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Properties of an acid-tolerant, persistent Cheddar cheese isolate, *Lacticaseibacillus paracasei* GCRL163

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Abstract: The distinctive flavours in hard cheeses are attributed largely to the activity of nonstarter lactic acid bacteria (NSLAB) which dominate the cheese matrix during maturation after lactose is consumed. Understanding how different strains of NSLAB survive, compete, and scavenge available nutrients is fundamental to selecting strains as potential adjunct starters which may influence product traits. Three *Lacticaseibacillus paracasei* isolates which dominated at different stages over 63-week maturation periods of Australian Cheddar cheeses had the same molecular biotype. They shared many phenotypic traits, including salt tolerance, optimum growth temperature, growth on *N*-acetylglucosamine and *N*-acetylgalactosamine plus delayed growth on *D*-ribose, carbon sources likely present in cheese due to bacterial autolysis. However, strains 124 and 163 (later named GCRL163) survived longer at low pH and grew on *D*-tagatose and *D*-mannitol, differentiating this phenotype from strain 122. When cultured on growth-limiting lactose (0.2%, wt/vol) in the presence of high concentrations of *L*-leucine and other amino acids, GCRL163 produced, and subsequently consumed lactate, forming acetic and formic acids, and demonstrated temporal accumulation of intermediates in pyruvate metabolism in long-term cultures. Strain GCRL163 grew in Tween 80-tryptone broths, a trait not shared by all *L. casei*-group dairy isolates screened in this study. Including citrate in this medium stimulated growth of GCRL163 above citrate alone, suggesting cometabolism of citrate and Tween 80. Proteomic analysis of cytosolic proteins indicated that growth in Tween 80 produced a higher stress state and increased relative abundance of three cell envelope proteinases (CEPs) (including PrtP and Dumpy), amongst over 230 differentially expressed proteins.

Keywords: *Lactobacillus*, Nutrient starvation, Proteomics, Tween 80

Introduction

Lactobacilli species, which are one of the many groups within the lactic acid bacteria (LAB), are generally regarded as safe for human consumption, play crucial roles in the production of a large variety of safe fermented foods and influence human health (Di Cagno et al., 2016; Orrhage & Nord, 2000). Specific species of *Lactobacillales*, such as members of the *Lacticaseibacillus* (formerly *Lactobacillus*) *casei* group (Zheng et al., 2020) which includes *L. casei*, *L. paracasei*, and *L. rhamnosus* (Gobbetti & Minervini, 2014), occur in the human mouth and gastrointestinal tract and have long been marketed as adjunct starter cultures and health-promoting probiotics (Gobbetti et al., 2015; Parvez et al., 2006; Peralta et al., 2017; Kleerebezem & Vaughan, 2009). While the core genome is conserved, there is variation in the pan-genomes within strains of the facultatively heterofermentative *L. casei* group resulting in phenotypic diversity (Duar et al., 2017; Toh et al.; 2013). However, *L. casei* group strains, like many other species of LAB, share phenotypic traits including acid and salt tolerance, production of many intracellular peptidases and cell-surface proteinases, plus synthesis of lactic acid as a primary metabolite, which aids food preservation and favours their dominance in acidic foods (Russell & Diez-Gonzalez, 1997).

Cheese manufacture initially involves adding starter LAB and proteolytic enzymes to acidify milk and form the curd in the presence of high salt concentrations. Subsequently, adventitious nonstarter lactic acid bacteria (NSLAB) proliferate to dominate the complex and dynamic microbiota during ripening stages that may last several months to years, particularly in hard cheeses such as Cheddar (McSweeney, 2007). NSLAB are opportunistically present due to persistence following pasteurisation (McAuley et al., 2012), carry-over from the indigenous milk microbiota in fermented artisanal products, and from cross-contamination during manufacturing (Gobbetti et al., 2015). The makeup of the NSLAB microbiota contributes to the distinctive organoleptic and physical properties of cheeses from different regions and factories. Their numbers increase from as few as 10–50 colony-forming units (CFU)/g in pasteurised milk at the start of Cheddar cheese manufacture to 10^7–10^9 CFU/g and remain high throughout ripening (Folkertsma et al., 1996; Gobbetti et al., 2015), with *L. casei* group strains dominating (Crow et al., 2001; Jordan & Cogan, 1993; Fitzsimons et al., 2001; Williams & Banks, 1997) and successions of biotypes observed (Coolbear et al., 2008). Starter LABs consume all the lactose in the cheese matrix in the first weeks of ripening, after which NSLAB grow in an environment of pH 5.0–5.5 at 8–12°C (McSweeney, 2007) on other nutrients present, potentially including: sugars released during autolysis of starter cultures (exopolysaccharides, glycolipids, peptidoglycan, and nucleic acids) (Møller et al., 2021; Thomas, 1986); amino acids or peptides released through proteolysis of the residual casein peptides due to intrinsic milk enzymes, the added coagulant and lysed starter bacteria (Kieronczyk et al., 2001); lipids (McSweeney & Sousa, 2000; Stefanovic et al., 2018); and citrate (Diaz-Muniz et al., 2006; Starrenburg & Hugenholtz,

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1991). Concentrations of free amino acids and fatty acids increase during ripening as do a complex mixture of compounds which contribute to the organoleptic properties of cheeses from NSLAB catabolism of proteins and lipids (McSweeney & Sousa, 2000; Stefanovic et al., 2018).

Because NSLAB may influence cheese quality traits both positively and negatively, there have been numerous studies on the composition of the microbiota of many types of cheese (see Gobbetti et al., 2015). The objectives of these studies included identifying strains with potential application as adjunct starters for accelerating ripening or improving consistency plus distinctiveness in cheese traits, particularly in regional artisanal cheeses made from unpasteurised milks. Strategies to select NSLAB for testing as potential adjunct starters normally involve assessing molecular diversity of strains isolated from ripening cheeses and establishing technological traits which may incur advantages in cheese manufacture, either through direct catabolic activity or by controlling the development of the adventitious microbiota. These traits include determining salt tolerance, acidification rates, establishing safety (screening for toxins and antibiotic resistance) and expression of peptidases that would contribute to amino acid metabolism and flavour development (e.g., see Meng et al., 2018; Randazzo et al., 2021; Stefanovic et al., 2018). The methods used for identification and differentiation of NSLAB strains have evolved over time, from conventional microbiological and biochemical testing (Jordon & Cogan, 1993; Williams & Banks, 1997) to increasing use of molecular biotyping based on 16S rRNA gene sequencing (Chandry et al., 1998; Stefanovic et al., 2017); randomly amplified polymorphic DNA analysis (RAPD) (Fitzsimons et al., 2001); genomic DNA fingerprinting by pulsed-field gel electrophoresis (PFGE) (Chandry et al., 1998; Crow et al., 2001; Stefanovic et al., 2017) and Rep-PCR (Chandry et al., 1998; Randazzo et al., 2021); and phylogenetic profiling using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Randazzo et al., 2021). A survey of six Australian Cheddar cheeses stored at 8°C and sampled at seven-weekly intervals over 63-week ripening periods identified 131 distinct molecular biotypes within the 774 *L. casei*-group strains isolated (Chandry et al., 2002), based on profiling using PFGE, Rep-PCR, and partial 16S rRNA gene sequencing. Three strains with identical PFGE profiles were dominant at different ripening times but were isolated 30 weeks apart, including strain 163 (later named GCRL163). The initial aim of the current study was to establish the technological and phenotypic traits of the three strains to assess their potential as adjuncts in local cheese manufacture. However, this revealed two distinct phenotypes, despite sharing similar genomic architecture, and differentiating traits were indicative of stress resistance. Consequently, strain GCRL163 was further characterised in terms of amino acid catabolism capacity and ability to scavenge fatty acids during nutritional depletion, which occur during Cheddar ripening in highly stressful environments (Papadimitriou et al., 2016). We have previously reported that GCRL163 (named as *L. casei*) survived long-term in media lacking lactose and produced signature flavour compounds while in stationary phase (Hussain et al., 2009b), including short-chain fatty acids indicative of fatty acid breakdown (Al-Naseri et al., 2013). Proteomic changes associated with lactose starvation, citrate utilisation (Al-Naseri et al, 2013; Hussain et al., 2009a) and suppression of fatty acid synthesis when culture media contained Tween® 80, a polyethoxylated sorbitan esterified with oleic acid, were also noted (Al-Naseri et al., 2013). The purpose of the current report was to establish the phylogenetic position of strain GCRL163 as *L. paracasei* and to document the key phenotypic traits of this stress-tolerant biotype relative to other dairy isolates and other well-studied strains of *L. casei/paracasei*, in context of the shifting nomenclature in this group (Zheng et al., 2020). Proteomic analysis of cytosolic preparations of GCRL163 cells was undertaken to provide insight into the underlying biochemistry of scavenging oleic acid from Tween 80 and how this impacts the physiology of cells under nutrient starvation, given that the media used in earlier experiments contained citrate as an alternative carbon source when Tween 80 was also in media (Al-Naseri et al., 2013). The differentiating traits of cometabolism of amino acids and oleic acid scavenging may be useful as biomarkers for predicting persistence in a cheese matrix beyond the technological and safety properties typically assessed.

Materials and Methods

Bacterial Strains and Routine Culture Conditions

Three *L. casei* group strains were isolated from ripening Australian Cheddar cheeses and characterised as genetically similar based on identical PFGE profiles (Chandry et al., 2002): strains 124 and 163 (GCRL163) were isolated 30 weeks apart whereas strain 122 was found in at a different timepoint. Strain MJA12 was isolated from a commercial probiotic milk drink (Yakult™) by purification on de Man, Rogosa, and Sharpe agar (MRS, Oxoid, Basingstoke, Hants, UK). The genomes of GCRL163 (= DSM 111790, = LMG32067) and MJA12 have been published (Nahar et al., 2017) (genome assemblies GCA_00209199.1 and GCA_002091975.1, respectively). *L. casei* ATCC393^T and *L. rhamnosus* ATCC7469^T were available in the Tasmanian Institute of Agriculture, University of Tasmania, culture collection. Other LAB strains were isolated from milk, cheese, or fermented food products. All strains were stored as described previously (Al-Naseri et al., 2013) and routinely cultivated on MRS media at 30°C or 37°C under anaerobic conditions (Anaerocult A, Sigma-Aldrich, Australia). Viable counts were normally made on MRS agar in triplicate after 48–72 hr incubation in anaerobic jars.

