



Selection, application and monitoring of *Lactobacillus paracasei* strains as adjunct cultures in the production of Gouda-type cheeses

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ARTICLE INFO

Article history:

Received 9 December 2009

Received in revised form 20 April 2010

Accepted 11 May 2010

Keywords:

Adjunct

Gouda cheese

Lactobacillus paracasei

DGGE

GC-MS

Sensory analysis

ABSTRACT

Raw milk cheeses have more intense flavours than cheeses made from pasteurized milk and harbour strains with potential adjunct properties. Two *Lactobacillus paracasei* strains, R-40926 and R-40937, were selected as potential adjunct cultures from a total of 734 isolates from good quality artisan raw milk Gouda-type cheeses on the basis of their prevalence in different cheese types and/or over several production batches, safety and technological parameters. Conventional culturing, isolation and identification and a combined PCR-DGGE approach using total cheese DNA extracts and DNA extracts obtained from culturable fractions were employed to monitor viability of the introduced adjuncts and their effect on the cheese microbiota. The control cheese made without adjuncts was dominated by members of the starter, i.e. *Lactococcus lactis* and *Leuconostoc pseudomesenteroides*. In the cheeses containing either R-40926 or R-40937, the respective adjuncts increased in number as ripening progressed indicating that both strains are well adapted to the cheese environment and can survive in a competitive environment in the presence of a commercial starter culture. Principal component analysis of cheese volatiles determined by steam distillation–extraction and gas chromatography–mass spectrometry could differentiate cheeses made with different concentrations of adjunct R-40926 from the control cheese, and these differences could be correlated to the proteolytic and lipolytic properties of this strain. Collectively, results from microbiological and metabolic analyses indicate that the screening procedure followed throughout this study was successful in delivering potential adjunct candidates to enrich or extend the flavour palette of artisan Gouda-type cheeses under more controlled conditions.

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1. Introduction

Flavour formation during cheese ripening is the result of a series of complex chemical and biochemical conversions of milk components (Smit et al., 2005). Essentially, the typical cheese flavour arises from three main processes i.e. proteolysis of milk caseins, lipolysis of milk fat and fermentation of (residual) lactose, lactate and citrate which collectively result in the production of a complex mixture of volatile aroma and non-volatile taste compounds (Marilley and Casey, 2004; Smit et al., 2005). Next to the action of enzymes from rennet and milk, microbial enzyme activities are largely responsible for the formation of flavour compounds during cheese ripening. For Gouda-type cheeses, micro-organisms involved in flavour development basically can be divided in two major groups, i.e. starter and non-starter lactic

acid bacteria (LAB). Although the main role of the starter culture is the production of lactic acid, it also largely contributes to proteolysis and lipolysis and hence flavour formation (Kieronczyk et al., 2003; Smit et al., 2005). Non-starter lactic acid bacteria (NSLAB), which may enter the cheese making process through the milk, the cheesemaking equipment and/or the production environment and which gradually become the dominant microbiota during ripening reaching numbers up to 10^7 – 10^8 cfu/g, also play an important role in the development of the organoleptic properties of mature cheese as a result of lipid and protein metabolism (Fox et al., 1998). As most LAB produce a range of proteinases, peptidases and amino acid converting enzymes, the proteolytic pathway is by far the most important process during flavour formation (McSweeney, 2004). In contrast, only moderate to little lipolysis occurs in Gouda and many other cheese types (Collins et al., 2003; McSweeney, 2004) probably due to a restricted or lack of lipolytic activity of most LAB (Broadbent and Steele, 2005).

Over the years, large-scale cheese production, the need for process and quality control, the application of a limited number of starter

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cultures exhibiting similar functional properties and the increased use of pasteurization processes intended to reduce the microbiological load of milk have resulted in a higher uniformity of many industrial cheeses (Wouters et al., 2002). In parallel to product consistency and quality, however, there is also a growing need for product diversification on the cheese market. To this end, the use of bacterial strains with attractive flavour-forming abilities is one of the possible strategies to answer to the increasing demand for cheeses with new or improved organoleptic properties. The much more diverse and heterogeneous microbiota and the more intense and stronger flavour of raw milk cheeses strongly suggest that NSLAB play a significant role during the ripening process and that their presence is beneficial for developing a full-flavoured cheese (Buchin et al., 1998; Grappin and Beuvier, 1997). Hence, raw milk cheeses can be an important source of adjunct cultures and the use of such adjuncts has already been investigated in several studies (Antonsson et al., 2003; Gómez-Ruiz et al., 2008; Lynch et al., 1996). On the other hand, the addition of adjunct cultures may also give rise to off-flavours or quality defects (Herreros et al., 2007; Lee et al., 1990), which highlights the relevance of careful screening of potential adjunct strains before their introduction in large-scale model fermentations.

