Rapid volatile metabolite profiling of *Lactobacillus casei* strains: selection of flavour producing cultures

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Abstract

A technique based on an electronic nose combined with multivariate analysis was used to select bacterial strains of *Lactobacillus casei* as flavour producing adjunct cultures. Volatile metabolites were extracted from a suspension of bacterial cultures incubated with amino acids using inside-needle extraction and headspace sampling. The extracts were then injected into a mass spectrometry-based electronic nose. Mass fragmentation patterns from the unresolved volatile components were subjected to multivariate data analysis. Principal component analysis (PCA) allowed differentiation of bacterial strains. Based on these results, bacterial strains were selected as adjunct cultures for cheesemaking. After a six-month ripening period, cheeses were analysed by gas chromatography and found to contain higher amounts of aroma contributing components than control cheeses.

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Introduction

Micro-organisms play an important role in cheese manufacture and ripening. They can be divided into two groups: starters and secondary flora (Beresford *et al.* 2001). The secondary microflora includes non-starter lactic acid bacteria (NSLAB), which do not contribute to acid production during manufacture, but generally play a significant role during ripening. In recent years, there has been increased interest in the use of NSLAB as adjuncts that contribute to an improved flavour quality.

At the end of the ripening period, *Lactobacillus casei* strains present an important part of the NSLAB of swiss-type cheeses such as gruyère and emmental (Demarigny *et al.* 1996; Thierry *et al.* 1998; Casey *et al.* 2006). NSLAB have been shown to play an important role for increased levels of proteolysis and enhanced flavour development (Puchades *et al.* 1989; Broome *et al.* 1990; Lynch *et al.* 1996; Crow *et al.* 2001).

Since there is a large genetic variability among the *Lactobacillus casei* strains found in gruyère cheese (Casey *et al.* 2006), the identification of flavour-producing strains is crucial for the selection of a desirable NSLAB population.

Recently, novel types of 'electronic noses' based on mass spectrometry have been developed. In contrast to classical mass spectrometer techniques, volatile compounds are analysed directly without separation (e.g. gas chromatography). Data are then analysed by multivariate data analysis. The advantages of electronic noses over the classic GC are the simplicity of use and higher speed. The greatest disadvantage is that the compounds present cannot be analytically identified.

The goal of this study was two-fold. First, to use an MS-based electronic nose to assess the ability of genetically different *Lactobacillus casei* strains to produce volatile compounds. Second, to select lactobacilli-producing volatiles as adjunct cultures for cheese manufacture.

Materials and methods

Microbial strains and chemicals

Lactobacillus casei strains from the Agroscope Liebefeld-Posieux collection were used in this study. They were cultured anaerobically in 10 mL of MRS (de Man *et al.* 1960) at 30°C.

Chemicals were purchased from Merck (VWR International AG, Dietikon, Switzerland) and Sigma-Aldrich (Buchs, Switzerland).

Species and strain identification

Species were identified by sequencing the 16S rRNA gene and comparing the DNA sequence obtained to those at the NCBI database. Strains were grouped into genotypes by a repetitive sequence based polymerase chain reaction (REP-PCR) that was performed on DNA extracts using the primer GTG5 (5'-GTGGTGGTGGTGGTG-3') as described previously (Versalovic *et al.* 1994).

Preparation of samples for electronic nose analysis

Three hundred and fifty millilitres of liquid MRS were inoculated with 350 µl of a bacterial suspension and incubated for 30 h at 30°C aerobically. Cells were harvested by centrifugation (4000 g, RT, 15 min), washed twice with 50 mM β-glycerophosphate (pH 7.5) and finally re-suspended in 6 mL 50 mM β-glycerophosphate (pH 7.5). The cultures were incubated as follows: the reaction mixture contained 0.5 mL concentrated biomass, 70 mM potassium phosphate (pH 5.5), 2 mM or 10 mM L-amino acid,

50 µM pyridoxal-5-phosphate and 1x RPMI 1640 Vitamins Solution (Sigma-Aldrich) in a final volume of 5 mL. To determine the influence of an amino acceptor, 10 mM 2-ketoglutarate was added. Incubation was performed in a 10 mL glass flask sealed with a grey butyl/PTFE septum without stirring for 7 d at 30°C.

