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Assessment of wild non-dairy lactococcal strains for flavour diversification in a mini-Gouda type cheese model

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ABSTRACT

Wild lactococci possess enhanced metabolic capabilities in comparison to industrial dairy strains, including increased amino acid-converting enzyme activities. A bank of *Lactococcus lactis* strains isolated from different non-dairy environments exhibited wider carbohydrate fermentation profiles in comparison to dairy lactococcal strains. In addition, these non-dairy lactococci had the ability to ferment lactose and produce diverse aroma profiles when grown in milk. Based on volatile analysis, five of these non-dairy strains were selected and investigated as adjuncts to diversify cheese flavour using a mini-Gouda cheesemaking process model and compared to a cheese manufactured with a commercial adjunct. In total, 8 different cheeses were evaluated in duplicate and ripened for 14 days at 12 °C, followed by 8 °C for 84 days thereafter. Physicochemical analysis of cheeses was performed at day 14 and sensory evaluation at day 84. Viable counts, intracellular enzyme activity and indices of proteolysis were monitored over ripening. The ability of the non-dairy strains to survive, lyse, and release intracellular enzymes and alter proteolysis was strain dependent. Some strains performed as well as the commercial adjunct in terms of secondary proteolysis although others were associated with bitterness and development of off-flavours and off-aromas. Attenuation of DPC6853 positively reduced its association with bitterness during ripening. It is evident that non-dairy strains have potential as adjuncts in semi-hard type cheeses, and could be harnessed to diversify flavour profiles in semi-hard cheese varieties.

Introduction

Flavour is a prime factor for consumer selection of cheeses, such as Gouda and Cheddar (Ross, Stanton, Hill, F Fitzgerald, & Coffey, 2000). The formation of cheese flavour arises from a series of chemical, biochemical and microbiological processes involving the breakdown of lactose (glycolysis), fat (lipolysis) and protein (proteolysis) (McSweeney & Sousa, 2000). For the generation of cheese aroma in semi-hard cheeses, it is widely agreed that the proteolytic system and amino acid-converting enzymes possessed by the starter cultures are of pivotal importance (Smit, Smit, & Engels, 2005). *Lactococcus lactis* strains are the most widely used starter cultures in the production of cheese. A few select strains are predominantly used as primary starters due to the technological attributes that they possess (Marshall, 1991). Although this has reduced inconsistencies in quality, it has resulted in a reduction in the diversity of flavour amongst commercial cheeses. A secondary starter or adjunct culture is often added to impart specific properties and generate characteristic flavour profiles associated with specific cheeses (Johnson, 2001).

While *L. lactis* is normally associated with dairy fermentations, the so-called 'wild strains' of this species can be isolated from raw milk and non-dairy environments (Centeno, Tomillo, Fernández-García, Gaya, & Nuñez, 2002; Nomura, Kobayashi, Narita, Kimoto-Nira, & Okamoto, 2006). Genome sequence analysis has revealed non-dairy lactococcal strains to be more metabolically diverse than dairy cultures (van Kranenburg et al., 2002). For example, *Lactococcus* strains from the wider environment have been shown to possess more active amino acid-converting enzymes than industrial dairy cultures and are auxotrophic for less amino acids than their dairy counterparts (Ayad, Verheul, de Jong, Wouters, & Smit, 1999). In lactococcal starter cultures, the formation of aroma compounds from amino acids begins with transamination and the conversion of an amino acid to its corresponding α-keto acid (Gao & Steele, 1998; Rijnen et al., 1999). This reaction is limited by the amount of α-ketoglutarate produced by the microorganism which acts as an amino group acceptor (Tanous, Kieronczyk, Helinck, Chambellon, & Yvon, 2002). Some studies have investigated the potential of lactic acid bacteria (LAB) strains from raw milk, raw milk cheeses and non-dairy niches to create cheeses of more diverse or intense flavour (Ayad et al., 1999; Centeno et al., 2002). Sensory analysis of cheeses made using wild strains has resulted in the use of atypical sensory descriptors (Morales, Fernández-García, Gaya, & Nuñez, 2003). Furthermore co-cultures of wild strains, leading to the completion of

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particular flavour formation pathways, may further diversify flavour profiles (Ayad, Verheul, Engels, Wouters, & Smit, 2001).

