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Short communication

Characterization of plant-derived lactococci on the basis of their volatile compounds profile when grown in milk

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ABSTRACT

A total of twelve strains of lactococci were isolated from grass and vegetables (baby corn and fresh green peas). Ten of the isolates were classified as *Lactococcus lactis* subsp. *lactis* and two as *Lactococcus lactis* subsp. *cremoris* based on 16S rDNA sequencing. Most of the plant-derived strains were capable of metabolising a wide range of carbohydrates in that they fermented D-mannitol, amygdalin, potassium gluconate, L-arabinose, D-xylose, sucrose and gentibiose. None of the dairy control strains (i.e. *L. lactis* subsp. *cremoris* HP, *L. lactis* subsp. *lactis* IL1403 and *Lactococcus lactis* 303) were able to utilize any of these carbohydrates. The technological potential of the isolates as flavour-producing lactococci was evaluated by analysing their growth in milk and their ability to produce volatile compounds using solid phase micro-extraction of the headspace coupled to gas chromatography–mass spectrometry (SPME GC–MS). Principal component analysis (PCA) of the volatile compounds clearly separated the dairy strains from the plant derived strains, with higher levels of most flavour rich compounds. The flavour compounds produced by the plant isolates among others included; fatty acids such as 2- and 3-methylbutanoic acids, and hexanoic acid, several esters (e.g. butyl acetate and ethyl butanoate) and ketones (e.g. acetoin, diacetyl and 2-heptanone), all of which have been associated with desirable and more mature flavours in cheese. As such the production of a larger number of volatile compounds is a distinguishing feature of plant-derived lactococci and might be a desirable trait for the production of dairy products with enhanced flavour and/or aroma.

1. Introduction

Lactic acid bacteria (LAB) have been used by mankind for centuries for the production of a variety of dairy-based fermented products. *Lactococcus lactis*, in particular, is a primary constituent of many starter cultures used for the manufacture of cheese, fermented milk, sour cream, and lactic casein (Ward et al., 2002; Klijn et al., 1995). Based on early investigations, there has been a strong belief that the cow and the milking equipment have been the main source for *Lactococcus* spp. in raw milk (Sandine et al., 1972). However, a number of studies have reported the isolation of *Lactococcus* spp. from sources other than raw milk. These studies have reported the isolation of strains of *Lactococcus* from various plant materials including fermented vegetables, minimally processed fresh fruits, vegetables, sprouted seeds, silage and other plants (Collins et al., 1983; Gutiérrez-Méndez et al., 2010; Kelly et al., 1998, 2000, 2010; Kimoto et al., 2004; Klijn et al., 1995; Noruma et al., 2006; Procópio et al., 2009; Salama et al., 1995; Siezen et al., 2008, 2010; Schultz and Breznak, 1978). *L. lactis* has also been isolated from soil (Klijn et al., 1995) and termite hindguts (Bauer et al., 2000).

Previous reports have also indicated that some lactococcal isolates of plant origin have exhibited technological characteristics such as; (1) flavour forming activity of key flavour compounds from amino acids that might be beneficial to the dairy industry (Smit et al., 2004, 2005; Tanous et al., 2002), (2) production of antimicrobial peptides or bacteriocins which generally kill or inhibit the growth of other closely related or unrelated bacterial strains and show potential use as food preservatives and pharmaceuticals (Cai et al., 1997; Kelly et al., 1998, 2000) and (3) displaying probiotic properties such as growth in the presence of 0.3% bile and removal of cholesterol during growth in vitro, a potential for use as probiotic strains (Kimoto et al., 2004).

Recent genomic analysis studies on plant derived strains of lactococci have confirmed the presence of gene clusters that code for the degradation of complex plant polymers such as arabinan, xylan, glucans and fructans and the uptake and conversion of plant cell wall degradation products such as α-galactosides β-glucosides, arabinose, xylose, galacturonate, glucuronate and gluconate as plant-derived energy sources (Siezen et al., 2010, 2011).

This report describes the isolation, identification and characterization of ten strains of *Lactococcus lactis* subsp. *lactis* and two strains of

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Lactococcus lactis subsp. *cremoris* isolated from plants: grass, baby corn and fresh green peas. These strains were clearly distinguishable from dairy starter strains based on the diversity of volatile compounds they produced when grown in milk.

