

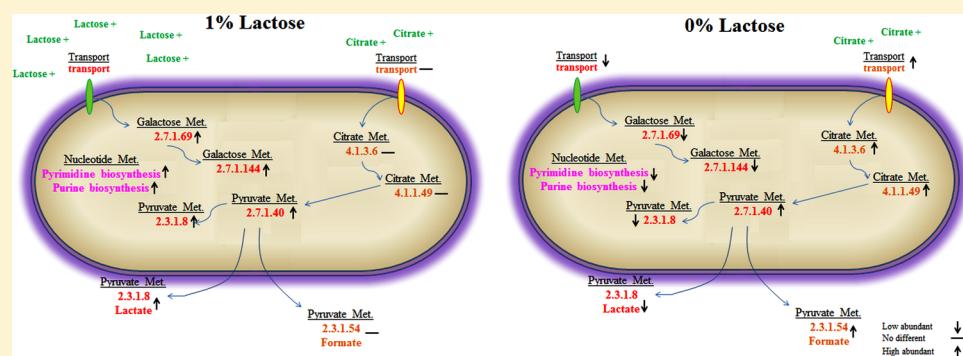
## Impact of Lactose Starvation on the Physiology of *Lactobacillus casei* GCRL163 in the Presence or Absence of Tween 80

Ali Al-Naseri,\*† John P. Bowman,† Richard Wilson,‡ Rolf E. Nilsson,† and Margaret L. Britz†

†Food Safety Centre, Tasmanian Institute of Agricultural Research, School of Agricultural Science, University of Tasmania, Private Bag 54, Hobart, Tasmania 7001, Australia

‡Central Science Laboratory, University of Tasmania, Hobart, Tasmania 7001, Australia

### Supporting Information



**ABSTRACT:** The global proteomic response of the nonstarter lactic acid bacteria *Lactobacillus casei* strain GCRL163 under carbohydrate depletion was investigated to understand aspects of its survival following cessation of fermentation. The proteome of *L. casei* GCRL163 was analyzed quantitatively after growth in modified MRS (with and without Tween 80) with different levels of lactose (0% lactose, starvation; 0.2% lactose, growth limiting; 1% lactose, non-growth-limited control) using gel-free proteomics. Results revealed that carbohydrate starvation lead to suppression of lactose and galactose catabolic pathways as well as pathways for nucleotide and protein synthesis. Enzymes of the glycolysis/gluconeogenesis pathway, amino acid synthesis, and pyruvate and citrate metabolism become more abundant as well as other carbohydrate catabolic pathways, suggesting increased optimization of intermediary metabolism and scavenging. Tween 80 did not affect growth yield; however, proteins related to fatty acid biosynthesis were repressed in the presence of Tween 80. The data suggest that *L. casei* adeptly switches to a scavenging mode, using both citrate and Tween 80, and efficiently adjusts energetic requirements when carbohydrate starved and thus can sustain survival for weeks to months. Explaining the adaptation of *L. casei* during lactose starvation will assist efforts to maintain viability of *L. casei* and extend its utility as a beneficial dietary adjunct and fermentation processing aid.

**KEYWORDS:** *Lactobacillus casei*, proteomics, starvation, Tween 80, lactic acid bacteria

## INTRODUCTION

Foods that are commonly used to deliver probiotic microorganisms to consumers include fermented dairy products (particularly fermented milk), yogurts, and cheese. A number of studies have investigated the potential of cheddar cheese as a probiotic delivery system because this product is a naturally rich source of probiotic bacterial genera and species.<sup>1–3</sup> Cheese-associated bacteria have been divided into two broad groups: starter lactic acid bacteria (SLAB) and secondary microorganisms.<sup>4</sup> SLAB are involved in acid production during manufacture and contribute to the curding and early ripening processes.<sup>4</sup> Secondary microorganisms do not substantially contribute to acid production; instead, they contribute to cheese ripening and flavor production.<sup>5</sup> Lactic acid bacteria are highly represented in this group and are commonly referred to as nonstarter lactic acid bacteria (NSLAB). *Lactobacillus casei* is a

dominant NSLAB in cheese, growing internally in most cheese varieties.<sup>6–8</sup>

The genus *Lactobacillus* comprises Gram-positive, non-spore-forming aerotolerant anaerobic bacteria.<sup>9</sup> *Lactobacillus* spp. are generally regarded as safe due to their long association with fermented foods and their natural presence in the small and large intestine,<sup>10</sup> and through transient adaptation to the mammalian host they can provide health benefits. These probiotic-related interactions are strain-specific,<sup>11</sup> and consequently research investigating strain-specific probiotic factors as well as the use of *Lactobacillus* spp. in food products, particularly cheese, has increased.