Isolation and Identification of LAB from Dairy and Fermented Food Products

Sample processing

Cheese, yogurt, and fermented vegetable samples were collected from local retail outlets in Hobart, Tasmania, Australia, and represented manufacturers from across the state. Milk samples were collected from the University of Tasmania's dairy farm in northern Tasmania. Supplementary Table S1 lists the source of the 65 samples processed for isolation of *L. casei* group strains. All samples of cheese and fermented vegetable products were transported on ice and processed the same day by aseptically collecting 13–20 g internal samples, shredding these into a fine consistency then 1 g samples plus 9 ml of sterile PBS placed into Nasco whirl pack sterile bags (Stennick Scientific, SA, Australia) for homogenisation (Stomacher 400 Circulator, Seward Limited, West Sussex, UK). Homogenates, milk and yogurt samples were plated onto LC agar prepared as described previously (Ramakanth & Shah, 1998). Plates were incubated anaerobically at 27°C for 72–96 hr before reviewing colony formation. Presumptive *L. casei*-group colonies were picked for purification by subculturing twice on MRS agar, Gram stained then isolates stored in glycerol broths at –20°C before molecular typing.

Presumptive Identification

DNA was extracted from all Gram-positive bacilli and coccobacilli isolates and control LAB using Isolate II Genomic DNA extraction kits (Bioline, Meridian Bioscience, Memphis, TN, USA) according to the manufacturer's directions for Gram-positive bacteria. PCR amplification of 16S rRNA genes used universal primers 16S-27F and 16S-1492R (Sigma-Aldrich, Sydney, Australia). Two sets of primers were selected from those described by Kwon et al. (2004) for multiplex typing of *Lactobacillus* species: an *L. casei*-group-specific primer IDL11F with a *Lactobacillus* genus conserved primer IDL03R (both primers binding within the 16S rRNA gene); and a *Lactobacillus* conserved primer IDL04F (binding within the 16S rRNA gene) with an *L. rhamnosus*-specific primer IDL73R (binding site within the 16S-23S intergenic space region) (primer sequences are shown in Supplementary Table S2). PCR reaction mixtures (25 μl) contained: 12.5 μl reaction mixture (Immomix II used for universal primers, My Taq HS Red Mix for the remainder, Bioline, USA), 1 μl each forward and reverse primer (each at 10 mM), 9.5 μl molecular grade deionised water, 1 μl DNA template (100 ng). Cycling conditions for 16S rRNA amplicons were: initial heating at 95°C for 1 min followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 52°C for 40 s, extension at 72°C for 10 s and final extension at 68°C for 7 min on a model C1000 thermocycler (Bio-Rad, Gladesville, NSW, Australia). PCR products were resolved on 2% agarose gels. Cycling conditions for *Lactobacillus* primer sets were: 10 min at 95°C, 46 cycles of 30 s at 95°C, 1 min at 55°C, 1 min at 72°C, 5 min at 72°C, and a final hold at 4°C. Products were resolved on 1.5% agarose gels. Positive controls were *L. casei* ATCC393, *L. rhamnosus* ATCC7469 and GCRL163.

Sequences for 16S rRNA amplicons (Macrogen, Seoul, Republic of Korea) were analysed using the Sequencher software programme (Sequenchergenetics, version 4.8) to remove ambiguities and then searched through the Basic Local Alignment Search Tool (BLAST) of the National Centre of Biotechnology Information (NCBI) to determine the closest matches with known 16S rRNA gene sequences. The data obtained provided presumptive identity of the bacterial strains and the BLAST results were compared with the PCR amplicon patterns to confirm species identity within the *L. casei*-group isolates.

Media and Growth Conditions for Determining Phenotypic Traits

Impact of temperature and salt on growth rates

Overnight starter cultures grown in MRS broth were diluted 15-fold into fresh medium and incubated anaerobically at temperatures between 3°C and 51°C. Samples were removed hourly and optical density measured at 620 nm (OD_{620}) using a microplate reader (Labsystems iEMS Reader MF, path length 0.50 cm, Thermo Electron Corporation, Milford, MA, USA). The maximum growth rates at each temperature, μ_{max} (hr^{-1}), were calculated from regression line analysis of $\log_{10}OD_{620}$ versus incubation time. Growth rate was calculated by standard principles $(3.322 \times \text{Log}[OD_2/OD_1])/(t_2\text{-}t_1)$, where OD_2 and OD_1 represented final and initial optical densities, and t_2 and t_1 represented the corresponding final and initial incubation times).

To determine the impact of NaCl concentration on growth, overnight cultures were diluted 10-fold in MRS broths supplemented with additional NaCl from 1% to 10% and growth rates at 30°C determined as above. Cultures were also serially diluted in PBS and plated for viable counts on MRS plates similarly supplemented with NaCl.

Carbohydrate utilisation patterns, amino acid, and nitrogen source auxotrophy

S3 medium has been described previously (Hussain et al., 2009b). Briefly, this contained 0.5% (wt/vol) tryptone (Oxoid, UK), mineral salts, 0.1% (vol/vol) Tween 80 (VWR International, Brisbane, Australia), nucleotides (10 mg/l each of adenine, guanine and uracil; 20 mg/l xanthine) and vitamins (4 mg/l pyridoxine-HCl; 1 mg/l riboflavin; 0.6 mg/l calcium pantothenate; 0.8 mg/l nicotinic acid; 0.1 mg/l folic acid; 2 mg/l *p*-aminobenzoic acid; 4 mg/l pyridoxal phosphate; 0.4 mg/l thiamine-HCl; 0.02 mg/l biotin; and 5 mg/l reduced glutathione). The broth was buffered at pH 6.5 (0.1 M or 0.35 M citrate-phosphate buffer or sodium phosphate buffer), and components varied depending on the experimental setup to determine auxotrophy.

Vitamin and nucleotide requirements were tested in S3 medium with 1% (wt/vol) *D*-glucose and 0.1 M citrate-phosphate buffer, pH 6.5. Growth in S3 lacking tryptone but containing a pool of amino acids in molar ratios expected in tryptone (Supplementary Table S3) was also used, to eliminate any impacts of vitamins potentially present in tryptone. Media were prepared to contain all the components except for the individual vitamin or nucleotide being examined. For each strain tested, a single colony from MRS plates was resuspended in 0.5 ml of sterile PBS and a 10 μl inoculated into 200 μl broth in standard flat-bottom 96-well micro-assay plates. Growth at 30°C was monitored at OD_{620} over 16 hr (Labsystems iEMS Reader MF) and final OD recorded.

To determine whether growth occurred on different sugars or metabolic intermediates, a series of media were prepared on the basis of S3 broth (tryptone, mineral salts, 0.1 M citrate-phosphate or sodium phosphate buffer, pH 6.5, and essential vitamins [pyridoxine-HCl, riboflavin, pantothenate, nicotinic acid, and folic acid] and xanthine). Sugars or metabolic intermediates were individually supplemented at 1% (wt/vol). Growth on *D*-glucose and media lacking an added carbon source were tested in each set of experiments for test-tube cultures (in anaerobic jars) or in sealed micro-assay plates with OD_{620} monitored as above for up to 12 days.

Amino acid auxotrophy was evaluated by culturing in S3 media with 1% (wt/vol) *D*-glucose and essential vitamins plus xanthine with 0.1 M citrate-phosphate, pH 6.5. Tryptone was replaced with a pool of amino acids (Supplementary Table S3) and growth on media lacking each individual amino acid was determined as above.

End Products Determination During Growth on Media With Elevated Amino Acids

S3 broths were prepared with tryptone, essential vitamins and xanthine with 0.35 M phosphate buffer, pH 6.5. Lactose (ultrapure, Sigma-Aldrich, St. Louis, MO, USA) was supplemented at 0.2% (growth-limiting) or 1% (not growth-limiting) (Hussain et al., 2009b). A series of S3-0.2% lactose broths were prepared containing 50 mM of *L*-glycine, *L*-alanine, *L*-valine or *L*-leucine, and two-way combinations of these amino acids; *L*-leucine was also supplemented alone in S3-0.2% lactose at 25 mM. A starter culture of *L. paracasei* GCRL163 was prepared in MRS broth, cells collected by centrifugation, washed in PBS, inoculated into each of the media and cultured at 30°C under anaerobic conditions as described previously (Hussain et al., 2009b). Samples were taken periodically for 46 days and end products detected by HPLC (C-610H column,

Supelco, Sigma-Aldrich, St. Louis, MO, USA) by refractive index and diode array (210 nm) scanning as described previously (Hussain et al., 2009b).

Evaluating Growth on Tween 80, Citrate, and Acetate

Flask cultures

The basis of modified MRS (mMRS) consisted of 1% (wt/vol) bacteriological peptone (Oxoid, UK), 0.5% (wt/vol) yeast extract (Oxoid, UK), 0.025% (wt/vol) $MgSO_4.7H_2O$, 0.005% (wt/vol) $MnSO_4.4H_2O$ in 0.1 M or 0.35 M potassium phosphate buffer at pH 6.5. This basal formulation was the control (Cont) in growth tests. The basal broth was supplemented with 0.1% (vol/vol) Tween 80 (mMRS-Tw), 0.5% (wt/vol) sodium acetate (mMRS-Ace) or 0.2% (wt/vol) triammonium citrate (mMRS-Cit) or combinations of these (mMRS-TwCit, mMRS-TwAce, and all three carbon sources).