2. Materials and methods

2.1. Bacterial strains and culture conditions

L. lactis subsp. *lactis* strains IL1403, and 303 and *L. lactis* subsp. *cremoris* strain HP were obtained from MFRC collection (Teagasc Food Research centre, Moorepark). Twenty grass varieties obtained from Moorepark animal feed study plots (Supplementary Table, ST1) and vegetables (fresh green peas, baby corn, broccoli and cucumber) obtained from local grocery stores were used as sources for the isolation of lactococcal strains. Cultures were grown in M17 broth supplemented with 0.5% of either glucose or lactose (as required) and incubated overnight at 30 °C. *Lactococcus* isolates were grown at different conditions (8 °C, 45 °C, in the presence of 4.0% NaCl, 6.5% NaCl and at pH 9.5) for up to seven days. Carbohydrate metabolism profiling was performed using API 50 CH kit (bioMérieux, Etoile, France). Growth of the isolates in milk was examined by culturing in 10% RSM (reconstituted skim milk) with or without glucose (0.5%) supplementation and incubation was at 30 °C for up to 5 days. Data presented are averages of three independent experiments.

2.2. Isolation and identification of Lactococcus strains

Grass or vegetable samples (10–15 g) were mixed with 100 ml sterile phosphate buffer (10 mM, pH 7.0) in a sampling plastic bag and mixed in a stomacher for 1 min. Serial decimal dilutions were made and 100 μl of the diluted sample was spread plated on GM17 agar plates. Plates were incubated anaerobically at 30 °C overnight and individual colonies were screened for catalase activity. Isolates identified as Gram positive cocci (appearing as diplococci and/or in chains) were transferred onto GM17 agar and incubated aerobically at 30 °C for 48 h. This serves to exclude strict anaerobic cocci from the study. One hundred and thirty nine isolates which were able to grow in both aerobic and anaerobic conditions were stored at 4 °C and subcultured once more before experimental use. Colony PCR was performed on these isolates using *L. lactis* species specific primers. To distinguish between subsp. *lactis* and subsp. *cremoris* strains a second PCR was performed using subspecies-specific primers (Table 1). All primers and PCR conditions were performed according to Pu et al. (2002). The complete 16S rDNA gene of the isolates identified as *L. lactis* was amplified using primers 27-F and 1492-R (Table 1) and PCR products were sequenced (Beckman Coulter Genomics, Essex, UK). DNA sequences were compared to those in the gene bank reference RNA sequence database (http://blast.ncbi.nlm.nih.gov/Blast/).

2.3. Plasmid profile analysis, and Pulsed Field Gel Electrophoresis (PFGE)

Plasmid profile analysis of the isolates was performed using the rapid mini-prep method of O'Sullivan and Klaenhammer (1993) and plasmid DNA was separated on 0.7% agarose gel. PFGE was performed according to Simpson et al. (2002) after restriction digestion of DNA was performed overnight in a restriction buffer containing 25 U of *Sma*I and an incubation temperature of 25 °C.

2.4. Volatile analysis of fermented milk

The volatile profiles produced by milk as well as dairy and plant lactococci isolates following overnight growth in 10% RSM supplemented with 0.5% glucose were assessed by solid phase micro-extraction of the headspace coupled to gas chromatography–mass spectrometry (SPME GC–MS). For volatile analysis, 5 ml of each culture following growth in 10% RSM was added to a 20 ml SPME vial (Apex Scientific Ltd., Maynooth, Co., Kildare, Ireland) and equilibrated to 40 °C for 5 min with pulsed agitation of 4 s at 250 rpm. Sample introduction was accomplished using a CTC Analytics CombiPal Autosampler (Agilent). A single 1 cm × 50/30 μm StableFlex divinylbenzene/Carboxen/polydimethylsiloxane (DVD/Carboxen/PDMS) fibre was used for all analysis (Supelco, Bellefonte, PA, USA). The SPME fibre was exposed to the headspace above the samples for 20 min at depth of 1 cm. The fibre was retracted and injected into the GC inlet at 250 °C and desorbed for 2 min. Splitless injections were made on a Varian 450 GC (Varian Analytical Instruments, Harbour City, California, USA) with a Zebron ZB-5msi (60 m × 0.25 mm ID × 0.25 μm) column (Phenomenex, Macclesfield, Cheshire, UK). Volatile compounds were separated under the following conditions: carrier gas: helium 1 ml min^{-1}, initial column temperature was −60 °C held for 2 min, heated to 20 °C at 50 °C min^{-1}, followed by heating to 110 °C at 4 °C min^{-1}, heating to 250 °C at 20 °C min^{-1} and finally holding for 5 min. The detector used was a Varian 320 triple quad mass spectrometer (Varian Analytical Instruments, Harbour City, California, USA) operating in the scan mode within a mass range of m/z 30–350 amu at 2.5 scans s^{-1}. Ionisation was performed by electron impact at 70 eV; calibration was performed by auto-tuning. Individual compounds were identified using mass spectral comparisons to the NIST 2005 mass spectral library. Individual compounds were assigned quantification and qualifier ions to ensure that only the individual compounds were identified and quantified. Quantification was performed by integrating the peak areas of the extracted ions using the Varian MS workstation, version 6.9.2 (Varian Analytical Instruments, Harbour City, California, USA). The results presented are the averages of two independent analyses.