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Environmental stresses such as osmotic pressure, cold, heat, oxidation, pH, and starvation can limit the growth of NSLAB during cheese ripening and in other manufacturing processes. Among these stresses, nutrient starvation, in particular, of fermentable substrates, is reportedly the most important limitation to growth.<sup>12</sup>

It is reported that cheese is deficient in lactose.<sup>13</sup> Lactobacilli, when faced with carbohydrate limitation, modulate several genetic regulons that are associated with different stress responses.<sup>14</sup> Bacteria adapt to nutrition limitation by assuming a physiological state that is characterized by the down-regulation of nucleic acid and protein synthesis and simultaneous up-regulation of protein degradation and amino acid synthesis.<sup>14,15</sup>

The physiological effect of lactose starvation and subsequent adaptations on the NSLAB was originally conducted by Hussain and colleagues<sup>14</sup> using a proteomic approach on *L. casei* GCRL163, originally a cheddar cheese isolate, which was reported by Chandry.<sup>16</sup> Their data were based on 2-D gel-based analyses, which have limits in the capacity to adequately quantify protein abundance and comprehensively examine a proteome.<sup>15</sup> Examination of metabolites produced during and after growth in a semidefined medium suggested that lipid degradation occurred as a consequence of starvation, which may have arisen from utilization of endogenous lipids through scavenging or from degradation of Tween 80. Tween 80 is usually added to media to optimize the growth of lactobacilli; however, it is not required for the growth of *L. casei*.<sup>17</sup> In the present study, the effect of different levels of lactose on the growth and physiological adaptations of *L. casei* strain GCRL163 to different levels of carbohydrate starvation was examined using gel-based and liquid chromatography/mass spectrometric-based proteomic approaches. The study also focuses on whether *L. casei* GCRL163 has the ability to utilize Tween 80 as a carbon source during starvation by breaking down the polyethoxylated sorbitan section of the molecule. The goal of the research was to understand more thoroughly the responses of a typical NSLAB cheese strain to carbohydrate starvation as part of the process to develop more effective ways to deliver food-associated probiotic microorganisms and to evaluate the metabolic capacity of NSLAB during starvation phases of fermented food products.

## MATERIAL AND METHODS

### Bacterial Strain and Growth Conditions

*L. casei* strain GCRL163, a cheddar cheese isolate,<sup>16</sup> was obtained from the Gilbert Chandler Research Laboratory (The University of Melbourne) strain collection and restored at the University of Tasmania under its original name and strain number. The strain was maintained in 40% glycerol in de Man Rogosa and Sharpe (MRS; Oxoid, Australia) broth at -80 °C. Appropriate dilutions of culture grown for 12 h in MRS broth were plated on MRS agar and incubated anaerobically using the Anaerocult A system (Oxoid, Australia) at 37 °C for 48 h. A single colony from the plate was resuspended in MRS broth. One individual colony represented one biological replicate. Cells were passaged through two sequential subcultures in MRS broth incubated at 37 °C for 12 h under anaerobic conditions. Bacterial cells were harvested during late stationary growth phase and washed twice with potassium phosphate buffer (PBS, pH 7.0) and subcultured in modified MRS (mMRS) consisting of 1% (w/v) bacteriological peptone, 0.5% (w/v) yeast extract, 0.1% (v/v) Tween 80, 0.5% (w/v) sodium acetate, 0.2% (w/v) triammonium citrate, 0.005% (w/v) MgSO<sub>4</sub>·7H<sub>2</sub>O, and 0.025% (w/v) MnSO<sub>4</sub>·4H<sub>2</sub>O in 0.35

M PBS at pH 6.2 ± 0.2 also containing either 0, 0.2 or 1% (w/v) lactose. All cultures were incubated at 30 °C for 12 h under anaerobic conditions.

### Starvation Experiments

Buffered mMRS with three different lactose concentrations (1, 0.2, and 0% w/v) were inoculated with *L. casei* GCRL163 to give an initial optical density (OD) of 0.021 ± 0.002 at 600 nm. The cultures were then incubated at 30 °C for 30 days under anaerobic conditions as previously described.<sup>14</sup> Tween 80 was included in the medium because it reportedly enhances survival. To test the impact of Tween 80 on survival in mMRS broth, media either containing or lacking Tween 80 were also used in these experiments.<sup>18</sup> Cell growth was monitored by measuring OD, determining viable counts on MRS agar and calculating biomass yield as dry weight.

