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Biochemistry of non-starter lactic acid bacteria isolate *Lactobacillus casei* GCRL163: Production of metabolites by stationary-phase cultures

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ABSTRACT

Physiological and metabolic traits of *Lactobacillus casei* strain GCRL163 were studied in a buffered semi-defined medium supplemented with different concentrations of lactose. The extent of growth (maximum OD_{600} reached) was limited by concentrations of $\leq$1% lactose in this medium. Survival of the strain during extended incubation periods (up to 30 days) in cultures without lactose was found to be better than in cultures supplemented with lactose. Important metabolites detected in lactose-starved (0% lactose) cultures were acetate, as a major end product, as well as ethanol, diacetyl, 3-methyl-1-butanol, benzyl alcohol, 1-butanol, isopropanol, 3-(methylthio)-propanoic acid and other compounds. Viability of recovered cells declined but metabolic products continued accumulating, when the pH was kept constant at pH 5.2. It was proposed that cells entered a non-culturable state but remained metabolically active. Results indicated the potential of this strain, as a possible non-starter lactic acid bacteria adjunct, to survive and utilize alternative available substrates as energy source in cheese curd.

1. Introduction

Flavour development in cheese occurs over extended periods of maturation and involves a complex microflora that varies over time, as exemplified by the known fluctuation in non-starter lactic acid bacteria (NSLAB) numbers in Cheddar cheese over typical maturation periods of several months up to several years. The dominant NSLAB species vary between manufacturers and countries, but representatives of the *Lactobacillus casei* group are often found in numbers that vary from 10^2 cfu g^{-1} initially to 10^8 cfu g^{-1} within weeks of production (Chandry, Moore, Davidson, & Hillier, 1998; Chandry, Moore, & Hillier, 2002; Crow, Curry, & Hayes, 2001; Fitzsimons, Cogan, Condon, & Beresford, 2001; Sheehan, Fenelon, Wilkinson, & McSweeney, 2007). Although the composition of this flora varies between manufacturing plants and within plants at different times (Berthier, Beuvier, Dasen, & Grappin, 2001; Jordan & Cogan, 1993; Somers, Johnson, & Wong, 2001; Williams & Banks, 1997), it is generally accepted that the NSLAB flora contributes to the development of distinctive flavours (Law, 1998; Lynch, McSweeney, Fox, Cogan, & Drinan, 1996; McSweeney, Fox, Lucey, Jordan, & Cogan, 1993; Puchades, Lemieux, & Simard, 1989). However, there is still considerable discussion on the underlying biochemistry responsible for production of specific flavours and the nature of the interactions that occur between members of the microflora in the process of formation of characteristic flavour profiles (Broadbent, Brotherson, Johnson, & Oberg, 2002; Steele, Budinich, Cai, Curtis, & Broadbent, 2006).

During Cheddar cheese maturation, the nutritional environment available to sustain growth and viability of the microflora varies considerably and NSLAB grow under sub-optimal growth conditions, including low temperature and a pH below that required for maximum growth and concentrations of salt that inhibit growth (Hussain & Britz, 2006; Rouch, Hillier, & Britz, 2002). Furthermore, lactose is depleted within weeks of manufacture, effectively starving the NSLAB microflora of a key substrate for growth (Martley & Crow, 1993; Shakeel-Ur-Rehman, Waldron, & Fox, 2004; Waldron, 1997). The action of proteinases and peptidases (either endogenous to milk or released from starter cultures) results in the slow degradation of specific casein types within the matrix and release of peptides and amino acids; these are then available for NSLAB metabolism (Fox, McSweeney, & Lynch, 1998). Degradation products of casein, peptides, and amino acids have been known to contribute to the development of cheese flavours (Ardö & Petterson, 1988; Broome, Krause, & Hickey, 1990; Puchades et al., 1989) and dominant NSLAB have been shown to contribute to an increase in free amino acid content in cheese and therefore influence flavour development. Free amino acids vary in their concentration over time and leucine has been reported to be dominant amino acid in Cheddar cheese after 6 months of maturation (Wallace & Fox, 1997).

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Reported investigations on LAB biochemistry and metabolism generally describe experiments undertaken in rich media such as de Man-Rogosa-Sharpe (MRS) broth or synthetic media containing high concentrations of carbon and energy sources, such as glucose or lactose and for short incubation periods (Lee, 2005; Swearingen, O'Sullivan, & Warthsehen, 2001; Torino, Taranto, & Font de Valdez, 2005). However, these experimental conditions do not represent the relatively stressful environments encountered in the cheese matrix, conditions that could significantly influence the survival and metabolism of members of the microflora. A few recent investigations focused on the use of starvation and/or energy-limited conditions simulating the depletion of lactose concentration during cheese ripening (Adamberg, Adamberg, Laht, Ardö, & Paalme, 2006; Díaz-Muñiz & Steele, 2006; Rouch & Britz, 2004).

The aim of the present study was to investigate the ability of *Lb. casei* GCRL163 to adapt, survive and grow under carbohydrate starvation conditions similar to those found during cheese ripening. A semi-defined synthetic medium was used, with the final pH of cultures maintained within 5–5.5, the pH range typically encountered in Cheddar cheese during maturation (Shakeel-Ur-Rehman et al., 2004). The incubation period was extended to 30 days to determine whether metabolites were formed after growth had ceased and the nature of these metabolites.