3. Results and discussion

3.1. Isolation of lactococci

In this study, 12 lactococcal strains were isolated from grass and vegetables based on 16S rDNA sequencing (Table 1). Ten of the isolates belonged to *L. lactis* subsp. *lactis* and two belonged to *L. lactis* subsp. *cremoris*. Six of the subsp. *lactis* strains were isolated from fresh green peas, three from grass and one from sweet corn, and the two subsp. *cremoris* strains were isolated from grass (Table 2 and ST1). The 16S rDNA sequence blast analysis results were consistent with those obtained using subspecies specific primers.

The plant derived lactococci isolates displayed a very broad adaptation like high salt (6.5%) and alkaline conditions (pH 9.5) (data not shown), which indicate that the strains are more suited to harsh

Table 1
Oligonucleotide primers used for PCR.

Primer designation	Target organism/gene	16s rRNA seq. target region	Sequence (5′–3′)
1RL	All *Lactococcus* sp.	1–19	TTTGAGAGTTTGATCCTGG
LacreR	*L. lactis*	219–238	GGGATCATCTTTGAGTGAT
LacF	*L. lactis* subsp. *lactis*	76–94	GTACTTGTACCGACTGGAT
CreF	*L. lactis* subsp. *cremoris*	76–94	GTGCTTGCACCGATTTGAA
27-F	16s rDNA gene	27–46	AGAGTTTGATCCTGGCTCAG
1492-R	16s rDNA gene	1470–1492	TACGGCTACCTTGTTACGACTT

Primer designs were according to Pu et al. (2002).

Table 2
Acidification of growth medium by plant derived *Lactococcus* isolates during growth in M17 supplemented with 0.5% glucose or lactose and in milk (10%RSM) with or without added glucose (0.5%). Data shown are averages of three independent fermentation experiments.

Lactococcus plant isolate	Source	pH 24 h culture		Coagulation of 10% RSM (pH)				Coagulation 10% RSM + 0.5% glucose (pH)	
		GM17 medium	LM17 medium	18 h	48 h	96 h	120 h	18 h	48 h
L. lactis subsp. *lactis* 144-S	Grass (AberDart)[a]	5.64	5.75	−	+(4.78)	NA	NA	−	+(4.48)
L. lactis subsp. *lactis* 144-L	Grass (AberDart)[a]	5.52	5.54	−	+(4.69)	NA	NA	−	+(4.60)
L. lactis subsp. *lactis* P-3	Green pea	5.58	6.06	−	+(4.75)	NA	NA	−	+(4.42)
L. lactis subsp. *lactis* P-5	Green pea	5.59	5.67	−	+(4.75)	NA	NA	−	+(4.58)
L. lactis subsp. *lactis* P-8	Green pea	5.59	5.70	−	+(4.80)	NA	NA	−	+(4.53)
L. lactis subsp. *lactis* P-21	Green pea	5.59	5.51	−	+(4.77)	NA	NA	−	+(4.58)
L. lactis subsp. *lactis* P-27	Green pea	5.58	5.85	−	+(4.6)	NA	NA	−	+(4.50)
L. lactis subsp. *lactis* P-29	Green pea	5.61	5.71	−	+(4.73)	NA	NA	−	+(4.52)
L. lactis subsp. *lactis* C-3	Baby corn	5.62	5.65	+(4.42)	NA	NA	NA	+(4.34)	N/A
L. lactis subsp. *lactis* M23-6	Grass (Greengold)[a]	5.62	5.64	+(4.56)	NA	NA	NA	+(4.57)	N/A
L. lactis subsp. *cremoris* M16-10	Grass (Dunluce)[a]	5.60	6.32	−	−	−	−(5.21)	+(4.52)	N/A
L. lactis subsp. *cremoris* M23-10	Grass (Greengold)[a]	5.66	6.47	−	−	−	−(5.10)	+(4.57)	N/A