Headspace analysis of volatile compounds by an electronic nose

SMart Nose (SMart Nose, Marin-Epagnier, Switzerland) is an electronic nose equipped with a quadrupole mass spectrometer QMS 422 (Inficon AG, Balzers, Liechtenstein), a programmable Combi PAL auto sampler (CTC Analytics AG, Zwingen, Switzerland) with a fully automatic capacity for 96 vials of 10 mL capacity and multivariate analysis software (SMart Nose 1.51) for data acquisition.

Before analysis, approximately 2.5 g solid NaCl were added to each sample and held at 10°C. Before headspace analysis, samples were transferred to a heated mixer and mixed at 60°C for 10 min. Two sampling techniques were used for headspace analyses. Either volatile compounds were pre-concentrated by inside-needle dynamic extraction (INDEx) or the headspace was transferred directly.

As regards INDEx pre-concentration, extraction was carried out using a modified 2.5 mL Hamilton syringe, where the needle was packed with permethylated cyclodextrin silicon derivative (SMart Nose, Marin-Epagnier, Switzerland). The needle temperature was 110°C. After sample incubation, the needle was inserted and 2.5 mL headspace was aspirated and ejected 15 times at a speed of 150 µL/s. After adsorption, the syringe was back-flushed 1 min with dry nitrogen to remove excess condensed water. Then, 70 µl of air were collected. Afterwards the needle was inserted into the injector set at 200°C, collected air was injected at 3 µL/s and the needle was left in the injector for an additional 2 min. The quadrupole mass spectrometer was used under EI ionisation (70 eV) in scanning mode. Three scanning cycles from 10 to 120 amu were performed at 0.5 amu/s. Then, the INDEx needle was cleaned in the injector under nitrogen flux for another 6 min while performing aspiration/ejection motions. The total analysis time per sample was about 24 min, taking into account the incubation time.

As regards headspace sampling, a fraction of the headspace (2.5 mL) was transferred into the mass spectrometer (injector temperature = 150°C). The quadrupole mass spectrometer was used under EI ionisation (70 eV) in multiple ion detection mode (MID). The MID scanning mode was performed with a set of 15 channels (ionic masses m/z = 40, 46, 48, 51, 57, 58, 60, 62, 64, 70, 71, 79, 86, 91, 94, 105) at a scanning rate of 2 s per m/z. All channels were scanned six times (six cycles per injection) after a delay of 20 s. The chamber was continuously purged with nitrogen except during measurement. The needle was back-flushed 1 min. The automatic sampler made it possible to inject the next sample immediately after the 1 min purge. So if the incubation time of the first two samples was not taken into account, the total analysis time per sample was 4 min and 32 s.

The mass intensity lists thus generated were processed by the data pattern recognition software of SMart Nose. The list was then transferred to The Unscrambler 9.5 software (CAMO Software AS, Oslo, Norway) for further data analysis.

Cheese manufacture

Model gruyère-type cheeses were produced from 120 L of pasteurised milk. After adding 120 mL each of fresh and an old starter culture, *Lactobacillus casei* strains were added to a final concentration of approximately 10^3 cfu/g milk. Coagulation occurred at 32°C after 40 min. After cutting and stirring at 32°C, the curd-whey mixture was scalded to 56°C for 30 min and the stirred at 56°C for 20 min. Then curd was filled into moulds. Brining was followed by ripening in the cold room (14-15°C, 90-96% relative humidity) for 10 d, then in a warm room (16-17°C, 90-96% relative humidity) for 50 d, and finally again at 14-15°C (90-96% relative humidity) for 120 d.