The aim of the present study was to evaluate a method to screen for potentially interesting LAB adjunct strains by determining the impact of two selected *Lactobacillus paracasei* strains on the microbiological and volatile composition of a Gouda-type model cheese. The potential effects of the cheese making process on growth, viability and survival of the introduced adjunct strains and the influence of the adjunct on the cheese microbiota were assessed using conventional culturing, identification and typing and PCR-DGGE analysis of total cheese DNA extracts and DNA extracts obtained from culturable LAB fractions. In parallel, steam distillation–extraction and gas chromatography–mass spectrometry (SDE–GC–MS) were employed to detect possible differences in the sensory profile between the control cheese and the adjunct-containing cheeses.

2. Material and methods

2.1. Initial selection of potential adjunct cultures

In the course of a previous study, the microbial diversity associated with the production and ripening of two Flemish artisan raw milk Gouda-type cheeses produced at the same location was assessed during two independent sampling campaigns using selective isolations and polyphasic identification (Van Hoorde et al., 2008b). Dereplication of the initial set of 734 isolates by (GTG)₃-PCR fingerprinting resulted in a subset of 298 genotypically unique isolates. The majority of these isolates ($n=244$) grouped in 25 genotypic clusters, whereas the other 54 isolates remained unclustered and belonged to species representing less than 1% of the total number of isolates. From clusters that contained isolates recovered from both cheeses and/or recovered during both sampling campaigns, two to three isolates were selected for genotyping using amplified fragment length polymorphism (AFLP) analysis as previously described (Van Hoorde et al., 2008a). Based on their unique AFLP fingerprints, a set of 19 mesophilic strains comprising *L. paracasei* ($n=10$), *Pedococcus pentosaceus* ($n=4$) and single strains of *Lactobacillus brevis*, *Lactobacillus curvatus*, *Lactobacillus perolens*, *Lactobacillus rhamnosus* and *Lactobacillus plantarum* was used for further taxonomic characterization, antibiotic susceptibility testing and functional screening.

2.2. Antibiotic susceptibility testing

Selected strains were screened for phenotypic resistance to erythromycin and tetracycline, i.e. the two most commonly found resistance phenotypes in non-enterococcal LAB (Klare et al., 2007; Van Hoek et al., 2008), using a modified version of the Kirby–Bauer disk diffusion method (Bauer et al., 1966). For this purpose, strains

were grown on the LAB Susceptibility test Medium (LSM) agar formulation (Klare et al., 2005) consisting of 90% Boserstest broth (CM0473, OXOID), 10% MRS broth (CM0359, OXOID, Basingstoke, UK) and 10 g/L General Purpose Agar N°2, (LAB M, Lancashire, UK). Prior to testing, isolates were subcultured in 5 mL MRS broth (CM0359, OXOID) for 24 h at 28 °C under anaerobic conditions. Subsequently, 100 µL of the broth culture was inoculated on LSM agar and grown overnight. For preparation of the inoculum, colonies were suspended in sterile saline solution (0.85% w/v NaCl) until an OD₅₅₀ equivalent of McFarland (McF) standard 1 was obtained. LSM agar plates were inoculated with the standardized McF 1 inoculum and paper disks (OXOID) impregnated with erythromycin (15 µg; CT0020) and tetracycline (30 µg; CT0054) were applied to the inoculated plates. Following anaerobic incubation for 48 h at 28 °C, inhibition zone diameters were measured using a digital calliper (Mausier Digital 2, Ludwigsburg, Germany). Interpretation of results to discriminate between resistant and susceptible phenotypes was based on the guidelines proposed by Charteris et al. (1998).

Strains displaying tetracycline resistance in disk diffusion were subsequently subjected to MIC determination as described previously (Masco et al., 2006) using LSM broth as inoculation medium. Strains for which phenotypic tetracycline resistance was confirmed were screened by PCR for the presence of tetracycline resistance genes *tet(M)*, *tet(W)*, *tet(O)* and *tet(T)* using the PCR primers and conditions as described by Masco et al. (2006).

2.3. Assessment of proteolytic and lipolytic properties

Protease and lipase activities were assessed on two differential media, milk plate count agar (MPCA) (OM0681, OXOID) and tributyrin agar (TA) (PM0004, OXOID, Basingstoke, UK), respectively. Isolates to be tested for the presence of protease and/or lipase activity were first recovered from MicroBank™ vials by incubating them aerobically in MRS broth for 24–48 h at 28 °C, after which 10 µL of the grown culture was plated on MRS agar and again incubated for 24–48 h at 28 °C under aerobic conditions. Subsequently, MPCA and TA plates were inoculated in duplicate and incubated under aerobic conditions at 14 °C, the temperature of ripening, and 28 °C, the optimal growth temperature of both isolates. Conversion of casein by proteolytic activity on MPCA plates resulted in the formation of clear zones around the grown colonies. Lipolytic activity was recognized through the formation of clear halos around colonies on TA plates resulting from hydrolysis of the ester bonds in the triglyceride tributyrin and the subsequent release of butyric acid.