2. Materials and methods

2.1. Bacterial strain and media

Lb. casei strain GCRL163 was isolated from Cheddar cheese provided by an Australian manufacturer (Chandry et al., 1998). Bacteria were routinely cultured using MRS (Oxoid, West Heidelberg, Australia) broth or plates at 30 °C, anaerobically (Oxoid jars with Gas Generating Kit BR038B). Stock cultures were prepared in glycerol storage broths (40% glycerol in MRS) and stored in cryo-vials under oxygen-free nitrogen at −20 °C as working stocks and −80 °C for permanent stocks. A tryptone-based defined medium (S3), developed in this work (based on the composition described by Rouch et al. (2002)), contained the following salts-tryptone (SS) components: 0.35 M phosphate buffer, pH 6.4 or 5.5; 0.5% tryptone; 0.025% $MgSO_4 \cdot 7H_2O$; 0.01% $CaCl_2$; 0.1% (v/v) Tween 80; 1 mL Vogel's trace elements solution (0.5% citric acid, 0.5% $ZnSO_4$, 0.1% $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.025% $CuSO_4$, 0.005% $MnSO_4$, 0.005% H_3BO_3, 0.005% Na_2MoO_4) per L of broth. SS broth was further supplemented with 10 mg L^{-1} each of adenine, guanine and uracil; 20 mg L^{-1} xanthine; 4 mg L^{-1} pyridoxine-HCl; 1 mg L^{-1} riboflavin; 0.6 mg L^{-1} calcium pantothenate; 0.8 mg L^{-1} nicotinic acid; 0.1 mg L^{-1} folic acid; 2 mg L^{-1} *p*-aminobenzoic acid; 4 mg L^{-1} pyridoxal phosphate; 0.4 mg L^{-1} thiamine-HCl; 0.02 mg L^{-1} biotin and 5 mg L^{-1} reduced glutathione to make S3 basal broth. L-Lactose (ultra-pure, Sigma–Aldrich, St. Louis, MO, USA) was added to this basal medium at concentrations up to 3% to determine growth-limiting concentrations of lactose, and media containing no lactose (S3-0%L), growth-limiting lactose (S3-0.2%L) and non-growth-limiting concentrations (S3-1%L) were used to determine the impact of lactose concentration on growth, survival and metabolite formation. S3 broths were buffered with 0.35 M phosphate buffer, which was required to maintain pH in the range of 5–5.5 in media containing 1% lactose. In preliminary experiments, S3 broth was used with 0.1 M phosphate buffer, which failed to maintain the pH in cultures with 1% lactose supplementation.

2.2. Isolation of rifampicin resistant mutants

Mutants spontaneously resistant to rifampicin (Rif) were isolated by plating 0.1 mL of overnight MRS cultures onto MRS plates containing 100 mg L^{-1} (MRS-Rif_{100}). Plates were incubated for 2–4 days at 30 °C anaerobically and Rif resistant colonies purified twice on the same medium to confirm the phenotype before storage in glycerol broths. Biochemical characteristics of the mutants were identical to the parents in terms of lactic acid production from lactose, growth rates and growth requirements. These Rif-mutants were used to monitor the growth of any contaminants in the cultures during 30 days incubation period.

2.3. Growth conditions and experimental designs

To determine the concentrations of lactose that limited growth, starter cultures were prepared by inoculating directly from glycerol storage broth into 20 mL of MRS broth and incubating anaerobically (30 °C) for 24 h. Aliquots of starter culture were transferred into 50 mL of S3 media (containing 0–3% lactose) in 100 mL Schott bottles to give an initial OD_{600} of ~0.1 and broths were placed immediately in anaerobic jars for incubation at 30 °C.

For large-scale experiments (up to 500 mL of medium) where the impact of lactose concentration on growth, survival and metabolite formation was determined, cells were collected from starter cultures by centrifugation, washed once with 10 mL of SS and resuspended in 5 mL of SS. Cells were subcultured in S3-1%L and incubated overnight under anaerobic conditions (30 °C). The OD_{600} of the cultures was recorded before cells were collected by centrifugation, washed and resuspended in SS. Calculated volumes of cell suspension were then used to inoculate S3-0%L, S3-0.2%L and S3-1%L to give a starting OD_{600} of 0.1 (as a high inoculums which corresponded to cell counts between 10^7 and 10^8 mL^{-1}) or 0.01 (as a low inoculums with cell counts between 10^6 and 10^7 mL^{-1}). The headspace was sparged with sterile oxygen-free nitrogen (in-line 0.22 μm filter) for 5 min, the bottles sealed and incubated at 30 °C in anaerobic jars. Samples were removed periodically under nitrogen gas flow and the headspace replaced before resealing the bottles.

Multiple controls were applied in these experimental conditions to determine any biotic or abiotic effects that may be responsible for the generation of any of the metabolites detected during incubation of cultures. S3 broth containing no cells was used as a negative control. Cells killed by autoclaving or addition of mercuric chloride were prepared as controls to assess whether biological activity was responsible for the metabolite formation (Juhasz, 1997).

2.4. Growth and viability measurements

Growth was monitored by measuring OD_{600}. Viable counts were performed in triplicate by plating 0.1 mL of serial dilutions in MRS broth onto MRS plates and, when using mutants, MRS-Rif_{100} plates were used. Colonies were enumerated after 72 h at 30 °C and counts expressed as colony forming units per mL (cfu mL^{-1}).

2.5. Lactose and lactate determination

Lactose and lactate concentrations were determined by high-performance liquid chromatography (HPLC) (Section 2.6) and using lactose/galactose and D-lactic acid/L-lactic acid enzymatic bio-analysis kits (Boehringer-Mannheim, Germany) according to manufacturer instructions. The total amount of lactic acid produced corresponded to the sum of the concentrations of both isomers.

2.6. Analysis of acidic metabolites

Cells were removed from culture samples by centrifugation (5 min, Eppendorf centrifuge) and the supernatant fluid filtered (0.22 μm, Millipore). Alternatively, the whole sample was placed in a 2 mL capacity screw-top plastic tube with 0.5 g of 0.1 mm glass