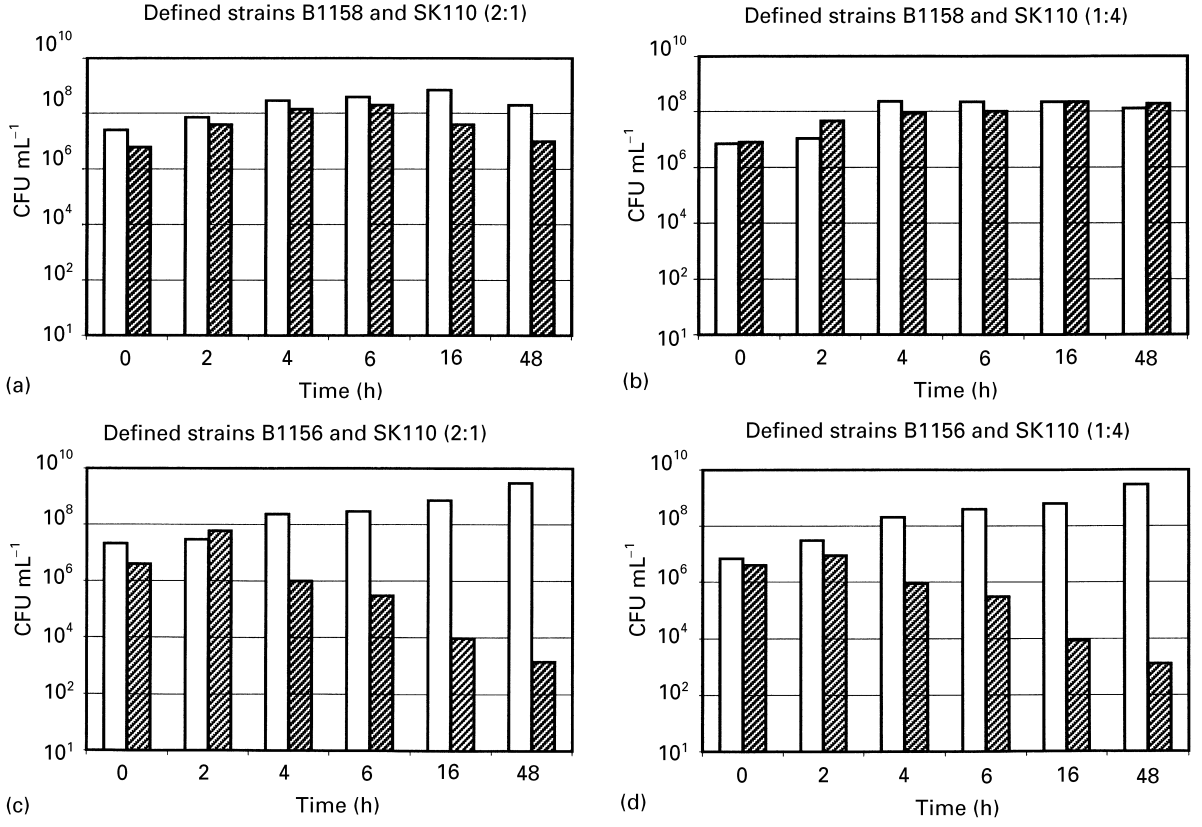

Fig. 2. Population dynamics of defined-strain starter cultures (open bars) with SK110 (filled bars), B1158 + SK110 (a and b) and B1156 + SK110 (c and d) in different combinations (2 : 1 and 1 : 4) during 48 h in milk cultures. Results are presented by means of two analyses.

to the difference in proteolytic activity between wild 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 cultures, 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). 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.

3.4. 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, Tagg & Ray, 1995), these antimicrobial activities are likely to be a consequence of bacteriocin production.

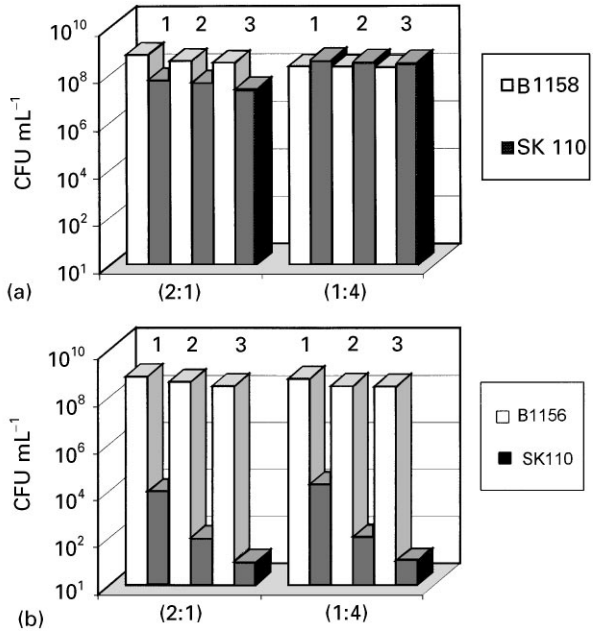

Fig. 3. Population dynamics of defined-strain starter cultures with SK110 (mean of duplicates), B1158 + SK110 (a) and B1156 + SK110 (b) in different combinations (2 : 1 and 1 : 4) in milk cultures after 16 h during three subcultivation.

The antibacterial compounds produced by tested wild strains were further characterized on the basis of their susceptibility to proteolytic enzymes (trypsin, proteinase K, α-chymotrypsin 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-chymotrypsin 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-chymotrypsin as proposed by Davey and Richardson (1981). B1152 produced an unknown bacteriocin which was characterized by heat sensitivity and its inactiviation by α-chymotrypsin only. Our findings agree with those of several others (Kozak, Baradonski & Dobrzarski, 1978; Scherwitz, Baldwin & Mckay 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, remains to be established.