Coagulation; +, positive; −, negative; NA, not applicable. pH of RSM at T 0 = 6.2 and M17 medium T 0 = 6.5.
[a] Grass variety.

environmental conditions in comparison to the dairy strains. It is believed that such adaptations are advantageous to the plant isolates where they are continuously exposed to unpredictable and harsh environmental conditions.

3.2. Utilization of carbohydrates, growth in milk and Pulsed Field Gel Electrophoresis

Most of the plant derived strains fermented D-mannitol, amygdaline, potassium gluconate, L-arabinose, D-xylose, sucrose and gentibiose, but none of the dairy control strains were able to utilize any of these carbohydrates (data not shown) A recent genomic analysis on plant derived *Lactococcus* confirmed the presence of gene clusters that code for the degradation of complex plant polymers such as arabinan, xylan, glucans and fructans and for the uptake and conversion of the plant cell wall degradation products, such as α-galactosides β-glucosides, arabinose, xylose, galacturonate, glucuronate and gluconate as plant derived energy sources (Siezen et al., 2010, 2011).

All subsp. *lactis* isolates were able to coagulate milk (10% RSM) although at different rates (18 to 48 h). The two subsp. *cremoris* strains (M23-10 and M16-10) failed to coagulate milk even after extended incubation of 7 days. However, these *cremoris* strains fermented milk in less than 18 h when the culture was supplemented with 0.5% glucose (Table 2). The results show that the two *cremoris* strains have a functioning proteolytic system but compromised lactose utilization ability. A similar result has been reported recently by Gutiérrez-Méndez et al. (2010) where plant *L. lactis* isolates showed the slowest growth rates and yield when lactose was used as energy source, compared to those obtained when glucose was used as carbohydrate source.

In ten of the plant lactococci isolates no plasmid was detected. One plasmid each was detected in two of the subsp. *lactis* isolates (P-21 and C-3) with size ranging between 80 and 90 kb (data not shown). Previous studies have clearly established that the majority of strains of dairy *L. lactis* depend on plasmids for lactose utilization, casein degradation, citrate utilization, bacteriocin production, bacteriophage resistance and slime formation (LeBlanc et al., 1980; McKay, 1983; Teuber, 1995; Mills et al., 2006).

Based on the PFGE DNA finger-printing data, the isolates could be identified as 10 distinct strains (Fig. 1). Two grass isolates 144-L and 144-S showed identical restriction pattern and thus assumed to be the same strain. The other two fresh green pea isolates, P-5 and P-8 also showed identical restriction patterns and growth characteristics and were assumed to be the same strain (Fig. 1).

3.3. Volatile flavour compounds

The formation of volatile flavour compounds in dairy products is a complex process resulting from glycolysis, lipolysis and proteolysis of milk components which is mediated by the enzymatic mechanisms of the microflora contained within the product (McSweeney and Sousa, 2000, Smit et al., 2004). However, the production of flavour compounds in fermented dairy products is strain dependent and therefore the composition of the starter mix can greatly influence the flavour profile (Kieronczyk et al., 2003). The volatile compounds produced by the plant lactococci isolates during milk fermentation were compared to that of the control strains. Supplementary Table 2 (ST2) lists the peak areas of 44 compounds identified in all of the samples (7 esters, 7 acids and fatty acids, 8 aldehydes, 8 ketones, 7 alcohols and 6 sulphur compounds and a diol compound). These compounds have frequently been reported to be present in cheddar cheese (Singh et al., 2003). The production of these and other flavour compounds result from diverse and very important biochemical reactions desired during the manufacturing and ripening of cheese (Fox et al., 1995). The perception of flavour is due to the balance in the type and concentrations of the

