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The genetic basis underlying variation in production of the flavour compound diacetyl by *Lactobacillus rhamnosus* strains in milk

Raquel Lo[a,b], Van Thi Thuy Ho[a,b], Nidhi Bansal[a,b], Mark S. Turner[a,b,c,*]

[a] The ARC Dairy Innovation Hub, The University of Queensland, St Lucia, QLD, Australia
[b] School of Agriculture and Food Sciences, The University of Queensland, St Lucia, QLD, Australia
[c] Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, QLD, Australia

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ABSTRACT

Diacetyl and the closely related compound acetoin impart desirable buttery flavour and odour to many foods including cheese and are generated through the metabolism of citrate by lactic acid bacteria (LAB). To increase the levels of these compounds, adjunct cultures capable of producing them can be added to cheese fermentations. In this study, we compared the diacetyl and acetoin producing abilities of 13 *Lactobacillus rhamnosus* strains from cheese sources. Diacetyl and acetoin production was found to be a common feature of *Lb. rhamnosus* grown in milk, with 12 strains producing these compounds. Whole genome sequencing of four strains revealed that genes encoding the citrate metabolising pathway present in other LAB are conserved in *Lb. rhamnosus*. One strain was, however, totally defective in diacetyl and acetoin production. This was likely due to an inability to produce the diacetyl/acetoin precursor compound acetolactate resulting from a frameshift mutation in the acetolactate synthase (*als*) gene. Complementation of this defective strain with a complete *als* gene from a diacetyl producing strain restored production of diacetyl and acetoin to levels equivalent to naturally high producing strains. Introduction of the same *als*-containing plasmid into the probiotic *Lb. rhamnosus* strain GG also increased diacetyl and acetoin levels. In model cheesemaking experiments, the *als*-complemented strain produced very high levels of diacetyl and acetoin over 35 days of ripening. These findings identify the genetic basis for natural variation in production of a key cheese flavour compound in *Lb. rhamnosus* strains.

1. Introduction

Diacetyl (2,3-butanedione) and the closely related acetoin (3-hydroxy-2-butanone) impart a desirable buttery aroma and both are key odorants in cheese, including Cheddar (Clark and Winter, 2015; Curioni and Bosset, 2002). Diacetyl is present only in small amounts in cheese, ranging from 0.02 to 13.68 ppm depending on the cheese type, while acetoin is generally present at concentrations 10–50-fold higher than those of diacetyl (Clark and Winter, 2015; McSweeney and Sousa, 2000). Diacetyl is generally regarded as more important for buttery flavour due to its low odour threshold (Smit et al., 2005). By comparison, the odour of acetoin is 100-fold weaker (Le Bars and Yvon, 2008). Diacetyl at 1.5–5 ppm is sufficient to impart a buttery flavour (Hemme, 2012).

Diacetyl is formed nonenzymatically in an oxidative reaction from acetolactate, which originates from pyruvate. Although pyruvate is a key metabolite that can be derived from sugars, diacetyl production is most often associated with citrate metabolism. *Lactococcus lactis* subsp. *lactis* biovar diacetylactis, *Leuconostoc* spp., *Lactobacillus paracasei* and *Lactobacillus rhamnosus* are among the few lactic acid bacteria (LAB) species that produce diacetyl from citrate (Díaz-Muñiz et al., 2006; Hugenholtz, 1993; Jyoti et al., 2003). The citrate to diacetyl/acetoin pathway has been extensively studied in *L. lactis* subsp. *lactis* biovar diacetylactis (García-Quintáns et al., 1998; García-Quintáns et al., 2008; Martín et al., 2004) and *Enterococcus faecalis* (Blancato et al., 2008; Repizo et al., 2013) and to some extent in *Lactobacillus casei* (Díaz-Muñiz et al., 2006; Mortera et al., 2013).

Diacetyl producing strains can be added into cheese fermentations as so called non-starter LAB or adjunct cultures to boost buttery flavour and odour in cheese. A diacetyl producing strain of *Lb. casei* used as an adjunct resulted in significantly higher concentrations of diacetyl and acetoin, and a significantly more buttery flavour as perceived in sensory analysis (Milesi et al., 2010). A high diacetyl producing strain of *L. lactis* subsp. *lactis* biovar diacetylactis was used to produce fresh Cheddar cheese curds, and diacetyl content was found to increase rapidly during manufacturing and storage (St-Gelais et al., 2009). In low fat Cheddar with added adjuncts (*L. lactis* subsp. *lactis* biovar diacetylactis and/or *Lactobacillus casei*) that produce high levels of diacetyl and acetoin,

* Corresponding author at: School of Agriculture and Food Sciences, Hartley Teakle Building (No. 83), The University of Queensland, St Lucia, QLD 4072, Australia.
E-mail address: m.turner2@uq.edu.au (M.S. Turner).

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sensory panellists were able to discern a more intense buttery flavour (Midje et al., 2000).