2.4. Preparation of adjunct cultures and manufacturing of model cheeses

Following recovery in MRS broth, subcultures of the two selected *L. paracasei* adjunct strains were transferred onto MRS agar and incubated aerobically at 28 °C. After 48 h, cells were harvested and suspended in sterile 0.85% w/v saline solution to obtain concentrations of 10^8 cfu/mL or 10^{10} cfu/mL. These suspensions were used to inoculate 100 L of cheese milk to obtain a final adjunct concentration of 10^3 or 10^5 cfu/mL.

Model Gouda-type cheeses were made in a pilot plant from pasteurized (15 s at 72 °C) milk using standard technology for the manufacturing of Gouda cheese (van den Berg, 2003). In total, four cheeses were produced on the same day from the same batch of pasteurized milk using the same basic ingredients to ensure that the respective cheeses only differed in the presence or absence of an adjunct culture. These four cheeses included a control cheese (i.e. G_C) without adjunct culture, a cheese with adjunct culture R-40937 in a final concentration of 10^3 cfu/mL (i.e. G₄₀₉₃₇), and two cheeses made with adjunct culture R-40926 in a final concentration of 10^3 and 10^5 cfu/mL (i.e. G₄₀₉₂₆₍₃₎ and G₄₀₉₂₆₍₅₎, respectively). Before adding starter and adjunct cultures, the temperature of the milk was brought to 30 °C.

3. Results

3.1. Selection of adjunct strains

From a set of 734 isolates collected and identified by the authors in a previous study (Van Hoorde et al., 2008a), 19 genotypically unique strains were selected as potential adjunct cultures. These included 10 *L. paracasei* and 4 *P. pentosaceus* strains and single strains of *L. brevis*, *L. curvatus*, *L. perolens*, *L. rhamnosus* and *L. plantarum*.

Antibiotic susceptibility testing by disk diffusion revealed that none of the 19 strains exhibited phenotypic resistance to erythromycin (i.e. all inhibition zone diameters ≥ 13 mm). In contrast, three strains, i.e. two strains of *P. pentosaceus* and the *L. curvatus* strain, displayed an inhibition zone diameter equal or smaller than 14 mm for tetracycline thus indicating phenotypic resistance to this antibiotic. This finding was subsequently confirmed by the MIC results (≥ 16 µg/ml, Klare et al., 2007) and by the presence of the corresponding *tet(M)* resistance gene. These three strains were omitted from further analysis.

The remaining 16 strains were assayed for the presence of proteolytic and lipolytic properties at 14 °C and 28 °C. None of the strains showed any proteolytic or lipolytic activity on MPCA or TA at 28 °C except for strain R-49026 belonging to *L. paracasei* which showed proteolytic activity at 28 °C and which was also the sole strain testing positive for lipase activity next to protease activity at 14 °C. Six other *L. paracasei* strains and one *L. perolens* strain exhibited protease activity only at 14 °C. Based on the above results, two strains were selected as adjunct culture for the manufacturing of model Gouda-type cheeses. *L. paracasei* R-49026 was selected because of its pronounced proteolytic and lipolytic properties. As second adjunct culture, *L. paracasei* R-40937 was selected on the basis of its proteolytic activity at 14 °C and because it was the only strain which was recovered from the raw milk and during ripening of the corresponding cheese batch produced from this milk (Van Hoorde et al., 2008a).

3.2. Viable counts of LAB

Four model Gouda-type cheeses with or without adjunct culture were manufactured from a single batch of pasteurized milk. Samples were taken from the curd (C), one-day old cheese (D1) and cheese ripened for 1, 3 and 5 week(s) and plated on the LAB-specific media

KAAA, LM17 and MSE. On all three media, but most explicitly on KAAA, lowest counts were observed for the control cheese G_C (Fig. 1). With respect to the cheeses supplemented with an adjunct culture, no pronounced differences in counts were observed except for the two cheeses supplemented with R-40926 that showed higher overall counts on KAAA medium compared to cheese G_C. As ripening progressed, all cheeses showed an overall decline in counts on LM17 and MSE. On KAAA, however, counts for the adjunct cheeses remained relatively stable (for G_40937) or even increased (for G_40926) during ripening as opposed to those for G_C which declined. Possibly, these observations can be attributed to the good growth of the respective adjuncts on this particular medium.