Strain GCRL163 was recovered from glycerol storage by two sequential subcultures in MRS broth at 37°C for 12 hr anaerobically then plating onto MRS agar. Single colonies were inoculated into MRS broths and cells harvested in early stationary phase by centrifugation, washed twice with Tris-HCl buffer (40 mM, pH 7.0) and sub-cultured into mMRS broths to give an initial OD_{600} of ~0.2. The cultures were then incubated at 30°C for 8 days under anaerobic conditions as described previously (Al-Naseri et al., 2013). Cell growth was monitored spectrophotometrically (BMG LABTECH GmbH, Spectrostar Nano, Germany) at OD_{600} and by viable counts in triplicate on MRS agar.

Cultures were sampled on days 1, 2, 4, 6 and 8 (150 ml each) and cells collected by centrifugation at 5,000 rpm for 10 min at 4°C and washed three times with Tris-HCl buffer (40 mM, pH 7.0). Cells were resuspended and concentrated in the same buffer (to an OD_{600} equivalent to 20) and stored at –80°C until selected for protein extraction. Three biological replicates were tested for each medium, each using a starter inoculum from a single colony.

Growth of fermented food isolates in mMRS with Tween 80 as carbon source

Single colonies on MRS plates of strains from dairy and fermented food products with presumptive identities in the *L. casei* group were inoculated into MRS broths and stationary phase cells collected as above. Cell suspensions were inoculated in triplicate into mMRS broths in Bioscreen plates to give a starting OD_{600} of 0.1–0.2. Growth was measured for the control (mMRS, no added citrate, acetate, or carbohydrate source) and mMRS-Tw (the control supplemented with Tween 80 only) by recording OD_{600} readings every hour over a period of 13 days at 37°C (Bioscreen C, Oy Growth Curves Ab Ltd., Helsinki, Finland).

Acid Tolerance

Long-term survival of Cheddar cheese isolates in buffers

Acid tolerance was measured from cell survival in 0.1 M citrate-phosphate buffers formulated to provide pH values of 3.0, 4.0, 5.0, 6.0, and 7.0. Overnight cultures in MRS broth were diluted 10-fold in fresh medium and incubated anaerobically at 30°C to obtain mid-exponential cultures. Cells were collected by centrifugation, washed in 0.85 % saline, and resuspended in the same volume of test buffer. Cell suspensions were incubated at 10°C (to simulate typical cheese ripening temperatures) and samples taken periodically up to 63 days. Cells were serially diluted in 0.8% saline and viable counts on MRS agar determined after 48 hr at 30°C. The variance between duplicate counts was <10%.

Survival in mMRS, pH 2.5, with or without Tween 80

mMRS broths at pH 2.5 (0.35 M citrate-phosphate) contained acetate, citrate, and 1% lactose either with or without 0.1% Tween 80. Starter cultures of strain GCRL163 in MRS were prepared from single colonies on MRS agar and used to inoculate each mMRS formulation in triplicate to give an initial OD_{600} of 0.21. Cultures were then incubated anerobically at 30°C and sampled every hour for 30 hr for viable counts in triplicate on MRS agar. Standard deviations were calculated.

Proteomic Analyses

Concentrated cells from early stationary phase cultures in mMRS-Tw and mMRS-TwCit were thawed on ice and 200 μl aliquots added to 0.5 g of zircon beads (0.1 mm, Daintree Scientific, TAS, Australia) in 2 mL screw-capped plastic tubes and cells lysed by beating in a TissueLyser II (Qiagen, Melbourne, VIC, Australia) for a total of 6 min, cooling on ice between 1 min bursts. Cellular debris was removed by centrifugation at 14,000 rpm for 30 min at 4°C (Eppendorf centrifuge 5417R, Crown Scientific Pty Ltd, Australia). The protein concentration in extracts was determined using BCA kits (Thermo Fisher Scientific, USA) with bovine serum albumin as a standard. Equal quantities of each sample (50 μg) were sequentially reduced, alkylated, and trypsin-digested, as previously described (Al-Naseri et al., 2013) and peptides separated by nano-liquid chromatography and analysed by tandem mass spectrometry (MS/MS) (LTQ-Orbitrap XL, Thermo Fisher Scientific, Australia). Operating conditions, data handling, normalisation of total spectral counts (SpCs) (to account for differences in trypsin digestion efficiency, protein concentration, and loading between samples), and database searching for protein identification have been described previously (Al-Naseri et al., 2013). Protein identity was accepted when two or more unique peptide sequences were detected and assigned a probability of >0.95 by the Protein Prophet algorithm (constraining the protein false discovery rate, FDR, to <1%) (Nesvizhskii et al., 2003). Gene locus descriptors for proteins were assigned from the genome of *L. paracasei* W56, a close phylogenetic relative of GCRL163 with a closed sequenced genome (UniProt proteome ID UP000003734). SpC data were $\log_2$ transformed and differences in relative abundance between proteins in extracts from mMRS-Tw relative to mMRS-TwCit were calculated based on average SpC. If proteins were not detected in a replicate, an imputed SpC value of 0.5 was used to enable statistical analysis. Significant differences (t-tests, p-value of <0.05 considered significant) in protein SpCs were calculated as described previously (Al-Naseri et al., 2013). Proteins were assigned to categories broadly based on clusters of orthologous groups (COGs) (Galperin et al., 2021) and T-Profiler methods used to generate T-values for these categories to compare relative abundance changes (Boorsma et al., 2005). Volcano plots ($-\log_{10}P$ against $\log_2$ differences in abundance, Excel scatter plot function) were used to visualise individual proteins with highly different abundances (p <0.05, $\delta\log_2$ >±1 or twofold differences in abundance). Protein domain analysis was done through UniProt (most recently accessed July 2021), the NCBI conserved domain database (CDD) and by BLASTP through the Kyoto Encyclopedia of Genes and Genomes (KEGG) common motif search tool to assign presumptive function to some uncharacterised protein.

Phylogenetic Classification of Strains and Bioinformatic Analyses

The sequenced genomes of strains GCRL163 and MJA12 were examined using NCBI Genome BLAST for *Lactobacillaceae* annotated

as *L. casei* (accessed November 2019 and July 2021) and tools available in the Integrated Microbial Genomics and Microbiomes (IMG/M) database (Chen et al., 2021) (https://img.jgi.doe.gov, accessed most recently in July 2021) for pairwise average nucleotide identity (ANI) and genome clustering analyses; phylogenetic tree construction (hierarchical clustering based on KEGG Orthology, KO, functions); gene layout; and obtaining sequences for intergenic regions associated with cell envelope proteinase (CEP) genes in GCRL163. Protein sequence similarity searches were undertaken using KEGG and NCBI tools for pairwise and multiple sequence alignment, and KEGG for gene layout in *L. paracasei* W56. The BPROM tool in the Softberry programme suite (www.softberry.com/) (Salamov & Solovyev, 2011) was used to predict sigma-70 promoter sites in intergenic regions of the *L. casei* GCRL163 genome for genes specifying CEPs and the Tomtom tool (Version 5.3.1) in the MEME programme suite (Gupta et al., 2007) for predicting regulatory motifs.

Results and Discussion

Phylogenetic Position of GCRL163 and Identity of Fermented Food Isolates

The phylogenetic position of isolates within the *L. casei* group has been under discussion for some time, given that 16S rRNA gene sequencing fails to differentiate between members within this group (Kim et al., 2020) and the type strain, ATCC393, was recognised as distinct from many strains previously named *L. casei* (Hill et al., 2018). Based on comparative genomic analyses of strains named *L. casei*, Ghosh et al. (2019) recommended reclassification of the majority of "*L. casei*" strains as *L. paracasei*, notably based on ANI and average amino acid identity similarities, including GCRL163 and MJA12. The emended description of the genus *Lactobacillus* resulted in 25 genera including the new genus *Lacticaseibacillus* (Zheng et al., 2020), encompassing the *L. casei* group (*casei*, *rhamnosus*, and *paracasei*) as a distinct cluster which includes *L. chiayiensis* (isolated originally from cow manure and closely related to *L. casei*, Huang et al., 2018). Consequently, the names of many *L. casei* group members are being updated on NCBI and other databases to reflect these changes. NCBI Genome BLAST for *Lactobacillaceae* strains named *L. casei* (Supplementary Fig. S1A) showed strains GCRL163 and MJA12 were distinct from *L. casei* ATCC393. Further genomic analysis (Supplementary Fig. S1B) showed strains GCRL163 and MJA12 are in the IMG/M ANI clique 5859, which contains strains currently named as *L. paracasei* and *L. casei* and is comprised of 152 genomes (accessed July 19, 2021): IMG/M hierarchical clustering based on KO function for this clique placed GCRL163 and MJA12 with several well-characterised probiotic strains isolated from fermented dairy products (Supplementary Fig. S1B). These data confirm the identity of GCRL163 and MJA12 as *L. paracasei* species.