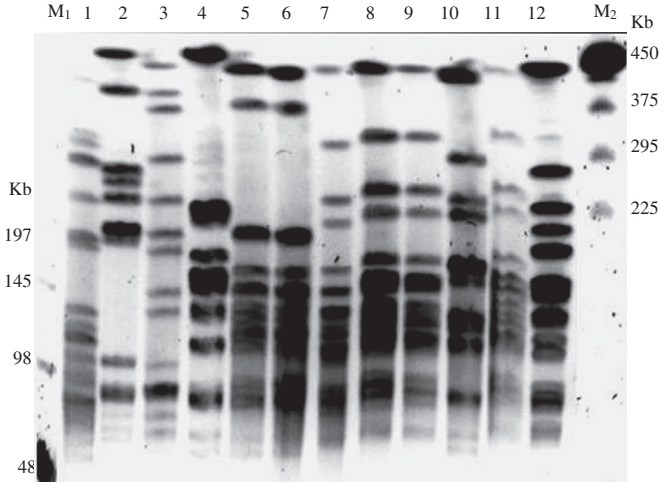

Fig. 1. PFGE restriction pattern of genomic DNA of *L. lactis* plant isolates. Genomic DNA in agarose block was digested with *Sma*I. Lanes 1 to 12 show DNA fingerprints of *L. lactis* C-3, M16-10, M23-10, M23-6, 144-L, 144-S, P-3, P-5, P-8, P-21, P-27 and P-29 respectively. M_1 and M_2 are low and high range molecular weight markers respectively. PFGE conditions were 6v, Pulse times 1–20 s for 22 h.

compounds present in the volatile profile of each strain (Bosset and Gauch, 1993). Hence, to interpret how the volatile profiles discriminated between the strains, PCA was performed and the results are presented in Fig. 2 principal component analyses. The first two PC's explained 49% of the variation. Fig. 2a shows the scores of the samples and Fig. 2b shows the loadings of the compounds. Three main groups were identified (Fig. 2a): group 1 contained the uninoculated milk sample, group 2 consisted of the control strains IL1403, HP and 303, and group 3 consisted of all the plant lactococci isolates. PCA analysis of the volatile profiles also clearly separated the control dairy strains from the plant lactococci isolates. This latter group was associated with a higher level of most of the volatile compounds detected suggesting the ability of the plant isolates to produce a wider variety of compounds (Fig. 2b). For example, the non-dairy strains produced higher levels of the branched chain aldehydes (2- and 3-methyl butanal and 2-methyl propanal) and their corresponding alcohols (2-and 3-methyl butanol and 2-methyl-1-propanol) which are the degradation products of leucine, isoleucine and valine respectively, and suggest that the non-dairy strains have enhanced amino acid catabolic abilities. In addition, higher levels of ethanol (and hence esters), diacetyl, acetoin and 2,3 butanediol were produced by the non-dairy strains. In dairy strains, these compounds are generally produced through citrate metabolism (McSweeney and Sousa, 2000). The broad ability of the plant lactococci isolates to utilize citrate is thus suggesting the higher potential that exists in these isolates to produce flavour compounds. The plant lactococci isolates were also associated with higher levels of sulphur