During a screen in our lab of several *Lactobacillus* species to identify candidate adjunct strains for enhancing cheese flavour, two strains of *Lb. rhamnosus* were found to produce much higher levels of diacetyl and acetoin than the other *Lactobacillus* species tested (data not shown). There is a considerable amount of research on diacetyl production in *Lb. rhamnosus*, most of which is on the type strain ATCC 7469 (Benito de Cardenas et al., 1992; de Cárdenas et al., 1987; Figueroa et al., 1996; Medina de Figueroa et al., 2001). However, the genetic basis of the diacetyl production pathway in *Lb. rhamnosus* has not been reported. In order to characterise the genes involved and determine the essential components in this pathway, we expanded the screen to more *Lb. rhamnosus* strains in search of naturally occurring low diacetyl producing strains. One such strain was found and examination of the diacetyl/acetoin pathway genes led to the finding that it has a truncated acetolactate synthase (*als*) gene. Restoration of diacetyl production in both milk and cheese by complementation with a full length *als* indicates that Als plays a crucial role in diacetyl formation in *Lb. rhamnosus*.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Lactococcus lactis* strains were routinely cultured in LM17 (M17 + 0.5% lactose) (BD, Franklin Lakes, NJ, USA) at 30 °C and *Lb. rhamnosus* in MRS (Oxoid, Basingstoke, UK) at 37 °C. *Lb. rhamnosus* strains containing the temperature-sensitive pG$^+$9 plasmid derivatives were incubated at 30 °C for 2 d in the presence of erythromycin (10 μg/mL). Agar plates inoculated with *L. lactis* and *Lb. rhamnosus* were incubated aerobically and anaerobically, respectively. Liquid cultures of both species were incubated in tubes with minimal headspace without agitation.

2.2. Genomic DNA extraction and whole genome sequencing

Two mL of stationary phase cultures in MRS were centrifuged to obtain cell pellets. The pellets were stored at − 20 °C until DNA extraction. DNA was extracted using a chloroform-isoamyl alcohol-based method as previously described (Prasad and Turner, 2011). The DNA

Table 1
Bacterial strains and plasmids used in this study.

Bacterial strain or plasmid	Abbreviation used	Relevant properties	Source a/o reference
Lactococcus lactis subsp. *lactis* ASCC 519[a]	S	Used as starter for cheesemaking.	Industrial strain
Lactococcus lactis subsp. *cremoris* ASCC 1263	S	Used as starter for cheesemaking.	Industrial strain
Lactobacillus rhamnosus ASCC 3018	3018	High diacetyl producer. Sent for whole genome sequencing.	Isolated from English double Gloucester cheese. Analysed by Desai et al. (2006).
Lactobacillus rhamnosus ASCC 3029	3029	High diacetyl producer. Sent for whole genome sequencing.	Isolated from commercial freeze-dried culture. Analysed by Desai et al. (2006).
Lactobacillus rhamnosus ASCC 1521	1521	High diacetyl producer. Sent for whole genome sequencing.	Type strain. Same strain as ATCC 7469[b] and DSM 20021[b] (Desai et al., 2006; Dicks et al., 1996).
Lactobacillus rhamnosus ASCC 3016	3016	Low diacetyl producer. Sent for whole genome sequencing.	Isolated from UK half-fat cheese.
Lactobacillus rhamnosus ASCC 277	277	High diacetyl producer	Same strain as NCIMB 8963[b]. Analysed by Desai et al. (2006).
Lactobacillus rhamnosus ASCC 1002	1002	High diacetyl producer	Isolated from UK farmhouse Cheddar. Analysed by Desai et al. (2006).
Lactobacillus rhamnosus ASCC 1520	1520	High diacetyl producer	Isolated from Mozzarella. Analysed by Desai et al. (2006).
Lactobacillus rhamnosus ASCC 3015	3015	High diacetyl producer	Isolated from New Zealand Cheddar.
Lactobacillus rhamnosus ASCC 3019	3019	High diacetyl producer	Isolated from New Zealand Cheddar.
Lactobacillus rhamnosus ASCC 3021	3021	High diacetyl producer	Isolated from commercial mixed probiotic.
Lactobacillus rhamnosus ASCC 3022	3022	High diacetyl producer	Isolated from Cheddar.
Lactobacillus rhamnosus ASCC 3023	3023	High diacetyl producer	Isolated from mixed culture.
Lactobacillus rhamnosus ASCC 3025	3025	High diacetyl producer	Same strain as CSCC 2607[b]. Isolated from Australian Mozzarella. Analysed by Desai et al. (2006).
Lactobacillus rhamnosus GG	GG	Known to have good transformation efficiency with protocol used. Used as transformation control and as another *Lb. rhamnosus* strain to test effect of pG$^+$-*als*.	Isolated from commercial probiotic tablet.
pG$^+$9-Cm		Contains both Em^r and Cm^r markers. pG$^+$9 (Maguin et al., 1996) with the ISS*1* element replaced with a EcoRI-SalI fragment from pNZ123 (De Vos, 1987) containing a Cm^r marker.	Same plasmid as pPNG904 (Lo et al., 2009).
pG$^+$9-*als*		Em^r. pG$^+$9-Cm containing full *als* gene from strain 3018 by cloning with PstI and XhoI, which removes the Cm^r marker.	This work

[a] ASCC, Australian Starter Culture Collection. These strains were provided by Dairy Innovation Australia Limited but are now owned by Chr. Hansen (Hørsholm, Denmark).
[b] ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen (Germany); NCIMB, National Collection of Industrial and Marine Bacteria (UK); CSCC, CSIRO starter culture collection. CSIRO, The Commonwealth Scientific and Industrial Research Organisation (Australia).