Purge & Trap gas chromatography – mass spectrometry

The cheeses (5 g) were suspended in distilled water (20 mL) and 10 g of the suspension were extracted by dynamic headspace (Purge & Trap) using a Tekmar 3100 instrument equipped with a tenax trap No. 1 (Tekmar Instruments, Cincinnati, OH, US). GC-MS was conducted using a SPB-1 sulphur capillary column (Supelco, 30 m x 0.32 mm x 4 µm film thickness) with helium as carrier gas (55 kPa) applying the following temperature program: 45°C (13 min) to 240°C at 5°C/min. Mass spectra were obtained in the EI ionisation mode at 70 eV and at a scan range from m/z = 26-250.

Results and discussion

Lactobacillus casei is the predominant species found in gruyère-type cheese after a ripening period of six months (Casey *et al.* 2006), so it is very likely that this species plays an important role in the development of cheese aroma compounds. Whereas information on flavour production by *Lactococcus* is increasingly available (Yvon and Rijnen 2001), data on metabolic pathways leading to volatile compound production by lactobacilli are much more limited. It is quite clear that amino acids, such as methionine, the branched-chain and aromatic amino acids are the most important sources for flavour production by lactic acid bacteria (Smit *et al.* 2005).

We used an electronic nose to study the ability of *Lb. casei* from the Agroscope Liebefeld-Posieux collection to produce volatile flavour compounds *in vivo*. We selected three genetically different *Lb. casei* strains (Figure 1). Each strain was incubated with leucine, methionine or phenylalanine in the absence or presence of 2-ketoglutarate, which is often used as an amino acceptor for the initial transamination reaction. We used the INDEx technique to concentrate the volatile compounds of the bacterial headspace. To identify background compounds, we analysed the headspace of water and a bacterial suspension in buffer. In the water headspace we found ions at m/z = 73, 77, 78, 89, 96, 103, 115 and 119 (data not shown). We concluded that they originated from the INDEx material and might be hexamethylcyclotrisiloxane, octamethylcyclotetrasiloxane, decamethylcyclopentasiloxane and fluorotrimethylsilane. The headspace of bacterial suspensions had high signal intensities at m/z = 51, 76 and 93. All these ions were excluded from data analysis.

Concerning the headspace measurements of bacteria incubated with amino acids, we first compared the spectra individually and finally performed a PCA (Figure 2). We found that none of the

bacterial strains produced volatile compounds during the incubation with methionine or phenylalanine. However, volatiles were produced after adding 2-ketoglutarate to the buffer. All three strains incubated on methionine then showed higher signal intensities at m/z = 47, 48, 64, 79 and 94. Incubation with phenylalanine and 2-ketoglutarate produced ions at m/z = 49, 50, 52, 53, 63, 65, 74, 75, 105 and 106. The PCA plot also showed that FAM 3228 incubated with leucine and 2-ketoglutarate produced volatiles with the masses 53, 54, 55, 56, 58, 59, 70 and 72. Only a closer examination of the spectra itself revealed that FAM 3228 and FAM 8407 also produced volatiles in the absence of 2-ketoglutarate. Additionally, ions at m/z = 61, 69 and 85 were found in the reaction mixtures containing bacteria and leucine and ions at m/z = 91 and 92 in the mixtures with bacteria, phenylalanine and 2-ketoglutarate.

We concluded that there are two metabolic pathways in *Lb. casei* that produce volatile components from amino acids. On the one hand, amino acid catabolism is initiated by the action of aminotransferases as was shown for *Lactococcus* (Yvon *et al.* 1997) and several *Lactobacillus* species (Amarita *et al.* 2001; Martinez-Cuesta *et al.* 2001; Thage *et al.* 2004a; Thage *et al.* 2004b). On the other hand, there are bacterial strains such as FAM 8407 and FAM 3228 that are able to catabolise leucine by a metabolic pathway independent of an amino acceptor.