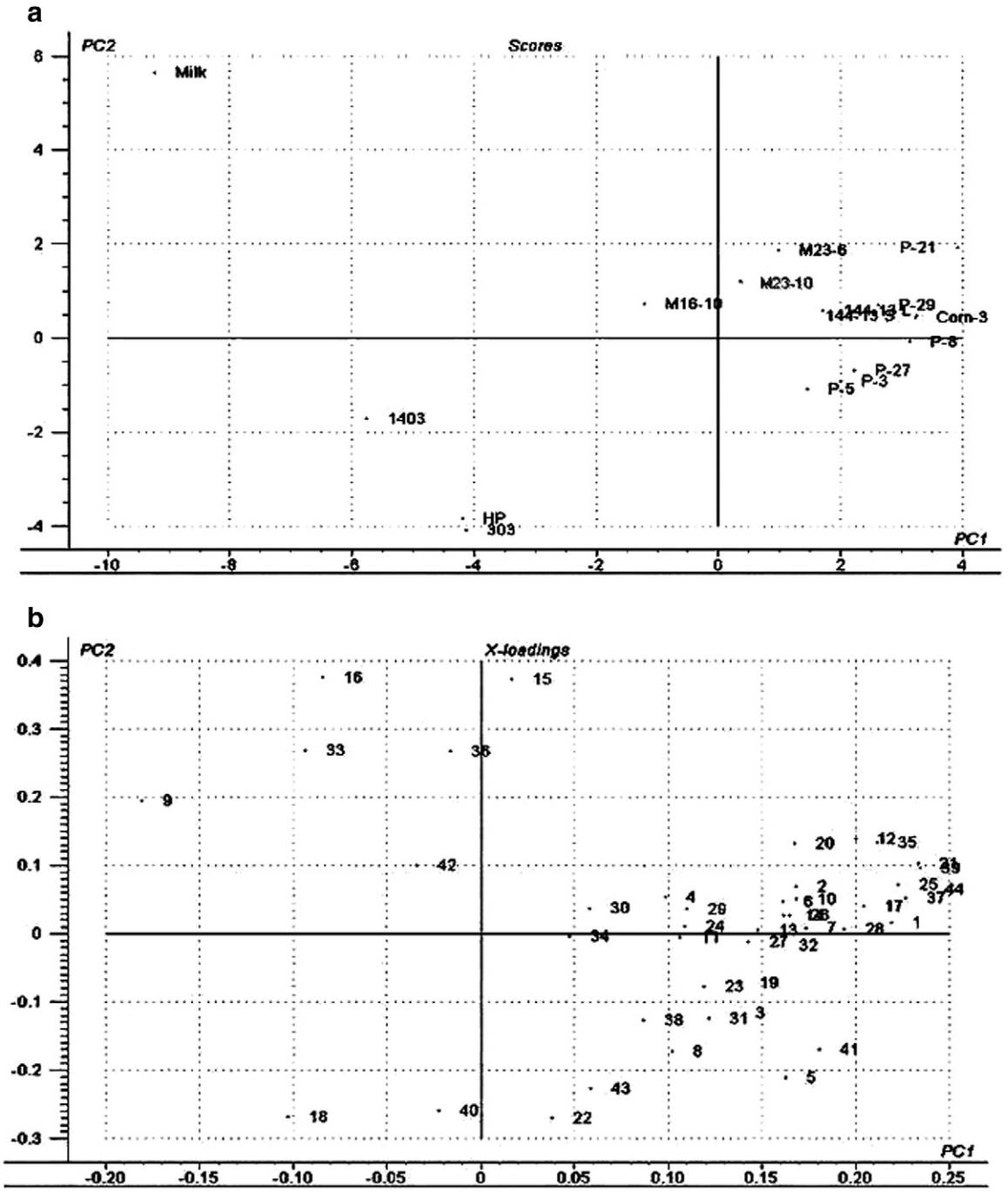

Fig. 2. Plots obtained from principal component analysis: (a) score plot of samples and (b) loadings of volatile compounds. Numbers of the volatile compounds correspond to numbers on ST2. Results presented are the averages of two independent analyses.

containing compounds (methanethiol, carbon disulphide, DMS, DMDS, DMTS and dimethyl-sulfone), suggesting an enhanced ability of these isolates to metabolise sulphur containing amino acids. The plant isolates were also associated with the fatty acid 2 and 3 methyl butanoic acids, several esters like butyl acetate and ethyl butanoate and ketones like acetoin, diacetyl, and 2-heptanone, all of which have been associated with desirable and more mature flavour in cheeses (for review see Singh et al., 2003). Almost all the plant isolates displayed increased production of the 23 (over 50%) volatile compounds detected in fermented milk compared to that of the dairy strains (part of Table ST2). The ability of the plant isolates to produce a more varied and diverse profile compared to the dairy isolates highlights the potential that these strains have to develop higher levels of a broader range of volatile compounds which could be used in dairy products to mask off flavours, create novel flavour profiles or enhance the development and reduce the time taken to develop the flavour of dairy products.

4. Conclusions

This study demonstrated that the plant-derived lactococci have an efficient ability to form high levels of a broad range of important volatile compounds associated with improved flavour in dairy products. The diverse abilities of the plant isolates to metabolise different substrates in milk and their ability to produce distinct flavours suggest their potential as starter adjuncts for the production of dairy products with more varied flavour characteristics and also their potential to be used as components in starter blends to create novel flavoured products or enhance the development and reduce the time taken to develop the flavour of dairy products. Nevertheless, much more analysis of the properties of these strains would be necessary before addition of these strains to starter blends was possible The study highlights the potential of volatile compounds based screening for the identification of plant lactococci isolates, which produce a wide range of volatile compounds associated with flavour, and suggests that a wider screening of strains using these techniques could be very fruitful for the isolation of novel cultures for the dairy industry.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2013.11.024.

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