3.3. Identification of LAB isolates

To monitor the introduction, growth and survival of the adjunct strains in the model cheeses, a total of 1147 randomly selected isolates recovered during the various sampling points on three LAB-specific media were identified and typed using a polyphasic approach. First, the collection of 1147 isolates was dereplicated based on clustering analysis of (GTG)₅-PCR fingerprints of crude alkaline extracts. For this purpose, separate dendrograms were constructed for each subset of isolates recovered from a specific cheese at a specific sampling point. By selecting one isolate per (GTG)₅-cluster, the initial collection was reduced to 130 isolates which were subsequently subjected to a second round of DNA extraction now using a modified version of the method of Pitcher and co-workers (Masco et al., 2003; Pitcher et al., 1989). This method yields DNA extracts that are of superior quality compared to the crude alkaline extracts for generating (GTG)₅-PCR that need to be compared with the user-generated BioNumerics database for identification. Partial 16S rRNA and/or *pheS* gene sequencing was performed when isolates could not be unambiguously identified by (GTG)₅-PCR profiling. Results of this polyphasic identification approach, including the individual prevalence of the two adjunct strains throughout ripening based on their different (GTG)₅-PCR profiles, are shown in Table 1. In all cheeses, *L. lactis* subsp. *lactis* and *L. pseudomesenteroides* were retrieved throughout ripening. Most probably, they originated from the starter as the corresponding (GTG)₅-PCR profiles were highly similar to those of *L. lactis* subsp. *lactis* and *L. pseudomesenteroides* isolates obtained from the starter. These

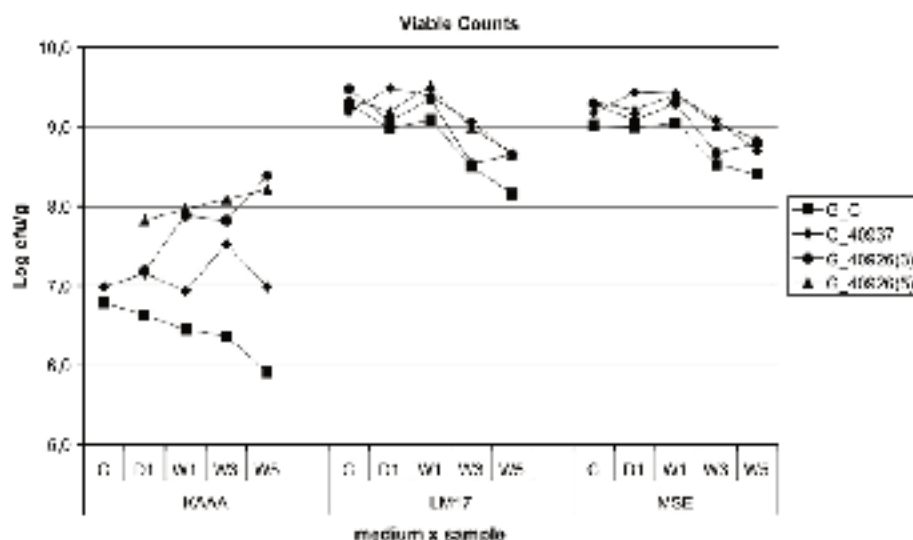


Fig. 1. Viable counts throughout production and ripening (curd (C), one-day old cheese (D1), one-week old cheese (W1) and cheese ripened for 3 and 5 weeks (W3 and W5, respectively) of the four cheeses (G_C, G_40937, G_40926(3) and G_40926(5)) as a function of the growth conditions (KAAA aerobic 37 °C, LM17 aerobic 37 °C and MSE aerobic 28 °C).

organisms were dominant in the curd and the early stages of ripening, but their number proportionally decreased as ripening progressed. In the curd and at each phase during ripening, higher numbers of the starter were retrieved in the control cheese compared to the cheeses supplemented with the adjunct strains. No representatives of the two adjunct strains or other strains of *L. paracasei* were retrieved in the control cheese. In cheeses G_40937, G_40926(3) and G_40926(5), all recovered *L. paracasei* isolates exclusively belonged to the corresponding adjunct cultures used and relative numbers of the adjunct strains increased with ripening. In addition to isolates assigned to the starter or adjunct used, also representatives of *L. curvatus*, *L. brevis* and *Enterococcus casseliflavus* were occasionally found in control and/or adjunct-containing cheeses. The latter two species were also recovered from the milk.