A total of 65 samples of raw milk and fermented food products (one Kimchi) were collected of which 48 showed growth on LC agar plates (Supplementary Table S1). While LC agar was originally developed for selectively enumerating *L. casei* in yogurt and fermented milk drinks (Ramakanth & Shah, 1998), other reports indicated that several LAB species could grow on this medium (see Karimi et al., 2012), an observation also made in the current study (Supplementary Tables S4 and S5). From 16S rRNA gene sequencing, species detected on LC plates included *Lactococcus lactis* subsp. *cremoris* and several lactobacilli: *L. curvatus*, *L. sakei*, *L. coryniformis*, from multiple raw milk samples, and *L. helveticus*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii* from cheese and fermented milk products, in addition to *L. casei* group members. Primers specific for *L. casei* group members strongly amplified the expected 727 bp fragment, consistent with 16S rRNA gene sequencing presumptive identification of isolates, whereas most other lactobacilli species showed no bands (Supplementary Fig. S2). However, under the assay conditions used faint bands of 727 bp were also seen for isolates presumptively identified as *L. curvatus*, *L. coryniformis*, and *L. sakei* but a smaller band (150 bp) was also amplified in parallel, distinguishing this pattern from *L. casei* group members. *L. rhamnose*-specific primers produced the signature 448 bp fragment for the control, ATCC7469, together with a larger, faint band and this banding pattern was able to distinguish isolates as *L. rhamnosus* (Supplementary Fig. S3). Again, faint smaller bands were also detected for non-*L. rhamnosus* isolates and, interestingly, ATCC393 showed the same signature pattern as the type *L. rhamnosus* strain, consistent with the convoluted phylogenetic placement of this strain (Hill et al., 2018). The primers selected from those described by Kwon et al. (2004) were therefore useful in screening for presumptive *L. casei* group isolates, particularly for differentiating between *L. rhamnosus* amongst *L. casei* group isolates for the purpose of the current study, although real-time PCR strategies based on species-specific genes may be of more utility for detecting *L. casei* group members in fermented foods in the future (Kim et al., 2020).

Technological Properties and Carbon Source Patterns of Cheddar Cheese Isolates

Strains 122, 124, and 163 were isolated from six Cheddar cheeses made by an Australian manufacturer at different times over one season and the microbiota monitored over 63-week ripening periods, with 20 isolates selected from each sample for PFGE fingerprinting (Chandry et al., 2002). From partial 16S rRNA gene sequencing, *L. casei* group strains dominated throughout ripening, as reported in many similar studies previously (Crow et al., 2001; Fitzsimons et al., 2001; Jordan & Cogan, 1993; Williams & Banks, 1997). The three strains had identical PFGE profiles and would be considered as closely related from overall genome architecture and as a persistent biotype, being isolated at different timepoints. They were indistinguishable in terms of optimum growth temperature (37°C) and salt tolerance, with growth detected at 6% NaCl on solid media but at much reduced μ_{max} when tested in broths with >2% NaCl (Supplementary Fig. S4) but could be differentiated based on carbon source utilisation and acid tolerance. Stefanovic et al. (2017) also noted metabolic diversity in *L. paracasei* strains with similar PFGE biotypes, concluding that PFGE patterns are not sufficiently discriminating for predicting metabolic capacity.

When screened for growth on 30 carbon sources (Table 1), strains 124 and 163 grew on *D*-tagatose and *D*-mannitol whereas strain 122 did not. Otherwise, all three strains grew on a range of carbohydrates, including *N*-acetylgalactosamine and *N*-acetylglucosamine, and failed to grow on *N*-acetylneuraminic acid (found in glycosylated caseins, Williams et al., 2000) and *N*-acetylmuramic acid (which is found in bacterial cell walls), carbon sources likely to be present in the cheese matrix. However, the strains showed an extended lag period prior to commencing growth on *D*-ribose (Fig. 1) which was not seen for other carbohydrates tested similarly. Given that the genomes of GCRL163 and MJA12 encode the genetic machinery associated with *D*-ribose catabolism (transporters and metabolic pathways, specifically nucleotide metabolism, amino acid synthesis requiring *D*-ribose and pentose phosphate pathways [PPP], involved in nucleotide synthesis and breakdown), the data suggested a period of adaptation was needed to utilise this carbon source. Variability in

Table 1. Carbohydrate growth profiles

Carbohydrate	Growth at 48 hr (OD at 620 nm[a])			Growth at 96 hr (OD at 620 nm[a])		
	122[b]	124[b]	163[b]	122[b]	124[b]	163[b]
D-Glucose[c]	0.437	0.508	0.521	-	-	-
N-Acetylgalactosamine[d]	0.496	0.463	0.510	-	-	-
D-Tagatose	0.041	0.420	0.142	0.086	0.370	0.304
D-Mannitol	0.043	0.106	0.492	0.062	0.324	0.418
D-Ribose	0.014	0.013	0.100	0.353	0.350	0.331
D-Xylulose[e]	0.018	0.015	0.010	0.019	0.018	0.013

[a]Optical density measured using a microplate reader, Labsystems iEMS Reader MF (path length 0.50 cm).
[b]Strain number.
[c]Similar growth was observed for other carbon sources: *D*-maltose, *D*-glucono-*δ*-lactone, *D*-gluconate, *D*-fructose, lactose, *D*-galactose and *D*-mannose (OD_{620} range of 0.185–0.72 after up to 6 days incubation). (-) means similar OD readings were recorded after 96 hr.
[d]Similar growth was seen with *N*-acetylglucosamine.
[e]Similar lack of growth occurred with other carbon sources: pyruvate, *L*-malate, glycerol, fumarate, succinate, *D*-raffinose, *D*-sorbitol, sucrose, *N*-acetylneuraminic acid, *N*-acetylmuramic acid, adonitol (ribitol), *L*-rhamnose and phosphorylated sugars 6-phospho-gluconic acid, *D*-fructose-6-phosphate, *D*-ribose-5-phosphate, *DL*-glyceraldehyde-3-phosphate (maximum OD_{620} < 0.05 after 96 hr incubation).

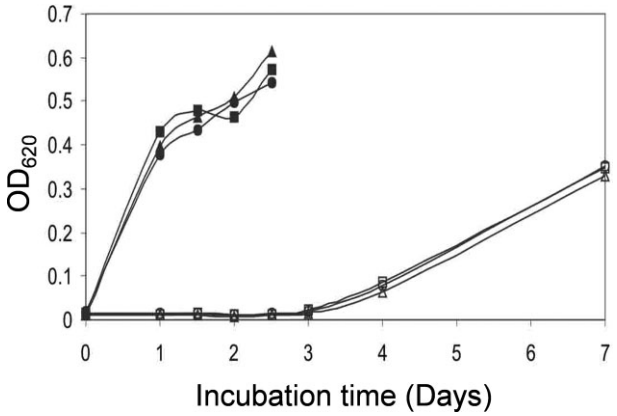

Fig. 1 Typical growth curves for *L. paracasei* of Cheddar cheese isolates 122 (●, ○) 124 (■, □) and 163 (▲, Δ) cultured in S3 broths supplemented with 1% (wt/vol) *N*-acetylgalactosamine (closed symbols) or *D*-ribose (open symbols).

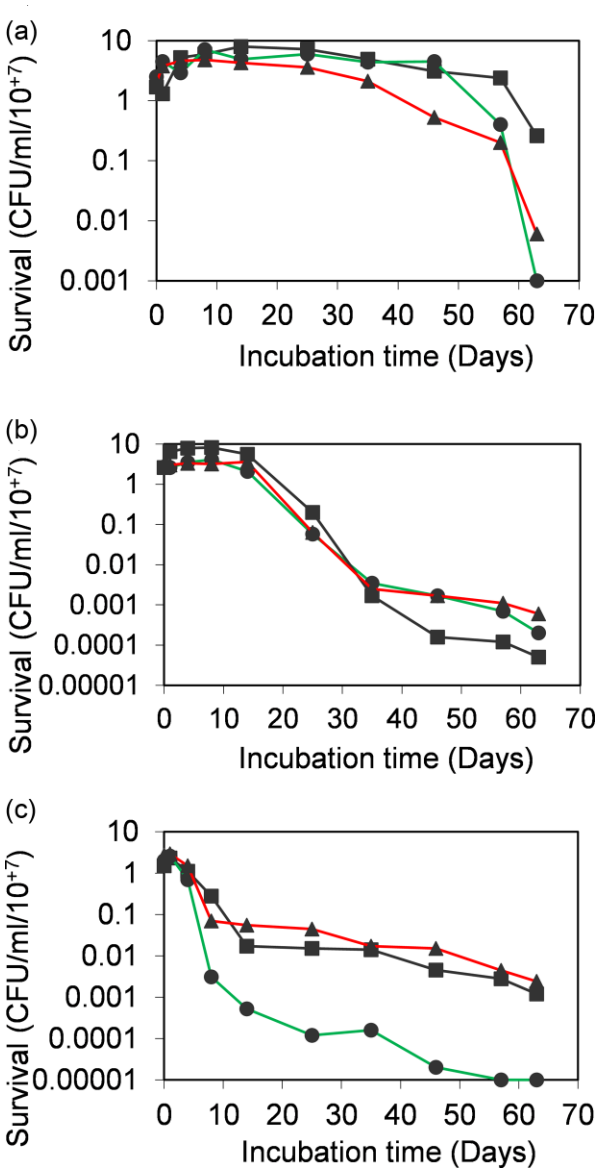

Fig. 2 Survival of *L. paracasei* strains 122 (●, green), 124 (■, black) and GCRL 163 (▲, red) incubated at 10°C in 0.1 M citrate-phosphate buffers for 63 days. (a), pH 5.0; (b), pH 4.0; (c), pH 3.0. Viable counts were on MRS agar plates, where duplicate counts were typically ±10% of the average. Survival rates at pH 6.0 and 7.0 were similar to pH 5.0.

carbohydrate utilisation by *L. casei* isolates from cheese, and between isolates from other sources, has been reported previously (Cai et al., 2007) and was attributed to niche-specific adaptation resulting from recombination occurring during evolution within different niches. However, strains were evaluated after 24 hr incubation in API kits (Broadbent et al., 2003) so that delayed use of carbon sources would not have been detected. Longer lag periods on *D*-ribose were reported by Adamberg et al. (2005) for *L. casei* group isolates and slower growth rates, likely due to the lower energy yield from *D*-ribose use. We previously reported the temporal induction of proteins during stationary phase of *L. paracasei* GCRL163 in media lacking an added carbohydrate source, including a xylulose-5-phosphate phosphoketolase which implicated the PPP in starvation stress responses (Hussain et al., 2013) and emphasises the need for long incubation periods to demonstrate the full metabolic capacity of isolates.