The aim of this work was to evaluate the flavour-forming ability of selected non-dairy lactococci as adjunct cultures in cheese. A bank of non-dairy strains was assessed for important technological characteristics including growth in milk, tolerance to salt and temperature, proteolytic activity and the ability to ferment different carbohydrates. In addition, the generation of volatile compounds in milk was also assessed for each strain. Non-dairy strains which formed diverse aroma profiles in milk were selected for use as adjunct cultures in the production of mini-Gouda type cheese. The resultant cheeses were assessed for physicochemical, microbial, biochemical, and sensory characteristics and compared to control cheeses produced using a commercially available adjunct derived from *Lactobacillus helveticus*.

Materials and methods

Bacterial strains and growth conditions

Lactococcus adjuncts used in this study were isolated from non-dairy sources. *L. lactis* ssp. *cremoris* strains DPC6854, DPC6857, DPC6858, DPC6859, DPC6860 and DPC6855 were isolated from grass samples sourced at the Animal and Grassland Research and Innovation Centre (Teagasc, Fermoy, Ireland). Strain DPC6853 was isolated from corn sourced at a local supermarket, while strain DPC6856 was isolated from a sample of bovine rumen content. Direct vat inoculation (DVI) cultures used in this study, CHOOZIT™ Classic 111 (a mix of 7 strains of *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*) and CHOOZIT™ Flav54 (a single Lactobacillus *helveticus* strain), were supplied by Du Pont-Danisco (Dangé St. Romain, France). Non-commercial lactococcal strains were cultured in M17 media (Oxoid, Hampshire, England) containing 5 g/L lactose monohydrate (L-M17) (VWR, Leuven, Belgium). *Lb. helveticus* cultures were enumerated on MRS (Oxoid, Hampshire, England) media adjusted to pH 5.3 (mMRS). Prior to the production of mini-Gouda type cheeses, whole cultures were grown in sterile (121 °C for 5 min) 10% reconstituted semi-skimmed milk (RSM) (Kerry Foods, Ireland) to 1×10^8 CFU/mL, pelletized in liquid nitrogen and stored at −80 °C. Non-dairy cultures underwent minimal subculturing in milk to maintain their natural metabolic characteristics.

L. lactis ssp. *lactis* DPC6853 was attenuated using a high pressure microfluidiser to increase the percentage of permeabilised or lysed cells within the population (Yarlagadda, Wilkinson, O'Sullivan, & Kilcawley, 2014). This was achieved by growing the strain overnight in M17 media containing 0.5 g/L lactose to 1×10^8 CFU/mL and passing 500 mL of culture through a Z chamber at 25,000 psi using a ME110EH Microfluidiser (Microfluidics, Newton, MA, USA). The attenuated cells were pelletized in liquid nitrogen and stored at −80 °C until required.

Technological characterisation of strains

Lactococcus cultures were tested for their ability to grow at 8–10 °C, 30 °C and 42 °C for 5 days in M17 broth (Oxoid) containing 0.5% lactose (VWR, Belgium). Growth at pH 9.2 and pH 9.6 and in the presence of 4 g/100 mL and 6.5 g/100 mL NaCl (Sigma Aldrich) was examined for 5 days at 30 °C in the same media. Casein breakdown was examined using skim-milk agar plates containing 10% RSM (Kerry Foods) and 1.5 g/100 mL bacteriological agar. The ability to ferment different carbohydrate substrates was assessed using the API 50 CH system (bioMérieux, Montalieu-Vercieu, France) according to the manufacturer's instructions. Carbohydrate fermentation was examined at 24 and 48 h.