3.4. PCR-DGGE analysis of total cheese DNA and bulk fractions

Analysis of DGGE profiles generated from total DNA extracts by band position analysis and band sequencing indicated the presence of members of *L. lactis* and *L. pseudomesenteroides* as the predominant species in all four model cheeses. In cheeses G_40926(3) and in particular G_40926(5), the presence of *L. paracasei* was also demonstrated (Table 1). In addition to the aforementioned species, analysis of V3-16S rRNA DGGE profiles of bulk fractions also confirmed the presence of *E. casseliflavus*, members of the *Enterococcus faecium*/*Enterococcus durans* group, *Enterococcus faecalis* and *P. pentosaceus* in various samples of all cheeses. In the DGGE profiles of culturable fractions grown on LM17 medium, bands assigned to *Staphylococcus* spp. and gamma-Proteobacteria were also found. DGGE profiles generated from the KAAA and MSE fractions clearly demonstrated the presence of *L. paracasei* in the three adjunct cheeses but not in the control cheese (Table 1). Discriminant analysis of the composite dataset encompassing the qualitative and quantitative diversity data of cheese samples obtained by culturing and DGGE allowed to discriminate between control cheese G_C, adjunct cheese G_40937 and the two adjunct cheeses G_40926(3) and G_40926(5) (Fig. 2). Discriminative characters could mainly be attributed to the presence of one or more DGGE bands assigned to *L. paracasei* and the recovery of isolates representing the two adjunct strains.

3.5. GC-MS profiling

The volatile composition of the analyzed Gouda-type cheeses was mainly characterized by aroma compounds derived from lipolysis, proteolysis and metabolism of (residual) lactose, lactate and citrate. The main cheese volatiles could be classified as methylketones, volatile acids, lactones, alcohols, and sulfur compounds (Table 2). The same set of aroma compounds was also previously identified in Gouda-type cheeses (Van Leuven et al., 2008). In this work, some differences were obtained in the volatile composition of the cheese made with adjunct G_40926 compared to the volatile composition of the control cheese sample G_C. In addition, differences were also observed in the amount of aroma compounds originating from lipolysis and proteolysis between cheeses made with the same adjunct R_40926, but in a different final concentration (10^3 and 10^5 cfu/mL). Smaller differences in volatile composition between the control sample and the cheese G_40937 were observed. This was confirmed by the PCA biplot of the volatile fractions analyzed by SDE-GC-MS, which indicated that the control cheese and G_40937 largely exhibited a similar volatile profile. However, the latter two cheeses could clearly be differentiated from the two cheeses made with R_40926 at different concentrations (Fig. 3). These Gouda-type cheeses were clearly characterized by higher amounts of cheese volatiles. Cheese G_40926(3) contained higher levels of compounds derived from proteolysis (2-methylpropanoic acid, 3-methylbutanoic acid and 2-methylbutanoic acid), whereas cheese G_40926(5) was

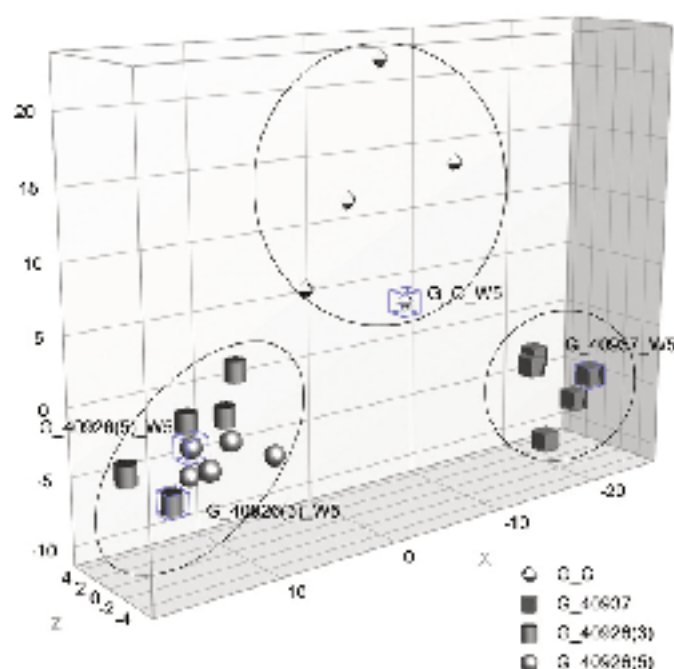


Fig. 2. Tri-dimensional discriminant analysis plot preserving the relative contributions of the three components, i.e. component 1 = X = 72.8%, component 2 = Y = 20.8% and component 3 = Z = 6.4%. Samples of 5-week old cheeses (WS) analyzed with SDE-GC-MS are indicated.

dominated by aroma components resulting from both lipolytic (tetradecanoic acid, hexadecanoic acid and lactones) and proteolytic (3-methylbutanal, 2-methylbutanal, 2-phenylethanol and methional) activities.