Cultures were harvested on days 1, 2, 4, 6, 8, 15, and 30 (150 mL each) using centrifugation at 5000 rpm for 10 min at 4 °C, and the cell pellets were washed three times with Tris-HCl buffer (pH 7.0). The cells were resuspended in Tris-HCl buffer (pH 7.0), concentrating to a standard OD. The samples were stored at -80 °C until selected for protein extraction. Three biological replicates were tested for each medium, triplicate cultures each set up from a single colony. Then samples were withdrawn at each time point for proteomic analysis.

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), analyses were performed on protein extracts for cells harvested after 1, 2, 4, 6, and 8 days of incubation to determine which samples would be used for subsequent analysis based on evaluating when cultures had entered stationary phase and when further visual changes in protein banding patterns thereafter were not evident. Proteomic analysis by nano HPLC and tandem mass spectrometry (nanoLC-MS/MS) was performed on cells harvested during the stationary growth phase (day 4 for 1 and 0.2% lactose; day 6 for 0% lactose).

### End-Product Analysis

The key end products of *L. casei* GCRL163 grown under different levels of lactose in the presence and absence of Tween 80 were quantified. Formic acid and lactic acid levels in culture supernatants were determined using an Acquity H-series UPLC-Xevo triple quadrupole mass spectrometer fitted with a BEH C18 column (2.1 × 100 mm × 1.7 μm particles) (Waters, Milford, MA). The solvent used for formate was 1% (v/v) acetic acid for 1 min, followed by a linear gradient to 20% acetonitrile at 4 min at a flow rate of 0.35 mL/min. For lactate, the initial hold with 1% acetic acid was 30 s with a gradient to 100% acetonitrile at 4 min. The column was held at 45 °C and the sample compartment was maintained at 6 °C. The mass spectrometer was operated in negative ion electrospray mode with a needle voltage of 2.3 kV, and selected ion monitoring was used for formate and selected reaction monitoring was used for lactate. The ion source temperature was 130 °C, the desolvation gas was nitrogen at 950 L/h, the cone gas flow was 50 L/h, and the desolvation temperature was 450 °C. The ion at *m/z* 45.0 was monitored for formic acid with a cone voltage of 30 V and a dwell time of 120 ms. A simultaneous negative ion full scan was acquired from *m/z* 40 to 300 in 200 ms. For lactate, the transition from *m/z* 87 to 43 was monitored with a cone voltage of 22 V, a collision energy of 7 V, and dwell of 50 ms. For external standardization calibration, a 200 μL aliquot of 10 different samples was pooled to create a solution for preparation of the matrix-matched spiked standard solutions.

Acetic acid, ethanol, caproic acid, and octanoic acid levels in culture supernatants were determined using a Varian HP-5 ms column ( $30\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$ ) on a Varian 3800 GC coupled to a Bruker-300 triple quadrupole mass spectrometer or a Varian 450-GC possessing a flame ionization detector. The flow rate used routinely was  $1.2\text{ mL/min}$ , the injector temperature was  $220\text{ }^\circ\text{C}$ , and  $1\text{ }\mu\text{L}$  injections split 10:1 were made. For acetic acid, the oven program was  $40\text{ }^\circ\text{C}$ , held for 2 min, followed by a ramp up to  $160\text{ }^\circ\text{C}$  at  $25\text{ }^\circ\text{C/min}$ . Ions from  $m/z$  35 to 200 were scanned every 0.18 s, and SIM ions at  $m/z$  31, 45, and 60 were monitored for 40 ms each. Quality control samples ( $n = 6$ ) at the 0.5% level were run after every 10 samples and gave an average of 0.47% with a standard deviation of 0.0098% (2.1% RSD). For ethanol, caproic acid, and octanoic acid, the injector temperature was raised to  $240\text{ }^\circ\text{C}$ , and oven program was  $45\text{ }^\circ\text{C}$  for 5 min, followed by a ramp up to  $160\text{ }^\circ\text{C}$  at  $25\text{ }^\circ\text{C/min}$ , and  $1\text{ }\mu\text{L}$  injections split 30:1 were made.