The other key phenotypic trait differentiating strains 124 and 163 was greater acid tolerance (Fig. 2), as both strains survived for longer periods during extended exposure to pHs < 5 at temperatures simulating cheese ripening temperature, suggesting that the GCRL163 phenotype was better stress adapted. The ability of LAB to cope with harsh environmental conditions encountered in food manufacturing has been well documented (Papadimitriou et al., 2016) although it is acknowledged that many contradictions in reported stress responses may arise due to the methodologies used and phenotypic variations. Commonly, LAB respond to rapid environmental changes, such as heat and acid shock, by induction of a common set of stress proteins including chaperones involved in nascent protein folding and management of misfolded proteins, incurring cross-protection from other stressors. How cells cope with multiple stressors or adapt during long-term exposure to threats to aid survival are less commonly documented. Palud et al. (2018) evaluated stress responses following transposon mutagenesis of *L. paracasei* after 24 hr exposure to stressors, identifying many genes not previously documented as stress-responsive and mapping which proteins were stress-specific or induced in responses to several different stimuli. We had also shown

that cultivation of GCRL163 at highly growth-inhibitory temperatures, in contrast to heat shock, caused changes in relative abundance of proteins both in cytosolic extracts (Adu et al., 2018) and at the cell surface (Adu et al., 2020), suggesting that there was considerable movement of proteins to and through the cell surface and identifying regulons not previously linked with heat stress adaptation. Yu et al. (2018) exposed *L. paracasei* Zhang to extended glucose starvation by daily sub-culturing in media with 0.02% or 2% glucose over a period of 8,000 generations (three years). Genome sequencing of survivors across different generations revealed a common set of multiple missense, deletion, and insertion mutations in structural genes and intergenic regions. Mutants performed better in competitive growth challenges with *L. plantarum* CHL as an indicator of improved fitness. Based on the level of mutation observed by Yu et al. (2018) during carbohydrate starvation, we speculate that the large numbers of biotypes that emerge during Cheddar cheese ripening from small numbers of starting NSLAB arise through adaptive evolution as the changing environment within cheese during ripening becomes increasingly harsh due to nutrient starvation and dehydration.

Auxotrophic Traits of GCRL163 and Growth on Amino Acids

L. paracasei GCRL163 displayed multiple amino acid auxotrophy, requiring arginine, threonine, branched-chain, and aromatic amino acids, and serine (not essential but improved growth). Vitamins required for growth were nicotinic and folic acids, riboflavin, pantothenate, and pyridoxine-HCl/pyridoxal. While xanthine was not required for growth, the combination of xanthine plus pyridoxine-HCl or pyridoxal improved growth in media where tryptone was the amino acid source (Supplementary Table S6). Multiple auxotrophic traits are predicted from IMG/G pathway maps for *L. paracasei* and are typical for the species (Gobbetti & Minervini, 2014).

Together with lipid degradation, the catabolism of amino acids is a key factor in producing flavour compounds in cheese and requires the concerted activity of several enzymes involved in transamination, decarboxylation, and deamination, which have been long studied in the LAB particularly in context of acid and nutritional stress responses (Papadimitriou et al., 2016). Degradation of the branched-chained amino acids in *L. casei* group strains involves IlvE which produces 4-methyl-2-oxopentanoic acid (α-ketoisocaproic acid) from *L*-leucine by transamination with a ketoacid (typically α-ketoglutarate), followed by oxidative decarboxylation to 3-methylbutanoyl-CoA (isovaleryl-CoA) by the Bfm complex (KEGG pathway hsa00280), which can subsequently be converted to flavour compounds (aldehydes, alcohols, and esters) (Díaz-Pérez et al., 2016; McSweeney & Sousa, 2000). Because *L*-leucine is both an essential amino acid in *L. paracasei* GCRL163 and a substrate for catabolism in forming cheese flavours, its metabolism is presumably carefully regulated to meet both demands. Wallace and Fox (1997) had shown that the inclusion of cas-amino acids during Cheddar manufacture produced superior flavour and texture relative to controls during ripening for six months, but outcomes depended on the concentration of added cas-amino acids. To determine the impact of amino acids on metabolite synthesised by GCRL163, S3 broths containing 0.2% (growth-limiting) or 1% (nongrowth-limiting) lactose plus high concentrations of individual amino acids (*L*-leucine, *L*-valine, *L*-glycine, or *L*-alanine) and combinations of these were cultured over 46 days (Supplementary Fig. S5). An extended incubation period was required previously to demonstrate the formation of volatile metabolites in S3 media with 0%, 0.2%, or 1% lactose (Hussain et al., 2009b). Combinations of amino acids were tested to determine whether cooperative catabolism occurred in *L. paracasei*, given the observation that Stickland reactions are responsible for amino acid breakdown in some strictly anaerobic Firmicutes including clostridial species and *Peptostreptococcus anaerobius* (Britz & Wilkinson, 1982). Stickland reactions involve one amino acid functioning as a proton donor which is oxidatively deaminated and decarboxylated (such as *L*-alanine, producing acetic acid) while reducing power generated in this reaction effects the reductive deamination of the amino acid proton acceptor (such as *L*-glycine, producing acetic acid), generating 1 mole of ATP for three amino acids consumed (Díaz-Pérez et al., 2016). *L*-Leucine was shown to function in both oxidative and reductive reactions catalysed by a cytosolic enzyme involved in transamination using pyruvate as a ketoacid, and requiring pyridoxal phosphate as cofactor, whereas the decarboxylating and reductive deamination enzymes were membrane- or surface-associated (Britz & Wilkinson, 1983). The original biochemistry for *L*-leucine degradation in these anaerobic species was demonstrated using cell suspensions and high concentrations of *L*-leucine (Britz & Wilkinson, 1982) and similar biochemistry in LAB may well have been undetected due to the membrane location of parts of this metabolic system. In the control cultures lacking additional amino acids, the major end-product of lactose metabolism by GCRL163 was lactate, which showed no change over 46 days for 1% lactose (pH 3.5–4.0) and 0.2% lactose (pH 6.0–6.4) cultures, although the latter produced formic and pyruvic acids as additional minor products likely related to metabolising amino acids in tryptone peptides in the S3 medium. For *L*-leucine supplemented broths, there was no evidence of reductive Stickland reactions, as the signature metabolite isocaproic acid was not detected under the analytical conditions employed. Trace amounts of isovaleric acid (signature for oxidative deamination of *L*-leucine and an expected product of the IlvE/Bfm system) were detected in day 46 in media supplemented with *L*-leucine, *L*-valine, *L*-alanine and the 1% lactose control, likely present in the latter two cultures due to endogenous *L*-leucine in the tryptone supplied in the S3 medium base. Acetic acid abundance increased over time and became a major metabolite by day 46 in all media supplemented with 50 mM of amino acids and the lactate initially produced from 0.2% lactose decreased over time. Transient accumulation of pyruvate and citrate occurred with *L*-leucine supplementation (Fig. 3a and Supplementary Fig. S5) and these trends were dependent on *L*-leucine concentration (Fig. 3b). Formic acid was detected as an early product in all amino acid supplemented media, declining with time for *L*-valine- and *L*-alanine-supplemented cultures but increasing in abundance in S3 media with both *L*-leucine and *L*-glycine. *L*-Leucine and *L*-glycine form a Stickland reaction pair with the former participating in oxidative decarboxylation and the latter in reductive deamination forming acetic acid: whether this paired reaction occurs in *L. paracasei* or synergy between catabolism of *L*-leucine and *L*-glycine is unlinked requires further experimental exploration. However, the notable trend with amino acid-supplement cultures was the initial formation of lactate and its subsequent utilisation, implicating lactate in amino acid catabolism. Speculatively, this may arise due to the conversion of lactate to pyruvate thence to acetic and formic acids (as observed) with possible involvement of pyruvate-formate lyase, as documented in *L. plantarum* under anaerobic conditions (Lindgren et al., 1990). If pyruvate is formed by lactate dehydrogenase, reducing power would also be available for other metabolic processes, such as reductive deamination, and pyruvate could serve

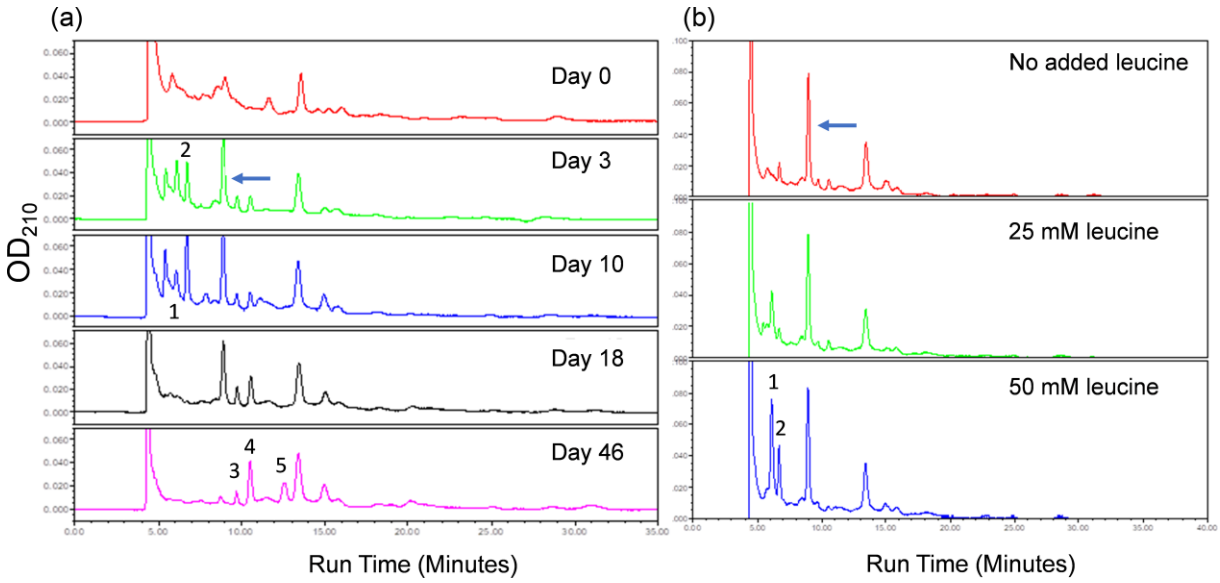

Fig. 3 Impact of high concentrations of *L*-leucine on metabolites. (a) shows a compilation of HPLC chromatograms (C-610H column) for *L. paracasei* GCRL 163 cultures in S3 broths supplemented 0.2% lactose with 50 mM *L*-leucine and sampled for up to 46 days and (b) for 7 days with S3-0.2% lactose with *L*-leucine at 25 or 50 mM. The peak corresponding to lactate is marked with an arrow and other peaks correspond to: 1, citrate; 2, pyruvate; 3, formate; 4, acetate; and 5, propionate. The relative abundance of major metabolites and their temporal detection in mMRS broths containing elevated concentrations of leucine, valine, glycine, or alanine are found in Fig. S5.