4. Discussion

Several criteria have been put forward for the selection of adjunct strains for use in cheese production. Adjuncts often originate from high-quality aged raw milk cheeses (Poveda et al., 2003; Randazzo et al., 2007), although also non-dairy environments such as sourdough, soil and grass are currently being explored as potential sources of interesting starters or adjuncts for cheese (Ayad et al., 1999; Di Cagno et al., 2006). Next, potential adjunct cultures should be able to reach and maintain high numbers during ripening without affecting the cheese manufacturing process. In addition, technological properties considered important for cheese making such as coagulation and acidifying activities (Randazzo et al., 2007), specific biochemical properties (Tungjaroenchai et al., 2001; van Hylckama Vlieg and Hugenoltz, 2007) and production of bacteriocins (Grattepanche et al., 2008) could be taken into account in the process of adjunct screening. Likewise, documentation of safety aspects such as the presence of acquired antibiotic resistance genes and/or potential virulence genes has gained importance in recent years during the selection of LAB strains for food and probiotic applications (Grattepanche et al., 2008). Finally, it is crucial that adjunct candidates should be taxonomically characterized up to the strain level to monitor their persistence and performance during the production of model cheeses.

For the present study, adjunct candidates were selected from an existing collection of 734 isolates previously obtained from two Flemish artisan raw milk Gouda-type cheeses (Van Hoorde et al., 2008a). An initial selection round, in which emphasis was put on strains that are well adapted to the cheese environment by selecting strain types prevalent in both cheese types and/or different production batches analyzed for a given cheese resulted in a total of 19 genotypically unique *Lactobacillus* and *Pedococcus* strains. Triggered by increasing concerns regarding the presence and transfer of antibiotic

Table 2
Volatiles of the control and adjunct cheeses as determined by SDE-GC-MS profiling.