#### SDS-PAGE

Cytosolic proteins were extracted by bead beating  $200\text{ }\mu\text{L}$  of the cell suspensions using  $0.5\text{ g}$  of zircon beads ( $0.1\text{ mm}$ ; Daintree Scientific, TAS, Australia) in a bead beater for 6 min. Cellular debris were removed by centrifugation at  $14\,000\text{ rpm}$  for 30 min at  $4\text{ }^\circ\text{C}$ . The protein concentration in extracts was determined using the BCA kit (Thermo Fisher Scientific, USA) using bovine serum albumin as a standard. Equal amounts of the sample buffer (1:1) were added to the protein samples and mixed by vortexing for 30 s, after which they were heated for 10 min. The samples were then centrifuged to remove any remaining cell debris. Six  $\mu\text{g}$  of protein was loaded in each lane of the SDS-polyacrylamide gels (resolving gel, 10%; stacking gel, 4%) and run at  $100\text{ V}$  for 90 min. Following electrophoresis, the gels were visualized by silver staining.<sup>19</sup>

#### nanoLC-LTQ-Orbitrap Tandem Mass Spectrometry

Protein samples were analyzed by nanoLC-MS/MS using an LTQ-Orbitrap XL (ThermoFisher Scientific). Equal quantities of each sample ( $50\text{ }\mu\text{g}$ ) were sequentially reduced, alkylated, and trypsin-digested, as previously described.<sup>20</sup> Normalization of total spectral counts (SpCs) for each biological sample was used to compensate for minor differences in protein concentration, variation in trypsin digestion efficiency between samples, and peptide loading. Aliquots of tryptic peptides equivalent to 50% of the in-solution digests were loaded at  $0.05\text{ mL/min}$  onto a C18 capillary trapping column (Peptide CapTrap, Michrom Bio-Resources) controlled by an Alliance 2690 Separations Module (Waters). Peptides were then separated on an analytical nanoHPLC column packed with  $5\text{ }\mu\text{m}$  C18 media (PicoFrit Column,  $15\text{ }\mu\text{m}$  i.d. pulled tip, 10 cm, New Objective) controlled using a Surveyor MS Pump Plus (ThermoFisher Scientific) at  $200\text{ nL/min}$  over a four-step gradient of 100% buffer A (5% acetonitrile in 0.2% formic acid) to 100% buffer B (90% acetonitrile in 0.2% formic acid) using the following steps: 0–10% B over 7.5 min; 10–25% B over 50 min; 25–55% B over 20 min; 55–100% B over 5 min; holding at 100% B for 15 min; and re-equilibration in 100% A for 15 min.

The LTQ-Orbitrap XL was controlled using Xcalibur 2.0 software (ThermoFisher Scientific) and operated in data-dependent acquisition mode, where survey scans were acquired in the Orbitrap using a resolving power of 60 000 (at  $400\text{ m/z}$ ). MS/MS spectra were concurrently acquired in the LTQ mass analyzer on the eight most intense ions from the FT survey scan. Charge state filtering, where unassigned and singly charged precursor ions were not selected for fragmentation, and dynamic

exclusion (repeat count 1, repeat duration 30 s, exclusion list size 500) were used. Fragmentation conditions in the LTQ were: 35% normalized collision energy, activation  $q$  of 0.25, 30 ms activation time, and minimum ion selection intensity of 500 counts.

#### Database Searching and Criteria for Protein Identification

Centroid mode spectra acquired were converted from .RAW files into .mzXML peak list files using the msConvert command (Proteowizard). The extracted MS/MS data were searched against the *L. casei* database of 25 421 protein entries downloaded from the National Center for Biotechnology Information on 19/7/2011. Semitryptic searches using parent ion and fragment ion mass tolerances of 10 ppm and 0.5 Da, respectively, were performed using X!Tandem running in the computational proteomics analysis system (CPAS), an open-source bioinformatics resource for analyzing large proteomics data sets.<sup>21</sup> S-Carboxamidomethylation of cysteine residues was specified as a fixed modification, and oxidation of methionine was specified as a variable modification. The peptide prophet and protein prophet algorithms were applied to the X!Tandem search results to assign probabilities to peptide and protein matches, respectively. Peptide–spectrum matches were accepted if the peptide was assigned a probability greater than 0.95, as specified by the peptide prophet algorithm. On the basis of the fit of the data to the predicted distributions of correct and incorrect matches, only peptide–spectrum matches for charge states +2 and +3 were accepted. Protein identifications were accepted if the protein contained two or more unique peptide sequences, and the protein was assigned a probability >0.95 by the Protein Prophet algorithm. This threshold will constrain the protein false discovery rate (FDR) to <1%.