as a ketoacid for transamination with amino acids, as seen for the IlvE/Bfm equivalent in clostridial species (Britz & Wilkinson, 1983). Metabolic pathway mapping in KEGG indicates that direct conversion of branched-chain amino acids into acetate does not occur and the underlying biochemistry of how high concentrations of amino acids in a carbohydrate-depleted environment impact pyruvate metabolism was not further explored in the current study.

Growth in the Presence of Tween 80, a Strain-Specific Trait?

Our prior proteomic analysis of lactose starvation involved long-term culture of *L. paracasei* GCRL163 in mMRS with no lactose, 0.2% or 1% lactose, either containing or lacking Tween 80, concluding that Tween 80 was weakly degraded under starvation conditions and suppressed expression of fatty acid synthesis genes in media lacking lactose (Al-Naseri et al., 2013). The media, however, contained citrate in all formulations and its metabolism was indicated by induction of citrate operon proteins and end-products detected. Greater abundance of proteins in the *myo*-inositol pathway was also detected, indicating relief of catabolite repression given that both operons are repressed in the presence of a fermentable carbohydrate (Langa et al., 2021; Montera et al., 2013). Consequently, it was not clear if Tween 80 acted as a growth substrate, although it has been included traditionally in LAB media to improve growth and provide a source of exogenous oleic acid (De Man et al., 1960). Fig. 4(c and d) shows that no significant growth occurred in the basal mMRS media (tryptone, yeast extract, and salts) (<0.5 $\log_{10}$ increase in CFU/ml) lacking any supplements and addition of acetate only marginally improved growth. Including citrate in the basal medium stimulated growth while addition of aetate failed to further increase viable counts. Citrate was considered as a poor growth substrate for *L. casei* group strains (Williams et al., 2000) and to be cometabolised in the presence of a carbohydrate at the later stages of growth, counteracting acidification (Montera et al., 2013). However, our data indicate that citrate acts as a carbon source in the absence of added carbohydrate, although cometabolism of peptides in the basal mMRS remains a possibility given that pyruvate was excreted and there is no proton motive force generated during Ca^{++}-citrate metabolism (Montera et al, 2013). Including Tween 80 in the basal medium (Fig. 4, a and b) resulted in a significant increase in CFU/ml and the addition of citrate (mMRS-TwCit) improved growth above Tween 80 alone and slightly improved growth above citrate alone.

Fig. 5 shows examples of growth kinetics for strains of *L. casei* group isolated from fermented food products when cultured on the basal mMRS medium or mMRS-Tw. While no growth was seen in the basal medium over 13 days and three of 14 strains tested failed to grow on mMRS-Tw, the remainder grew on mMRS-Tw but with different kinetics: they varied in the maximum OD achieved, some showed rapid lysis and one commenced growth after an extended lag period (Supplementary Table S7). However, there was no obvious relationship between the kinetics seen and species within the *L. casei* group or source. This may suggest that the ability to scavenge carbon from lipids, including the incorporation of oleic acid into membrane lipids (Corcoran et al., 2007; Zotta et al., 2017), may be a differentiating phenotypic trait for potential starter NSLAB.

Proteomic Analysis Reveals Elevated Stress and Increased Proteolysis in Cells Cultured in mMRS Supplemented Only With Tween 80

As there was insufficient biomass generated in the control cultures lacking either Tween 80 or citrate (Fig. 4), proteomic analyses were conducted on cytosolic fractions of stationary-phase cells from mMRS-Tw and mMRS-TwCit cultures (Fig. 6; SpC data plus statistical analyses are shown in Supplementary Tables S8–S10). This analysis complements our prior report on lactose starvation in GCRL163, which effectively compared mMRS-Cit and mMRS-TwCit (Al-Naseri et al., 2013) in the absence of lactose. Given that Tween 80 was in both conditions tested in the current report and the combination of Tween 80 plus citrate improved growth above

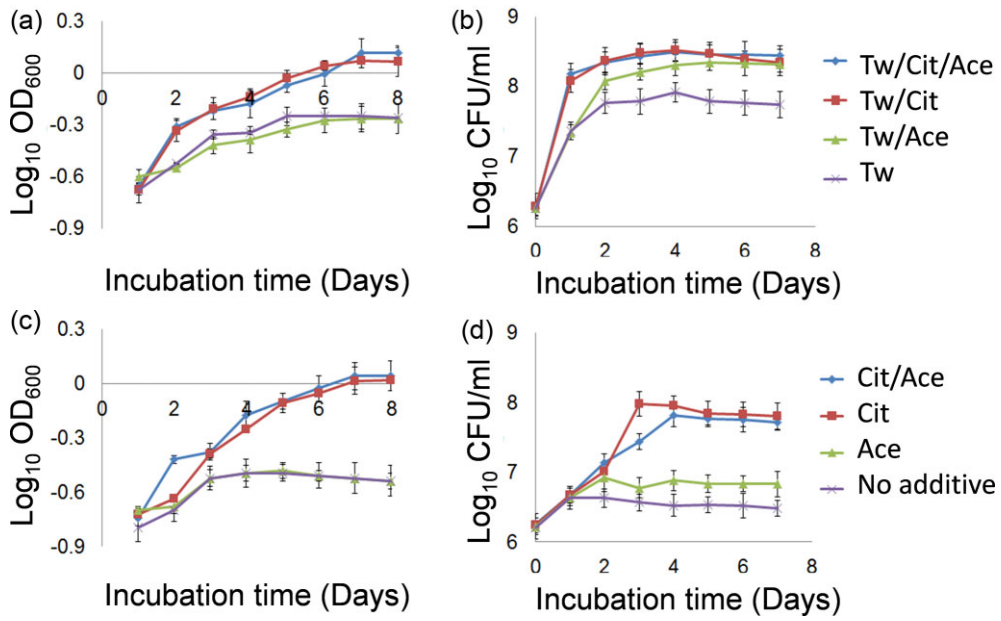

Fig. 4 Growth kinetics of strain GCRL163 in mMRS containing Tween 80 (Tw), triammonium citrate (Cit), acetate (Ace), and combinations of these supplements. The basal medium contained bacteriological peptone, yeast extract and mineral salts without an added carbohydrate source. Panels a and b: growth curves from OD_{600} readings and viable counts on MRS plates during culture in media containing Tween 80. Panels c and d: growth curves for media lacking Tween 80. Standard deviations for triplicate cultures are shown.

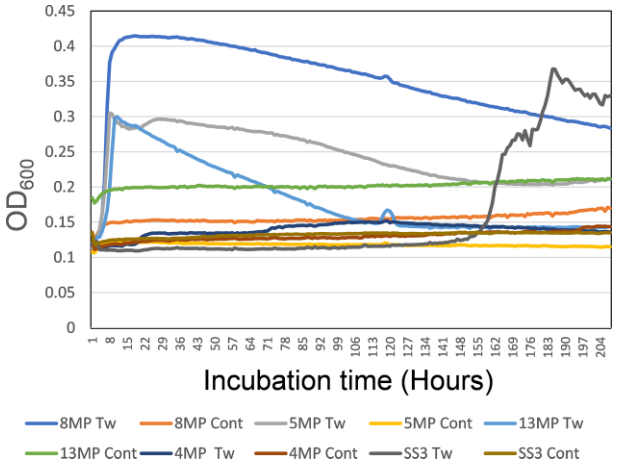

Fig. 5 Variation in growth kinetics of selected dairy isolates of *L. casei*-group isolates in media supplemented with Tween 80. The mMRS broth control contained the basal salts solution, tryptone and yeast extract (control, Cont) or this medium supplemented with 0.1% (vol/vol) Tween 80 (Tw). The origin of the isolates and their species are documented in Supplementary Tables S1 and S5; Table S7 summarizes the kinetics for all strains tested. Strains 8MP and SS3 are *L. rhamnosus* and the remainder are *L. casei/paracasei* strains.

either supplement alone, comparing mMRS-Tw with mMRS-TwCit data would presumably detect changes in relative abundance of proteins impacted by cooperative metabolism of Tween 80 plus citrate and altered expression of proteins in the absence of citrate as an added carbon source.