Aromatic compounds ^{a,b,c}	Cheese			
	G_C	C_40973	C_40926(3)	C_40926(5)
Amino acids				
<i>Aldehydes (branched chain and aromatic)</i>				
3-Methylbutanal	9.83 ± 1.10	11.21 ± 1.43	22.50 ± 3.14	26.70 ± 0.76
2-Methylbutanal	1.10 ± 0.03	1.38 ± 0.22	1.99 ± 0.28	2.77 ± 0.02
Benzoaldehyde	16.09 ± 1.52	16.81 ± 2.96	15.34 ± 0.71	13.23 ± 0.00
Phenylacetaldehyde	36.03 ± 1.77	32.04 ± 1.74	38.81 ± 0.66	31.85 ± 0.27
Sum	63.04 ± 2.16	60.43 ± 0.01	78.63 ± 2.05	74.54 ± 0.47
<i>Sulfur compounds</i>				
Methional	3.14 ± 0.32	5.68 ± 1.15	4.35 ± 0.26	5.43 ± 0.03
<i>Branched-chain acids</i>				
2-Methylpropanoic acid			8.24 ± 0.35	
3-Methylbutanoic acid			215.60 ± 31.10	
2-Methylbutanoic acid			42.97 ± 7.04	
Sum			266.81 ± 37.79	
<i>Alcohols</i>				
1-Butanol	9.38 ± 0.94	0.94 ± 0.45	19.87 ± 1.51	21.88 ± 0.23
3-Methyl-1-butanol	3.94 ± 0.40	10.67 ± 0.01	35.15 ± 6.99	26.91 ± 1.27
2-Methyl-1-butanol	n.q.	n.q.	12.90 ± 3.10	11.44 ± 0.85
2-Phenylethanol	2.53 ± 0.56	13.41 ± 0.03	4.62 ± 0.01	11.40 ± 1.20
Sum	15.84 ± 1.90	34.04 ± 0.49	72.52 ± 11.61	71.63 ± 1.15
1H-Indole	6.93 ± 0.41	5.09 ± 0.64	4.57 ± 0.05	2.88 ± 0.11
Carbonyl compounds				
<i>Ketones</i>				
3-Hydroxy-2-butanone	653.68 ± 97.46	215.50 ± 3.18	546.67 ± 154.65	358.88 ± 79.01
2,3-Pentanedione	4.66 ± 0.45	8.08 ± 2.93	4.97 ± 0.05	4.88 ± 0.49
Sum	658.34 ± 97.91	223.58 ± 0.25	551.63 ± 154.71	363.76 ± 79.50
<i>Alcohols</i>				
2,3-Butanediol	23.09 ± 4.78	36.80 ± 7.25	21.44 ± 6.31	28.19 ± 7.22
Other				
Furfural	70.96 ± 3.32	52.85 ± 2.67	65.60 ± 4.41	54.43 ± 0.44
2-Pentylfuran	12.43 ± 0.04	6.51 ± 0.97	9.99 ± 1.46	7.06 ± 0.30
α -Pinene	1.58 ± 0.08	2.77	3.76 ± 0.18	2.83 ± 0.16
Limonene	6.84 ± 0.44	6.99 ± 0.37	7.48 ± 0.30	6.39 ± 0.35
9-Decene-2-on	29.80 ± 5.61	27.25 ± 0.48	27.65 ± 0.05	30.29 ± 1.97
Sum	121.60 ± 9.25	94.92 ± 6.44	114.47 ± 6.43	101.00 ± 1.75
Lipids				
<i>Linear aldehydes</i>				
Pentanal	3.88 ± 0.32	3.21 ± 0.22	3.89 ± 0.34	3.35 ± 0.02
Hexanal	25.18 ± 0.87	0.87 ± 2.02	24.15 ± 1.87	13.27 ± 2.09
Heptanal	5.77 ± 0.74	4.71 ± 0.64	6.18 ± 0.62	6.56 ± 0.95
Nonanal	35.11 ± 7.77	25.60 ± 3.06	35.55 ± 3.62	36.99 ± 4.51
Decanal	7.62 ± 1.82	3.15 ± 0.23	3.61 ± 0.54	3.93 ± 0.47
Dodecanal	33.49 ± 3.71	32.08 ± 3.34	27.28 ± 0.84	26.55 ± 1.24
Tetradecanal	149.36 ± 10.06	122.11 ± 20.32	113.88 ± 14.03	112.96 ± 0.72
<i>trans</i> -2,4-Decadienal	9.27 ± 0.95	8.26 ± 2.38	8.02 ± 0.64	7.41 ± 1.15
2-Octenal	3.54 ± 0.30	2.61 ± 0.21	3.34 ± 0.11	2.73 ± 0.27
2-Nonenal	6.27 ± 1.12	4.50 ± 0.93	5.75 ± 0.50	5.81 ± 0.99
2-Decenal	21.13 ± 3.49	n.q.	19.14 ± 2.97	15.91 ± 2.31
Sum	300.99 ± 29.41	219.25 ± 26.66	250.77 ± 11.96	235.45 ± 13.28
<i>Methyl ketones</i>				
2-Pentanone	628.41 ± 17.00	631.04 ± 0.18	692.72 ± 18.09	637.13 ± 44.80
2-Hexanone	37.64 ± 3.11	35.92 ± 0.10	40.36 ± 2.72	36.93 ± 3.89
2-Heptanone	2063.03 ± 108.22	2003.05 ± 16.18	2133.17 ± 40.60	2082.60 ± 107.32
2-Octanone	36.60 ± 2.64	29.49 ± 0.25	34.64 ± 4.62	31.36 ± 1.88
2-Nonanone	1701.42 ± 183.95	1580.55 ± 11.58	1653.32 ± 38.58	1675.30 ± 14.09
2-Decanone	32.51 ± 4.35	28.16 ± 0.83	30.65 ± 0.31	30.91 ± 1.55
2-Undecanone	1999.46 ± 327.36	1881.30 ± 21.86	1783.03 ± 29.90	2080.46 ± 7.50
2-Dodecanone	58.28 ± 5.81	40.09 ± 1.93	50.15 ± 3.54	62.33 ± 2.42
2-Tridecanone	2367.49 ± 431.61	2212.99 ± 147.85	1956.87 ± 139.95	2412.66 ± 43.92
2-Tetradecanone	59.68 ± 10.54	55.32 ± 5.28	49.50 ± 4.29	59.66 ± 0.60
2-Pentadecanone	2684.50 ± 395.20	2489.95 ± 319.45	2103.60 ± 313.50	2575.06 ± 108.14
Sum	11,689.01 ± 1479.51	10,996.44 ± 469.12	10,527.99 ± 386.87	11,584.38 ± 31.99
<i>Secondary alcohols</i>				
2-Butanol	11.98 ± 1.21	33.27 ± 0.59	12.65 ± 0.77	26.81 ± 3.30
2-Pentanol	10.82 ± 0.50	41.79 ± 7.32	33.14 ± 6.98	31.52 ± 2.72
2-Hexanol	5.71 ± 0.50	14.42 ± 0.48	13.62 ± 2.21	14.73 ± 1.72
Sum	28.50 ± 0.21	89.48 ± 7.20	59.41 ± 3.95	73.05 ± 2.29
<i>Volatile fatty acids</i>				
Butanoic acid	60.79 ± 24.37		235.01 ± 42.09	
Hexanoic acid	261.64 ± 14.57	1684 ± 4.16	528.25 ± 18.70	
Octanoic acid	914.58 ± 214.76	296.38 ± 119.93	1422.71 ± 71.40	65.70 ± 2.64
<i>Volatile fatty acids</i>				
Decanoic acid	5857.36 ± 1648.86	5168.54 ± 1039.81	6843.04 ± 113.21	3851.88 ± 199.92
Undecanoic acid	51.69 ± 16.10	51.57 ± 15.81	67.58 ± 0.01	47.06 ± 2.14