For the interpretation of the spectra, we consulted the NIST Chemistry WebBook (http://webbook.nist.gov/chemistry/). We concluded that *Lb. casei* could catabolise leucine to 3-methylbutanal and 3-methylbutanol, methionine to methional and dimethyldisulphide and phenylalanine to benzaldehyde, phenylacetaldehyde and/or 2-phenylethanol. Many of these compounds have been described as key odorants of different cheese varieties (Rychlik and Bosset 2001; Curioni and Bosset 2002). These findings and the representative masses for aroma compounds are summarised in Table 1.

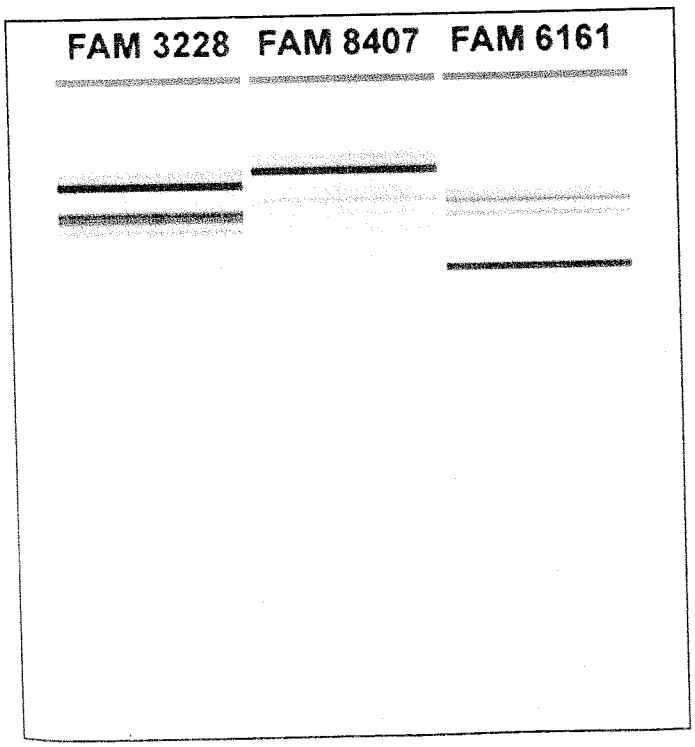

Figure 1: REP-PCR patterns from different *Lb. casei* genotypes.

In order to assess the transfer of these results to the cheese matrix, we selected, as adjunct cultures, FAM 8407 and FAM 3228 as strong producers and FAM 6161 as a weak producer of volatile compounds from leucine. Hard cheeses were prepared by adding *Lb. casei* cultures together with the starter culture to pasteurised milk. Cheese manufacture was repeated three times. After six months ripening it was found that bacteria of the adjunct cultures grew from 10^3 cfu/g to 10^8 cfu/g. Cheeses were then analysed by GC-MS. It was found that the cheeses with the *Lb. casei*