#### Statistical Analysis of LTQ-Orbitrap Mass Spectrometry Data

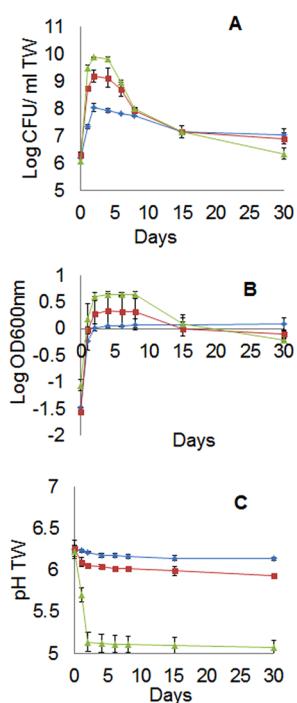
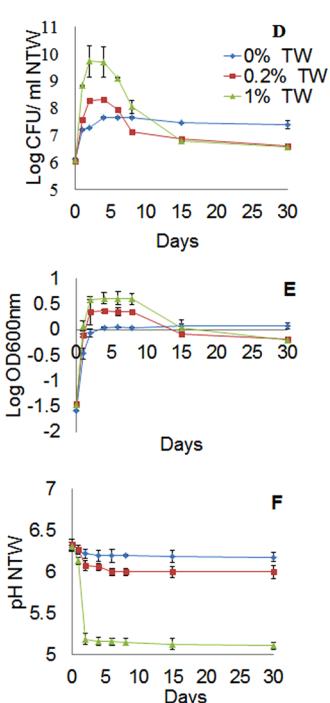
The SpC, a sampling statistic output of Protein Prophet, was used to determine relative protein abundance.<sup>23,24</sup> Differences in the expression level of proteins and their significance was then determined for soluble fractions. Log<sub>2</sub> fold changes in protein expression under each treatment condition were calculated based on the average SpC, according to the method described by Old.<sup>25</sup> Significant differences ( $P$  value of <0.05) in protein SpC were normalized and tested for significant differences using the  $\beta$ -binomial distribution test implemented in R. The total spectral count (TSpC) normalization approach was used as described by Gokce.<sup>26</sup>

## RESULTS AND DISCUSSION

*L. casei* GCRL163 was grown at three different levels of lactose with the pH of mMRS controlled using PBS. Bacterial cells were harvested at stationary phase (Figure 1). It has been previously documented that the bacteria generate a general stress response upon entering into the stationary growth phase and become stress cross-protected.<sup>22–24</sup> To understand better how NSLABs adapt to conditions where they are carbohydrate-starved, this study examined growth in media with and without Tween 80. It has been documented that presence of Tween 80 in the media enhances the growth or survival of lactic acid bacteria.<sup>18,27–29</sup>

#### *L. casei* GCRL163 Grows Slowly but Retains Viability during Lactose Starvation

The growth of *L. casei* GCRL163 in mMRS supplemented with Tween 80 and containing no added lactose reached maximum viable count after 2 days (Log CFU 8.02); then, viability

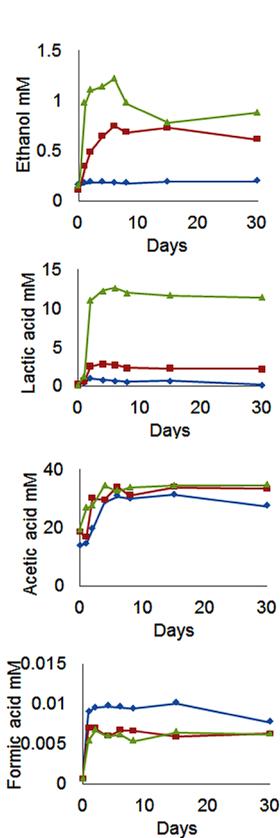
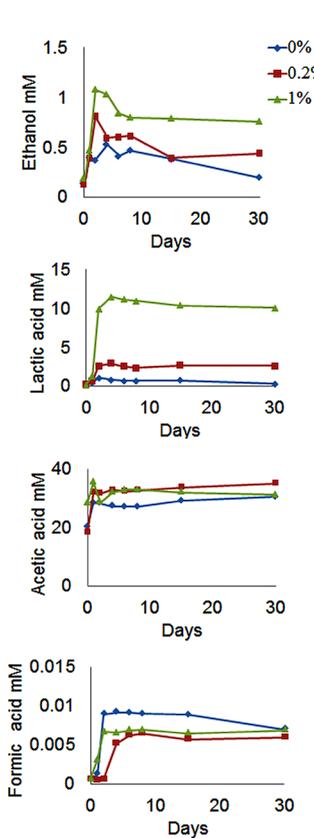
**Tween 80****No Tween 80**