with the results from culture-independent analysis which clearly showed the presence of predominant bands assigned to members of the starter in the DGGE profiles of total cheese DNA extracts in all four cheeses. (GTG)₂-PCR profiling of isolates from cheeses G_40937 and G_40926(3)/(5) confirmed the presence of adjunct strains *L. paracasei* R-40926 and R-40937 in the cheeses in which they were respectively used. The observation that their relative numbers increased as ripening progressed indicates that both strains are well adapted to the cheese environment and can survive in a competitive environment in the presence of a commercial starter culture. DGGE analysis obtained from total DNA extracts of the adjunct-containing cheeses revealed the presence of *L. paracasei* in only a few samples, whereas DGGE profiles of the corresponding culturable fractions, especially those grown on KAAA and MSE, clearly demonstrated the presence of *L. paracasei*. Possibly, preferential amplification and differences in amplification efficiency (Suzuki and Giovannoni, 1996) or the possibility that some of the media used clearly favored growth of *L. paracasei* could explain these differences between culture-dependent and culture-independent approaches. Although overall differences in diversity were minimal, the control cheese could be differentiated from the adjunct-containing cheeses by discriminant analysis on the basis of the absence or presence of isolates and bands assigned to *L. paracasei*. Moreover, cheeses G_40937 and G_40926(3)/(5) could also be discriminated based on differences in the (GTG)₂-profiles of the corresponding *L. paracasei* adjunct isolates. For cheeses supplemented with strain R-40926, no concentration effect on the overall diversity could be demonstrated. To verify whether this is due to a normalization of both concentrations during cheese production or the absence of a true concentration effect, absolute numbers of the adjuncts in the cheeses would need to be determined e.g. by using species- or strain-specific primers in a real-time PCR assay.

In previous studies, the use of *L. paracasei* strains as adjuncts and its different effects on the volatile composition of the ripened cheese have been documented. For Danish Danbo cheeses, it has been shown that either beneficial effects or off-flavours were obtained depending on the *L. paracasei* adjunct strain added (Antonsson et al., 2003). Lynch et al. (1999) investigated the influence of amongst others *L. paracasei* subsp. *paracasei* on the ripening of Cheddar cheeses and observed an increase in flavour intensity and bitter flavour whereas the creaminess and milky flavour decreased. Probiotic cheeses made with *L. paracasei* were perceived significantly different from the probiotic free cheeses, with higher scores for bitterness (Org et al., 2007). In the present study, a descriptive sensory analysis of the four cheeses according to the method previously described by Van Leuven et al. (2008) revealed that the scores for the descriptor 'aroma intensity' increased in the cheese to which adjunct culture R-40926, irrespective of the concentration, was added (ANOVA: 0.05 < P < 0.10; data not shown). Furthermore, PCA of the volatile compounds could clearly differentiate cheeses made with adjunct R-40926, in particular G_40926(3), from the control cheese G_C which showed only small differences with the cheese made with adjunct R-40937. The cheese made with adjunct R-40926(3) was mainly characterized by volatiles derived from proteolysis (branched-chain volatile acids), while the cheese made with adjunct R-40926(5) was dominated by fat-related compounds such as free fatty acids and γ - and δ -lactones, of which the concentration generally correlates with the extend of lipolysis (Collins et al., 2003), next to some amino acid-related compounds (branched-chain aldehydes and sulfur compounds). These results are in line with the observed technological properties of R-40926 which showed next to proteolytic also lipolytic properties, whereas R-40937 only showed evidence of some proteolytic activity on milk plate count agar.

The use of adjunct cultures in the production of new types of artisan Gouda-type cheeses is a promising strategy to compensate for flavour uniformities due to a loss of biodiversity in the cheeses. In this study, we propose one possible approach to screen well-characterized NSLAB with clear predominance in raw milk cheeses for basic safety

and technological parameters with the aim of using a subset of selected cultures as adjuncts in model cheese production. Although outside the scope of this study, additional functional screening for the presence of specific flavour-forming enzymes and their metabolic products could further contribute to this selection process. Together with a better understanding of the population dynamics with respect to interactions of the introduced adjuncts with other bacteria predominating the cheese ecosystem, this strategy could pave the way to diversify the current range of Gouda-type and other semi-hard type cheeses by enriching or extending their flavour palette.

Acknowledgements

This research was supported by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen). The fund for Scientific Research-Flanders (FWO-Vlaanderen) is acknowledged for the postdoctoral fellowship of Geert Huys. We thank Willy Deville for his technical advice and assistance. We are also grateful to the cheese manufacturer workers for their cooperation, for the use of their equipment and for providing the raw material and samples used in this study.

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