Following overnight growth of individual strains in 10% RSM, 0.5 mL cultures were added to individual 10 mL of RSM and incubated for 24 h at 30 °C. The production of volatile compounds in milk was assessed using a headspace (HS) solid phase micro-extraction (SPME) method as described by Yarlagadda, Wilkinson, Ryan, et al. (2014). For analysis the detector was set in electron ionisation mode at a mass/charge (m/z) range between 35 and 350 Da. Mass spectra for compounds were compared to the NIST 2005 spectra library to identify individual compounds and from an internally generated compound list. Compounds were each assigned a distinct quantification ion to ensure accuracy in identification and quantification. Compounds were semi-quantified by calculating the area under each individual peak. An auto-tune was performed at the start of each sample run to ensure that conditions remained constant for each run. All analyses were carried out in duplicate and data was analysed using Unscrambler Software, version 9.7 (CAMO ASA, Trondheim, Norway).

Mini-cheese production

Sixteen mini-Gouda type cheeses were manufactured with pasteurised milk using the vat in place (VIP) mini-cheese system at Du Pont-Danisco (Dangé St. Romain, France) with and without the addition of adjunct cultures. Cheeses were produced under conditions designed to minimise the growth of non-starter lactic acid bacteria (NSLAB) which may impact the flavour profile of the cheese. All cheeses were made using Classic 111 as a starter culture. A control cheese containing the starter only was produced and each non-dairy adjunct strain was also compared to a commercial adjunct, Flav54. Experimental and control cheeses were made from the same batch of milk in duplicate over two days. Milk was sourced from a local co-operative and pasteurised before production. For cheesemaking, 2 L of milk was preheated at 32 °C and inoculated with the starter Classic 111 (Du Pont-Danisco, France) at 7 direct culture units (DCU) and non-dairy adjunct cultures were added at 35 g of pelleted frozen culture/L. Flav54 adjunct cultures were added at 10 g/L. Carlina™ calf chymosin (Du Pont-Danisco, France) was added (4 mL of 200 g/L) 1 h after starter/adjunct addition at pH ~6.55. The coagulum was allowed to form for ~40 min. The curd was cut and stirred for 5 min followed by whey removal (700 mL) and addition of 600 mL of distilled water at 32 °C. Each vat was maintained at 36 °C for 20 min with constant stirring. Cheeses were pre-pressed for 30 min under a 1 kg weight in a circular mould. Samples were transferred for final pressing at 36 °C under 50 kPa. Cheese pH was monitored at constant intervals until a pH of 5.3 was reached when cheeses were placed in 150 mL of brine solution 300 g/L NaCl containing 0.5 g/L Natamax™ anti-mould agent (Du Pont-Danisco, France) for 1 h. Finally, cheeses (200 g) were allowed to dry at room temperature for 10 min before vacuum packing. Cheeses were ripened at 12 °C for 14 days and samples taken with cheeses were subsequently ripened at 8 °C thereafter until 84 days. Cheese sampling and analysis were performed at days 14, 42, and 84.

Cheese physicochemical and microbiological analyses

Cheese physicochemical analysis was performed at day 14. The moisture, NaCl, fat, pH and protein were determined as described elsewhere (Hickey, Kilcawley, Beresford, Sheehan, & Wilkinson, 2007). Analysis of the microbiological content of cheeses was carried out in duplicate at each sampling time. Cheese samples were mixed with tri-sodium citrate (2% wt./vol.) at a 1:10 (wt./vol.) dilution and homogenised for 5 min using an IUL stomacher (Lennox Lab. Supplies Ltd., Dublin, Ireland). Starter culture cell counts were enumerated on M17 agar (Oxoid, Hampshire, England) containing 0.5% wt./vol. lactose monohydrate (VWR, Leuven, Belgium) after 3 days of incubation at 30 °C. NSLAB were enumerated on LBS agar (Difco, Detroit, Michigan, USA) after 5 days of incubation at 37 °C under anaerobic conditions.

Cheese extract preparation

A homogenised cheese extract was prepared at each sampling point to determine post-proline aminopeptidase (PEPX), aminopeptidase N (PEPN) and lactate dehydrogenase (LDH) activities. To generate an