**Figure 1.** Growth of *Lactobacillus casei* GCRL163 in modified buffered MRS broth. Containing 0% (●), 0.2% (■), or 1% (△) lactose and either supplemented with Tween 80 (A–C) or lacking Tween 80 (D–F). Growth levels are shown in the form of viable counts (A,D) and optical readings at 600 nm (B,E), while pH change is shown in the bottom graphs (C,F).

gradually declined over the 30 day incubation period (final count Log CFU 7.04), while OD remained constant. This indicated that cells remained intact but recovery of culturable cells declined. Dry weight data were consistent with this (Supporting Information, Figure 1). Viability was higher in cultures lacking Tween 80 and lactose. In contrast, cultures containing 0.2 and 1% lactose reached maximum cell density after 2 days (Log CFU 9.18 and Log CFU 9.87 for 0.2 and 1%, respectively); however, OD and viability declined substantially after 6–8 days of incubation (Figure 1A,B). A similar growth pattern was observed in the absence of Tween 80 (Figure 1D,E). A decline in pH with 1 and 0.2% lactose growth due to organic acid production (principally lactate, Figure 2) paralleled the changes in OD and cell viability (Figure 1C,F). Tween 80 did not substantially affect either growth yields or survival whether the media was lactose replete or not.

#### Key End-Products of Starved *Lactobacillus casei* GCRL163

The key end products of *L. casei* GCRL163 grown on three different levels of lactose in the presence and absence of Tween 80 were measured over an incubation period of 30 days. Lactate and ethanol were the major end products (Figure 2) in media containing 0.2 and 1% (w/v) lactose, reaching maximum levels when stationary growth phase was attained. In the presence of Tween 80, this was found to occur after 6 days; however, without Tween 80, the time to reach maximal levels was only 4 days. Lactate and ethanol occurred in an approximately a 10:1 ratio; however, when Tween 80 was present, ethanol levels were slightly reduced, most evident in cultures grown without lactose. The presence of lactate and the reduced pH of the medium may have affected the survival of *L. casei* GCRL163 when grown in the

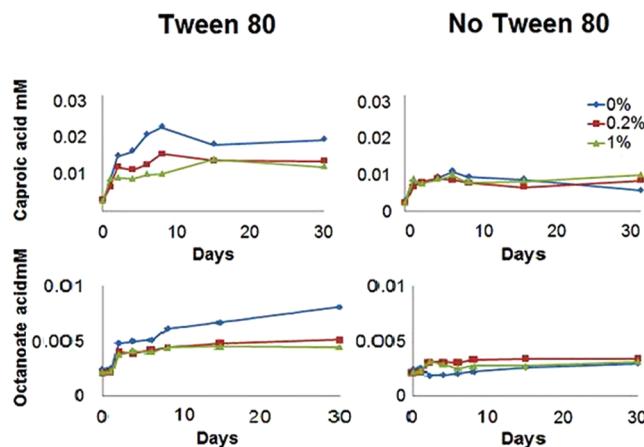
**Tween 80****No Tween 80**

**Figure 2.** Metabolic products formed by *Lactobacillus casei* GCRL163 cultures grown in mMRS with different levels of lactose (0, 0.2, and 1% w/v) with and without Tween 80.

presence of high levels of lactose, as previously shown by Hussain<sup>30</sup> using a semidefined medium.

Sustained survival of *L. casei* GCRL163 under lactose starvation suggests the use of an alternative carbon source to acquire energy or the cells physiologically adjust to tolerate carbohydrate starvation. The main potential carbon sources in 0% lactose mMRS are citrate, amino acids, acetate, and trace carbohydrates in the yeast extract. Formic acid was measured in culture supernatants, and data show that the amount of formic acid produced when cultures lacked lactose, either in the presence or absence of Tween 80, was increased (Figure 2). This is particularly of note given that the biomass in cultures lacking lactose was significantly lower than that for cultures containing 1% lactose.

Hussain<sup>30</sup> showed that long-chain fatty acid compounds, including octanoic and caproic acids, were detected after growth of GCLR163 in a semidefined medium. The possible source of these long-chain fatty acids is Tween 80, and in this study the mMRS supplied with Tween 80 compared with mMRS without Tween 80 shows increases in levels of octanoic and caproic acids over the incubation period (Figure 3). This suggests that *L. casei* GCRL163 has the ability to weakly degrade Tween 80, but there is no evidence that Tween 80 can directly support any significant growth. Although the concentration of these end-products is low, higher levels of both acids may impact cell viability in cultures containing Tween 80.



**Figure 3.** Caproic acid and octanoate acid of *Lactobacillus casei* GCRL163 cultures grown in mMRS with different levels of lactose (0, 0.2, and 1% w/v) with and without Tween 80.