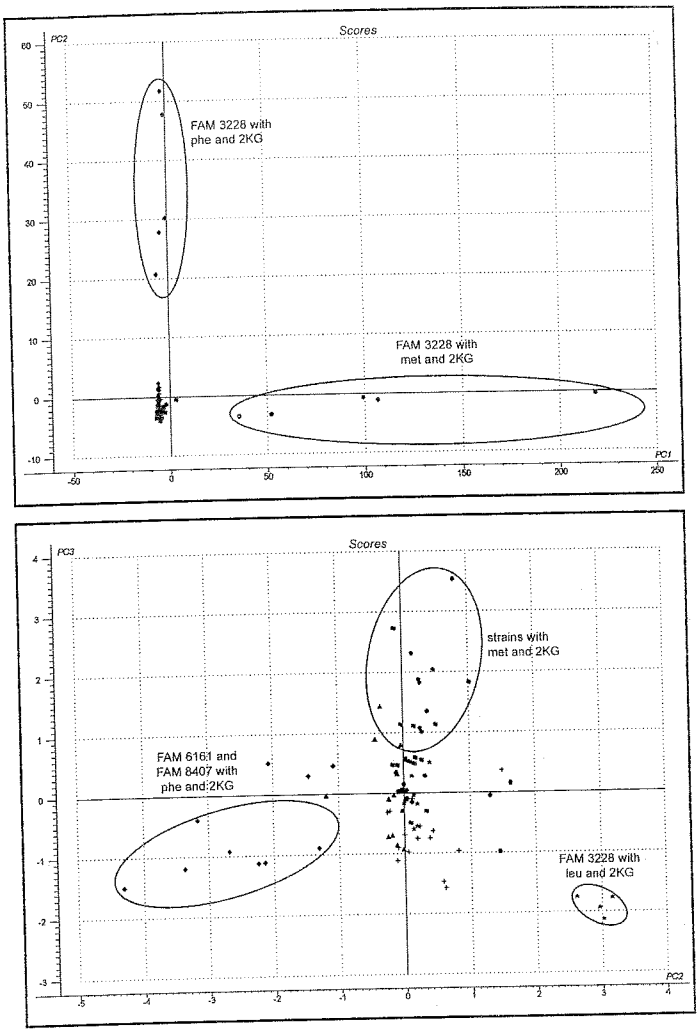

Figure 2:. Volatile compounds produced by the strains FAM 8407, FAM 3228 and FAM 6161 were preconcentrated by INDEx sampling. The figure illustrates principal component analysis of the signal intensities measured by scanning from 47 to 120 amu and the exclusion of masses 51, 73, 76, 77, 78, 89, 93, 96, 103, 115, and 119.

A. PC 1 accounted for 90% and PC2 for 9% of the variance. The first principal component is positively correlated with the masses 47, 48, 64, 79, and 94, the second principal component is positively correlated with the masses 49, 50, 52, 53, 63, 65, 74, 75, 105 and 106.

B. PCA was repeated after excluding the samples with FAM 3228 incubated on methionine and 2-ketoglutarate and incubated with phenylalanine and 2-ketoglutarate. PC2 and PC3 were plotted to show clustering more clearly. PC2 accounted for 17%, PC3 accounted for 14% of the variance.
(met: methionine, phe: phenylalanine, leu: leucine, 2KG: 2-ketoglutarate)

Table 1: Summary of the most intense ions found in the headspace of *Lb. casei* strains by using INDEx headspace sampling. Ions in brackets could not be explained. leu: leucine, met: methionine, phe: phenylalanine, 2KG: 2-ketoglutarate.

Substrate	FAM 6161	FAM 8407	FAM 3228	Ions (m/z)	Putative compounds
leu	-	+	+	53, 54, 55, 56, 57, 58, (61), (69), 70, 71, (72), (85)	3-methylbutanal 3-methylbutanol
leu + 2KG	+	+	+	53, 54, 55, 56, 57, 58, (61), (69), 70, 71, (72), (85)	3-methylbutanal 3-methylbutanol
met	–	–	–	–	–
met + 2KG	(+)	(+)	+	47, 48, 64, 79, 94	methanethiol dimethyldisulphide
phe	–	–	–	–	–
phe + 2KG	+	+	+	49, 50, 52, 53, 63, 65 74, 75, 91, 92, 105, 106	benzaldehyde phenylacetaldehyde 2-phenylethanol

FAM 8407 and FAM 3228 showed significantly higher signals for 3-methylbutanal//3-methylbutanol, 2-methylbutanal/ 2-methylbutanol and 2-methylpropanal/2-methylpropanol (Figure 3). The latter compounds are certainly derived from isoleucine and valine, respectively, and may be catabolised by the same metabolic pathway as leucine (McSweeney 2004).