Over 1,800 proteins were detected in at least one replicate of the two growth conditions and many of the proteins were functionally uncharacterised (Supplementary Table S8). Statistical analyses of functional classes were conducted by filtering for proteins detected in a minimum of two replicates (Fig. 6). Most functional classes remained largely unchanged between the two culture conditions (T-value differences $<\log_2 = 1$). However, when citrate was present functions associated with greater glycolytic flux and carbohydrate metabolism, protein synthesis (ribosomes, tRNA aminoacyl synthesis) and the tricarboxylic acid pathway increased, consistent with higher growth rate, flux towards peptidoglycan synthesis (indicated by higher relative abundance of GlmS) and citrate utilisation. In contrast, cells from mMRS-Tw showed higher abundance in lipid-related metabolism, cell-surface proteins/internalins, DNA repair/recombination, and transporters (combined ABC-type and phosphotransferase systems) (Fig. 6a). This was reflected in relative abundance changes for individual proteins which were significantly highly up- or downregulated (Fig. 6b; Supplementary Table S9, which shows the statistical analysis of 554 proteins detected in a minimum of three replicates in one condition, and Table S10, which summarises highly differentially expressed proteins where $\delta\log_2 > \pm 0.9$, total 238 proteins). Some of the most highly upregulated proteins during culture in mMRS-TwCit were associated with pyruvate metabolism, including pyruvate oxidase (Pox5), acetate kinase (AckA), pyruvate formate lyase (PflB), and the pyruvate formate-lyase activating enzyme (PflA) (Supplementary Table S11), which is consistent with the enzymology of citrate catabolism in this species (Montera et al., 2013), and our prior study on proteomics and end-products synthesised during lactose starvation (Al-Naseri et al., 2013). Interestingly, while citrate operon proteins were upregulated in cytosolic preparation from cells cultured in mMRS-TwCit (Supplementary Table S11), all of these proteins were still readily detectable in preparations from cells cultured in mMRS-Tw, in addition to the upregulation of fumarate reductase flavoprotein subunit (IfcA). In the absence of citrate, glycerol catabolism proteins (GlpK and GlpD), pentose phosphate (XpaK and SgaU) and fructose/mannose catabolism (tagatose-6-phosphate kinase, FruK) pathway proteins and the glycolytic protein phosphoglycerate kinase (Pgk) were all highly more abundant ($\delta\log_2 = 1.16$–5.60) (Supplementary Table S11). This

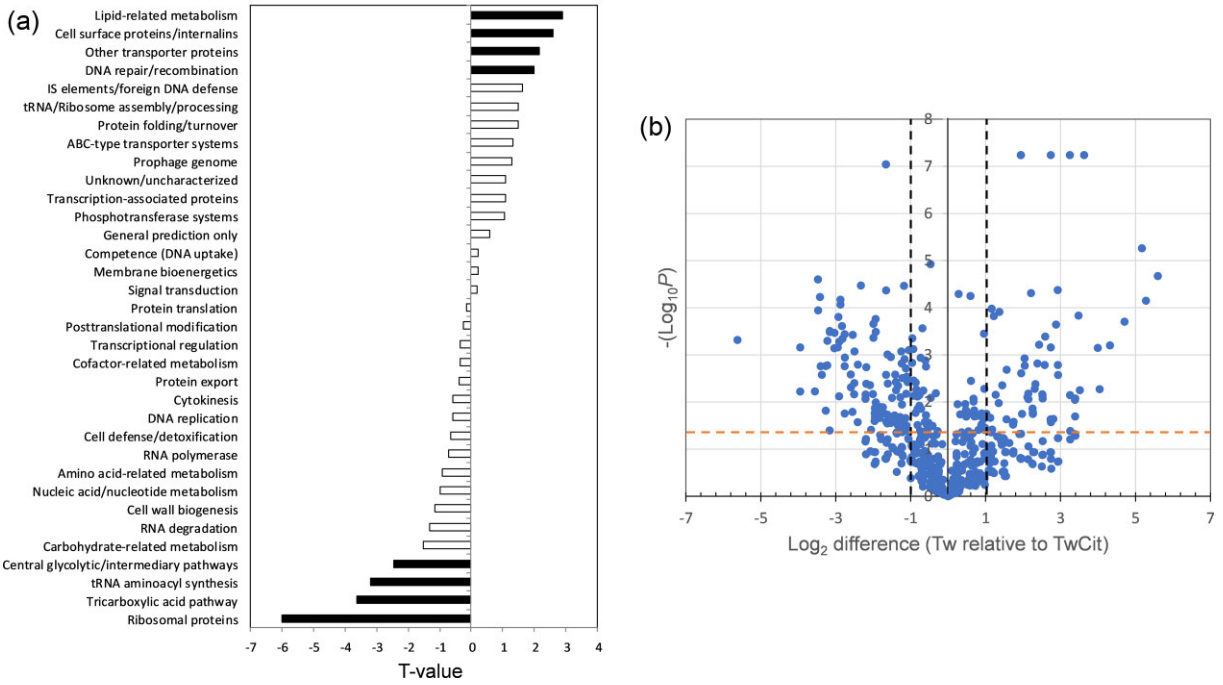

Fig. 6 Relative abundance differences for proteins extracted from cells of *L. paracasei* GCRL 163 cultured in mMRS broths supplement with Tween 80 relative to cells cultured in Tween 80 plus citrate. Panel (a) shows T-value differences for proteins grouped into functional classes ($p < 0.05$) with differences >±2 highlighted (black bars). The volcano plot (b) was constructed using all proteins with peptides detected in a minimum of two replicates in either growth condition (total 974 proteins). The black hatched line indicates $\log_2 = \pm 1$ (twofold) differences and the orange hatched line indicates $p = -\log_{10}(0.05)$.

was accompanied by increased relative abundance of transport proteins associated with ribose, mannitol, mannose, and fructose uptake (Table 2). Notably, genes encoding FruA3 and FruK are in the same operon and therefore are ostensibly coregulated. Whether these observations are linked directly to Tween 80 metabolism, or represent a response to nutrient starvation more generally, remains to be established, given that modulation of a variety of transporters occurs for many stressors (Papadimitriou et al., 2016) and BglP (beta-glucoside uptake, $\delta\log_2 = 2.15$, Table 2) was shown to be a general stress indicator in *L. paracasei* (Palud et al., 2018).

While numerous peptidases were detected in cytosolic extracts from both growth conditions, their relative abundance differences were not significant (Supplementary Table S9). Table 2 summarises the relative abundance differences ($\delta\log_2$ >±1) for amino acid-related proteins, highlighting the relatively greater abundance of three CEPs in extracts prepared from cells cultured in mMRS-Tw as a key difference, in addition to other peptidases which are differentially detected between growth conditions. CEPs in lactobacilli and other Gram-positive species are large, modular proteins which may contain domains that include: signal peptides (typically the rarer KxYKxGKxW type), cell wall anchor motifs, peptidase_S8 (subtilase family), fn3_5 motif (fibronectin-like domain), and other domains. They vary in molecular weight and domain composition. The PrtP protein in *L. paracasei* and PrtR in *L. rhamnosus* are well characterised and are involved in casein degradation (Ji et al., 2021), although the annotation is often not clear, as seen for the gene in *L. paracasei* W56 named Dumpy. The Dp gene is located in the same genomic region as PepN but on the opposite strand. Dp is adjacent to a second Prt-like protein but separated by an intergenic region, which may imply differential regulation (Supplementary Fig. S6). The two CEP genes in this region (Dp, BN194_05390, and BN194_05400) are distinguished based on size (encoding proteins with 1 836 and 2 239 amino acids) but share a similar domain architecture, 40% sequence similarity, and are distinct from PrtP (BN194_24060, domain structures differ and 12–15% sequence similarity is seen between Dp and BN194_05400 and PrtP). Although several possible transcriptional factors have been proposed in regulation of the proteolytic systems of *Lactobacillus* (Alcántara et al., 2016; Brown et al., 2017), the intergenic region of *prtP* contains a *cre* site, indicating catabolite control regulation of expression (Catone et al., 2021). We mapped the intergenic region for strain GCRL163 and confirmed the same layout of promoter and *cre* site, implicating CcpA regulation of expression of PrtP in the presence of citrate. Mapping the intergenic region between the Dp gene and *pepN* indicated two possible transcriptional factor-binding regions, one (BPROM data) well upstream from the initiation codon for Dp and a second (Tomtom data) immediately upstream (with similarity to a TetR regulator-binding motif) (Supplementary Fig. S7). Given the relatively lower abundance of the CEPs and some peptidases in cells from mMRS-TwCit cultures, and greater abundance of transporters associated with transporters of polar amino acids in mMRS-Tw (Table 2), citrate likely represses expression of peptide degradation, suggesting a complex regulatory network for citrate catabolism where its own degradation is CcpA controlled in the presence of fermentable sugars but its presence may also engage regulatory networks that employ CcpA. This remains to be investigated experimentally, although several regulators were differentially detected in the two growth conditions (Supplementary Table S11), including higher abundance of CcpA, ArlR, and Rex in cells cultured in mMRS-TwCit.