#### Effect of Lactose Starvation Is Reflected in Broad Changes in the Proteome of *L. casei* GCRL163

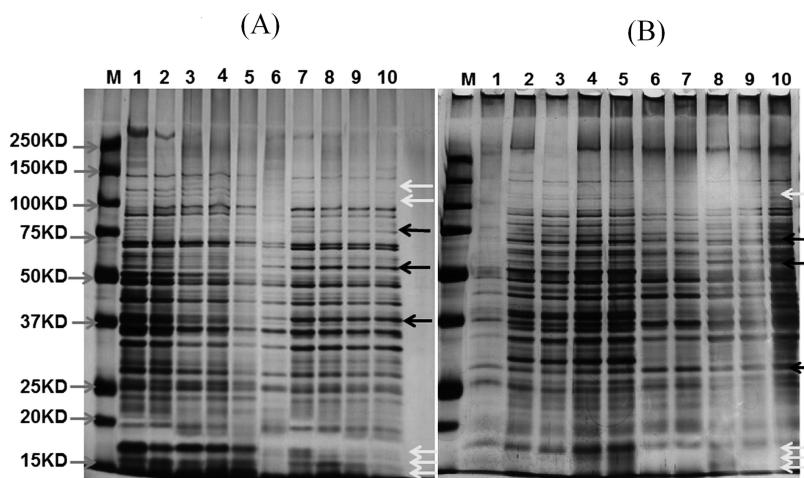
Gel-based protein profiles of *L. casei* GCRL163 grown in mMRS with different levels of lactose with and without Tween 80, respectively, revealed significantly different expression of a number of proteins (Figure 4A,B). This included changes in the intensity of several protein bands, particularly in lactose-starved cultures grown with and without Tween 80 in relation to cultures grown with 1% lactose. A consistent set of proteins was differentially increased in abundance in response to starvation that had molecular weight ranges between ~55 and ~80 kDa, while several protein bands of 15 to 20, 45, and 120 kDa became less abundant. The SDS-PAGE data demonstrate that lactose starvation resulted in qualitative changes to the overall proteome of *L. casei* GCRL163. To determine the nature of these difference within the proteome, specifically LC/tandem MS-MS analysis was based on in-solution cytosolic protein samples, allowing discernment of relative abundance changes in detectable proteins

within the proteome. Samples from stationary growth phase were chosen for these analyses.

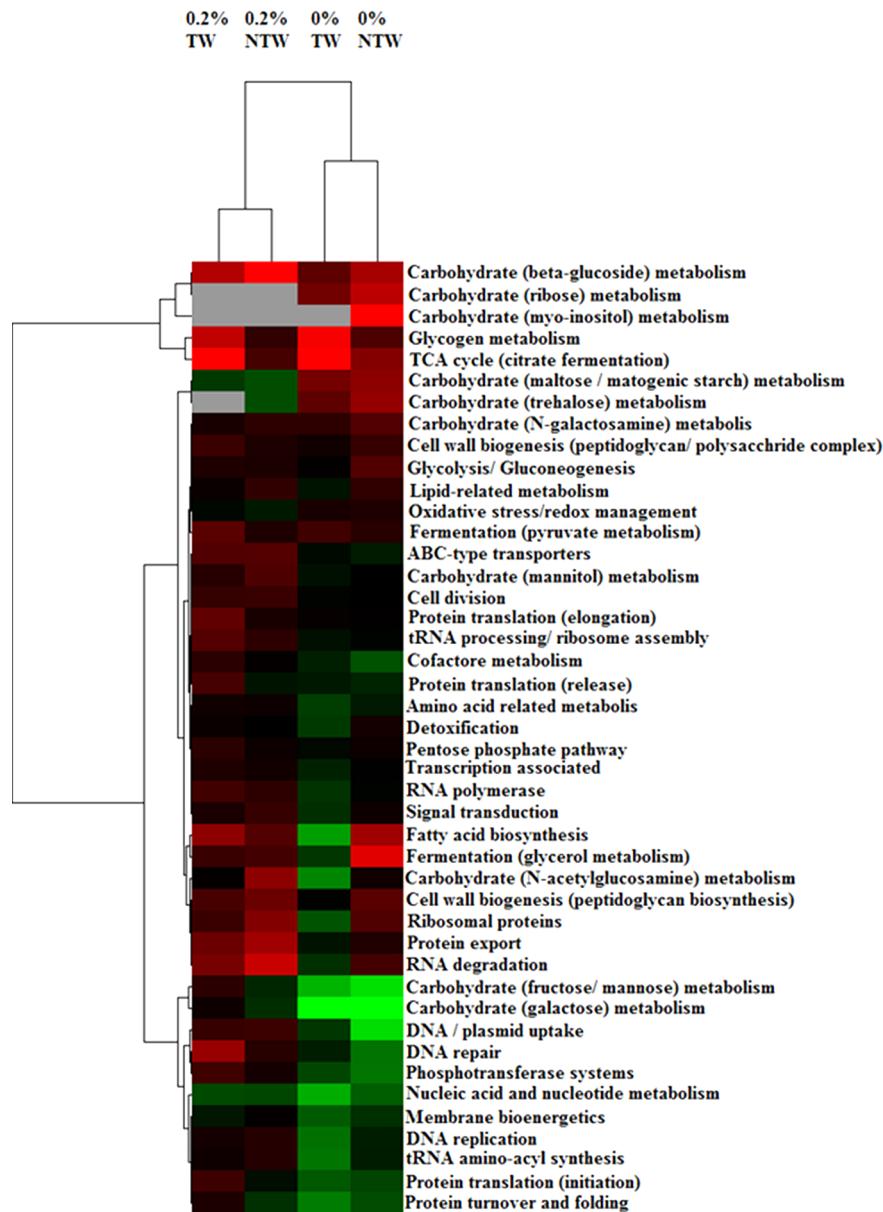
#### Starvation Leads to Repressed Lactose Metabolism and Phosphotransferase Abundance