Thus, it was clear that the electronic nose could be used as a screening tool for the identification of flavour-producing bacteria. We decided to screen 81 *Lb. casei* strains that were found to be genetically different by REP-PCR (data not shown). Since we found that the needle openings of the INDEx needle often got clogged by small particles, which hampered gas flow, and that this kind of measurement took quite a long time, we changed to headspace sampling. Headspace sampling has the advantage of being simple, reliable and a fast way to measure volatile compounds in liquid and solid matrices. In addition, we used a mixture of amino acids with slightly increased concentrations and added citrate as a potential substrate for production of diacetyl. We selected ion mass fragments that were representative for 3-methylbutanal, 3-methybutanol, dimethyldisulphide, benzaldehyde and diacetyl. Since we observed no production of diacetyl (representative ion at m/z = 86), this ion mass was used for normalisation of the data before PCA. Data were then standardised to ensure that they were expressed in comparable units. Figure 4 shows the resulting PCA plot. Bacterial strains could clearly be grouped according to the production of dimethyldisulphide (PC1) and benzaldehyde (PC2). PC3 accounted for 10% of the variance and correlated positively with the masses 70, 71 and 91 (data not shown). We interpretated PC1, 2 and 3 as indicators of the catabolism of methionine, phenylalanine and leucine, respectively. Furthermore, we concluded that there are strains which could metabolise phenylalanine and methionine in the absence of an amino acceptor. Currently, we are investigating these catabolic pathways which do not necessitate a transaminaton step. Additionally, these cultures are being used as adjunct cultures to study their influence on cheese ripening.

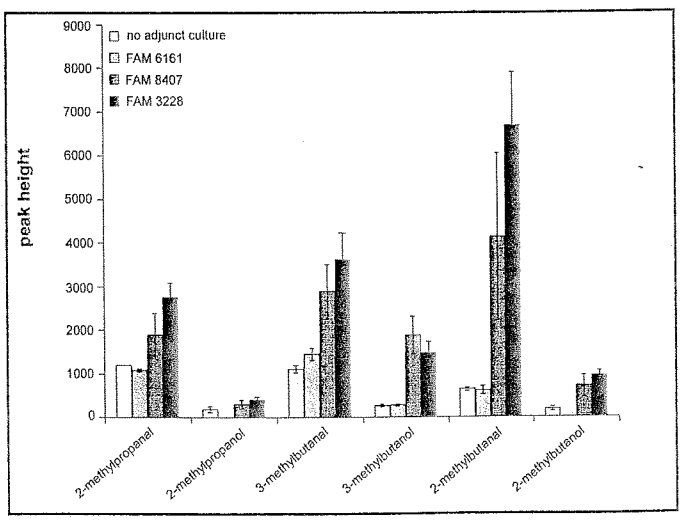

Figure 3: Volatile compounds found in hard cooked cheese after six months of ripening and identified by Purge & Trap gas chromatography. The error bars indicate standard deviation of cheeses manufactured on three different days.

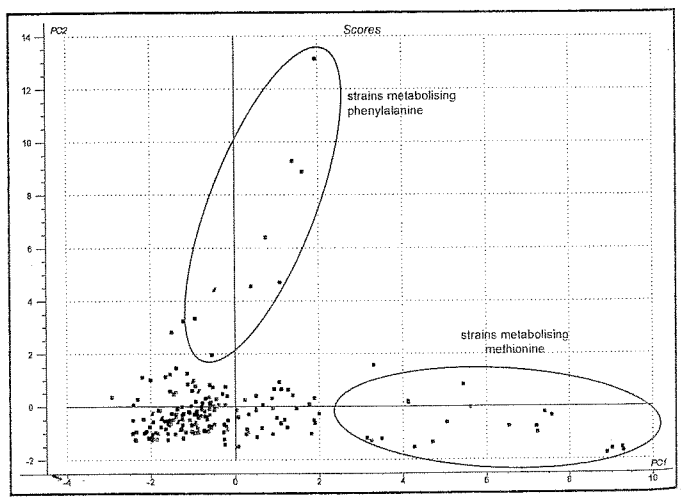

Figure 4: Screening of 81 *Lb. casei* strains for the production of volatile components originating from amino acids. Principal component analysis of signal intensities measured using headspace sampling. PC1 accounted for 41% and PC2 for 22% of the variance. PC1 indicated that bacteria produced dimethyldisulphide (positive correlation with ion masses 62, 64, 79 and 94 amu), PC2 indicated that bacteria produced benzaldehyde (positive correlation with ion masses 51 and 105).

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