International Journal of Food Microbiology 172 (2014) 57–61

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Short communication

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

Debebe Alemayehu [a,b], John A. Hannon [a], Olivia McAuliffe [a], R. Paul Ross [a,b,*]

[a] Teagasc Food Research Centre, Moorepark, Cork, Ireland
[b] Alimentary Pharmabiotic Centre, University College Cork, Ireland

ARTICLE INFO

Article history:
Received 11 July 2013
Received in revised form 13 November 2013
Accepted 24 November 2013
Available online 3 December 2013

Keywords:
Lactococcus lactis
Sugar metabolism
Plasmid
PCR
PFGE
Gas chromatography–mass spectrometry (GC–MS)

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

* Corresponding author at: Teagasc Food Research Centre, Moorepark, Fermoy, Co., Cork, Ireland. Tel.: +353 25 42229; fax: +353 25 42340.
E-mail address: paul.ross@teagasc.ie (R.P. Ross).

0168-1605/$ – see front matter © 2013 The Authors. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijfoodmicro.2013.11.024

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).