An interesting observation was that the relative abundance of fatty acid synthesis proteins was higher in mMRS-Tw,

Table 2. Relative abundance differences of cytosolic proteins of *L. paracasei* GCRL 163 following culture in mMRS supplemented with Tween 80 or mMRS supplemented with Tween 80 plus citrate

Gene locus	Gene/protein symbol	Protein name	Function	δLog$_2$[a]	*P*	negative log$_{10}$*P*[b]
Amino acid-related metabolism						
BN194_24060	PrtP	PII-type proteinase	protein degradation (casein)	2.809	0.023022	1.638
BN194_16390	BfmBAB	2-oxoisovalerate dehydrogenase subunit beta	BC-AA degradation/BCFA primer synthesis	1.636	0.074153	1.130
BN194_05400	BN194_05400	cell-envelope associated proteinase	peptidase for amino acid acquisition	1.557	0.023372	1.631
BN194_24670	PepC	cysteinyl aminopeptidase, peptidase C1 family	peptidase for amino acid acquisition	1.492	0.060279	1.220
BN194_05390	Dp	large cell wall associated protein with peptidase domain	peptidase for amino acid acquisition	1.414	0.066889	1.175
BN194_18260	YghT	PepP_like Xaa-Pro aminopeptidase,	peptidase for amino acid acquisition	1.152	0.044253	1.354
BN194_02530	YjbG	oligoendopeptidase F	peptidase for amino acid acquisition	−1.278	0.020914	1.680
BN194_13540	GlyA	serine hydroxymethyltransferase	glycine biosynthesis; folate biosynthesis; one carbon pool by folate	−1.391	0.003797	2.420
BN194_09790	PatB2	cystathionine beta-lyase PatB	L-methionine biosynthesis	−1.622	0.018477	1.733
BN194_05410	PepN	aminopeptidase N	peptidase for amino acid acquisition	−1.738	0.013007	1.886
BN194_21160	FpaP3	Proline iminopeptidase	peptidase for amino acid acquisition	−1.812	0.050407	1.298
BN194_24910	ProB	glutamate 5-kinase	L-proline biosynthesis	−2.206	0.069196	1.160
BN194_08130	YjbG2	oligoendopeptidase F	peptidase for amino acid acquisition	−2.592	0.004919	2.308
Protein folding and stress-related						
BN194_29440	BN194_29440	Acid shock protein, alpha-crystallin domain heat shock protein	protein disaggregation (during rapid growth)	5.172	5.51E-06	5.259
BN194_06990	Csp	Cold shock protein 1	RNA chaperone	2.923	4.23E-05	4.374
BN194_25470	AldA	lactaldehyde dehydrogenase/glycolaldehyde dehydrogenase	detoxification of aldehydes	2.740	5.78E-08	7.238
BN194_04740	Npr	NADH peroxidase	oxidative stress management	2.592	0.000411	3.386
BN194_23760	GroES	class I heat-shock protein (chaperonin) small subunit	nascent protein folding	2.385	0.001533	2.814
BN194_19450	ClpE	ATP-dependent Clp protease ATP-binding subunit ClpE	recycling defective proteins	2.118	0.007895	2.103
BN194_01950	Asp23_2	alkaline shock protein 23 family protein	Cell surface maintenance?	2.046	0.001703	2.769
BN194_12460	CspLA	Cold shock-like protein CspLA	RNA chaperone	2.039	0.00119	2.924
BN194_19460	ClpB	ATP-dependent chaperone/Clp protease	recycling defective proteins	0.975	0.039465	1.404
BN194_17460	DnaK	chaperone protein DnaK	nascent protein folding; protein rescue	0.944	0.023068	1.637
BN194_16010	HslU	ATP-dependent protease ATPase subunit HslU	recycling defective proteins	−1.431	0.019383	1.712
BN194_04910	BN194_04910	penicillin acylase family protein	detoxification (aminohydrolase)	−1.613	0.061791	1.209
BN194_22870	BN194_22870	universal stress protein, UspA superfamily	stress related	−2.184	0.00412	2.385
BN194_30010	Maa	maltose O-acetyltransferase family protein	detoxification	−3.033	0.000342	3.466
BN194_07010	BN194_07010	Cys-based peroxiredoxin, OsmC superfamily	hydroperoxide removal	−3.371	0.00265	2.577
Phosphotransferase systems						
BN194_30430	MtlA_2	PTS (Fructose/Mannitol family) subunit IIBC	mannitol uptake	2.254	0.013915	1.857
BN194_06940	BglP	PTS system beta-glucoside-specific transporter subunit IIBCA	beta-glucoside uptake	2.145	0.008774	2.057
BN194_15410	FruA3	PTS (fructose family) subunit IIABC	fructose uptake	1.729	0.028136	1.551
BN194_07330	LacF2	PTS(Lactose family) IIA subunit	lactose uptake	1.343	0.054111	1.267
BN194_03000	LevD/ManX	PTS(Glucose/Mannose family) IIA	glucose/mannose uptake	1.268	0.007048	2.152
BN194_29720	ManX	PTS (Mannose/Fructose/Sorbose family) IIAB	glucose/mannose uptake	−1.082	0.003784	2.422
BN194_02970	LevE/ManX	PTS(Glucose/Mannose family) IIB	glucose/mannose uptake	−1.222	0.043717	1.359
BN194_19660	CelA	PTS system (Lactose/DACB/beta-glucoside family) IIB	cellobiose uptake	−1.950	0.02112	1.675
BN194_19410-20	PtsI	phosphoenolpyruvate-protein phosphotransferase	PTS general components	−3.265	0.001734	2.761

Table 2. Continued.

ABC-type transporter systems						
BN194_07710	BN194_07710	ABC-type transporter, substrate-binding protein	unknown transport	3.255	0.007175	2.144
BN194_03290	RbsB	ribose ABC-type transport system, substrate-binding protein	ribose uptake	2.934	0.00268	2.572
BN194_00680	OpuCA	glycine betaine/carnitine/choline ABC-type transporter, ATP-binding protein	carnitine/choline/glycine betaine uptake	2.931	0.058279	1.234
BN194_21150	YjbQ	Trk-type K+ transport ATPase	potassium ion uptake	2.314	0.005291	2.276
BN194_29910	GlnH4	polar amino acid ABC-type importer, permease component	polar amino acid uptake	2.264	0.017898	1.747
BN194_04050	BN194_04050	ABC-type sugar transport system, auxiliary component	unknown function	2.261	0.016756	1.775
BN194_29920	GlnH5	polar amino acid ABC-type importer, permease component	polar amino acid uptake	2.254	0.013915	1.856
BN194_07220	YkpA	ABC transporter ATP-binding protein YkpA	unknown transport	1.736	0.03015	1.520
BN194_05030	YceJ	MFS-type transporter	unknown transport	1.152	0.044253	1.354
BN194_11200	MdxE/MalE	maltose/maltodextrin ABC-type transporter, substrate binding protein	maltose/maltodextrin uptake	−1.61	0.0041	2.387
BN194_29810	BN194_29810	sugar/glycerol-3-phosphate uptake ABC-type importer, substrate-binding protein	sugar/glycerol-3-phosphate uptake	−1.784	0.015518	1.809
BN194_11260	UgpB	glycerol-3-phosphate ABC transporter substrate-binding protein	sugar/glycerol uptake	−2.183	0.00184	2.735

[a]Highest abundance changes are highlighted in red, grading to lowest in green for proteins with $\log_2$ differences >±1 $\log_2$ for cells from mMRS-Tw relative to mMRS-TwCit.
[b]Most significant changes are graded in blue, least significant changes graded in yellow with mid-point (no colour) set for $-\log_{10}P$ when $p = 0.05$.

contradicting our previous findings (Al-Naseri et al., 2013), the reported incorporation of Tween 80 into cytoplasmic membranes (Zotta et al., 2017) and reported suppression of fatty acid synthesis genes by Tween 80 in *L. plantarum* (Reitermayer et al., 2018). Several of the fatty acid synthesis proteins were detected with very low SpC in extracts from cells cultured in mMRS-TwCit. Given that Tween 80 was present in both media, it is likely that fatty acid synthesis repression was greater in the presence of an alternative fermentation substrate such as citrate. When cultured in mMRS broths with 1% lactose buffered at pH 2.5, either in the presence or absence of Tween 80, the survival of strain GCRL163 was significantly improved in the presence of Tween 80 (Supplementary Fig. S8), suggesting that even in the presence of lactose, Tween 80 is scavenged into cell membranes to protect against acid stress.

Table 2 shows that culturing in mMRS-Tw resulted in relatively higher abundance of stress-related proteins, although chaperone proteins GroES, DnaJ, and GrpE, and other Clp proteins, were not significantly changed in abundance (Supplementary Table S9). However, the greater abundance of other stress proteins (notably the acid shock protein, $\delta\log_2 = 5.17$; cold shock proteins and an Asp23/alkaline shock protein associated with cell wall stress, Yu et al., 2018), and NADH peroxidase (Npr) indicates a greater stress level of cells in mMRS-Tw that is reminiscent of oxidative stress management (Zotta et al., 2017).

Overall, the proteomic data suggest that culturing in Tween 80 in the absence of a fermentable carbon source, such as citrate, induces a stress state that increases proteolysis and amino acid metabolism. This is linked to catabolite control regulation and accords with reports in *L. plantarum* that Tween 80 influences the metabolic profile in favour of producing aroma compounds from amino acid catabolism (Parlindunga et al, 2019). Evaluating the regulons impacted during nutrient starvation and how Tween 80 modulates protein expression is the subject of our continuing investigations.

Conclusion

Selecting NSLAB strains that may contribute to the final product traits of ripening cheese often relies on evaluating technical properties which are largely shared within species. We found that genetic profiling based on PFGE was not sufficient to predict the subtle phenotypic differences in three *L. paracasei* strains isolated at different timepoints during Cheddar cheese ripening and that longer cultivation periods were required to demonstrate the full metabolic capacity of isolates, particularly for carbohydrate utilisation and survival at low pH. Long lag periods were observed before commencing growth on both *D*-ribose and, for some other cheese isolates, on Tween 80, indicating adaptation was required to cope with new nutritional environments. Given that strain GCRL163 survived exposure to low pH longer, we concluded that this phenotype was better stress adapted. The ability to scavenge carbon during nutrient starvation, including degradation of peptides and assimilation of fatty acids, may be more indicative traits when screening cheese isolates for their potential as adjunct NSLAB. Proteomic analysis established that in the absence of a fermentable carbon source, such as citrate, stress proteins, but not chaperones, were induced and proteolysis due to CEPs increased. Whether these observations are linked directly to Tween 80 catabolism or to a general nutritional stress response remains to be established.

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Supplementary Material

Supplementary material is available online at *JIMB* (*www.academic.oup.com/jimb*).

Author Contributions

Conceptualisation: Britz M.L.; methodology: Baker, A.L., Wilson, R., Bowman, J.P., and Britz, M.L.; data curation: Wilson, R., Bowman, J.P., and Britz, M.L.; investigation: Al-Naseri, A., Shah S.S, Rouch, D.;

writing-original draft preparation: all; writing-review and editing: all; project administration: Britz, M.L. and Bowman, J.P.

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Conflict of Interest

The authors declare no conflict of interest.

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