The production of these bacteriocin 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.

3.5. 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

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.

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 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). 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) shoud 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, De Jong, Teisman & Ellen, 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.

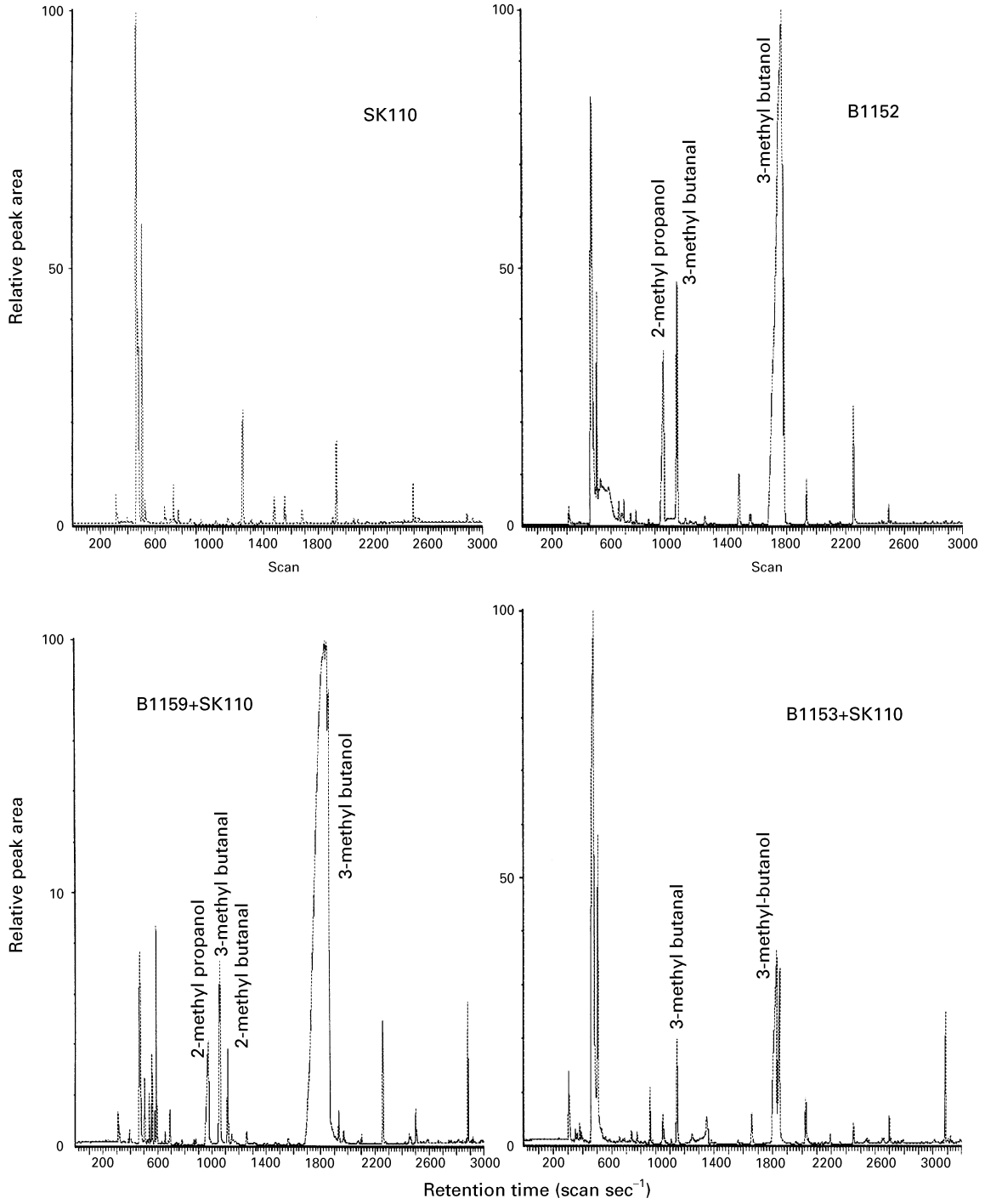

Fig. 4. GC-MS aroma profiles of volatile compounds purged from 3-month-old cheese prepared with industrial strain SK110 and wild starter cultures mixed with SK110.

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,

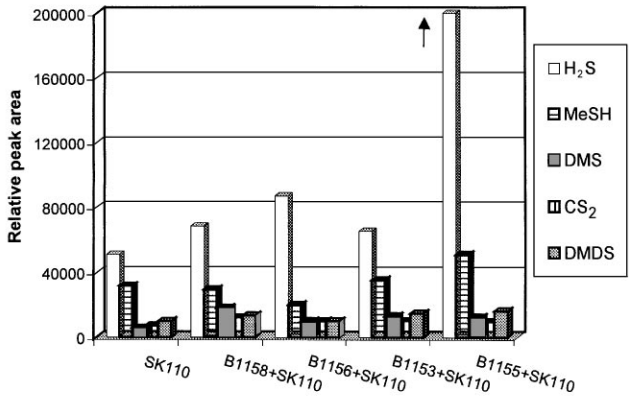

Fig. 5. Relative amounts of sulphur compounds formed during ripening of cheese after 3 months. H_2S, hydrogen sulphide; MeSH, methanethiol; DMS, dimethylsulphide; CS_2, carbon disulphide; DMDS, dimethyldisulphide.

B1155 + SK110, B1156 + SK110 and B1153 + SK110 and with SK110 (control cheese) after 3 months of ripening by using HS-FPD method (Fig. 5). 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. 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 (Urbach, 1993; Parliment, Kolor & Rizzo, 1982) and can be formed by enzymatic degradation of methionine by *L. lactis* (Engels et al., 1997).

4. Conclusions

Wild strains generally showed 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 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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