A total of 649 different proteins were detected that passed stringent filtration criteria. The contribution of observed proteins organized on the basis of functional groups is included in Figure S2 in the Supporting Information. A heat map based on the relative SpC abundance of proteins grouped into functionally allied protein sets (Figure 5), defined on an ontological basis<sup>20,31,32</sup> was created to delineate global proteomic changes against cultures that were provided 1% (w/v) lactose. The highest proportions of proteins detected were associated with carbohydrate uptake and subsequent fermentation (18% of proteins identified), and these were separated into distinct sets to better reflect details of the fermentative metabolism. Proteins required for lactose uptake and catabolism via D-galactose as well as proteins involved in their activation were strongly repressed in lactose starved cultures (Figure 6).

Franci<sup>33</sup> demonstrated that *L. gasseri* ATCC 33323 uses PTS systems to import lactose. Lactose-specific PTS proteins, key enzymes required for lactose catabolism (6-phospho- $\beta$ -galactosidase, galactose-6-phosphate isomerase LacA and LacB subunits, tagatose 6-phosphate kinase, and tagatose 1,6-diphosphate aldolase, 1-phosphofructokinase), a lactose transport regulator (equivalent to LSEI\_0681), and the transcriptional antiterminator LacT were 5–20 times less abundant compared with cultures that contained lactose when cultured either with or without Tween 80. When grown with 0.2% lactose, *L. casei* GCL163 showed virtually no deviation in the abundance (0.7 to 1.3 ratio to the control) of these proteins. Accompanying the decline in lactose PTS systems, several other PTS protein subunits were also less abundant, likely reflecting the dearth of phosphoenolpyruvate (PEP) occurring during carbohydrate starvation. The SpC data indicated that a 5- to 6-fold increase occurs in the abundance of fructose 1,6-biphosphatase type III, a key regulator of gluconeogenesis.<sup>34</sup> Gluconeogenesis would be necessary for the creation of key cellular building blocks in the



**Figure 4.** (A) SDS-PAGE of cytosolic protein fraction from *Lactobacillus casei* GCRL163 grown in mMRS broth with or without lactose with Tween 80. The black arrows are protein bands that are up-regulated. The white arrows are protein bands that are down-regulated. (B) SDS-PAGE of cytosolic protein fraction from *Lactobacillus casei* GCRL163 grown in mMRS broth with or without lactose without Tween 80. The black arrows are protein bands that show qualitative increased concentration. The white arrows are protein bands that show qualitatively reduced concentration. Lanes 1–5 are samples from cells cultured in modified MRS with 1% lactose for 1, 2, 4, 6, and 8 days and lanes 6–10 are for the cell cultured in modified MRS with 0% lactose harvested at the same days.



**Figure 5.** Heat map generated from spectral count data organized by functional groups using Cluster v. 3.0.38. Unsupervised hierarchical clustering on the basis of uncentered correlation was used to compare the overall proteome data sets (top dendrogram) and the protein functional groups (left-hand dendrogram)a. TW: Tween 80, NTW: No Tween 80. Percentages refer to the lactose concentration in mMRS broths.

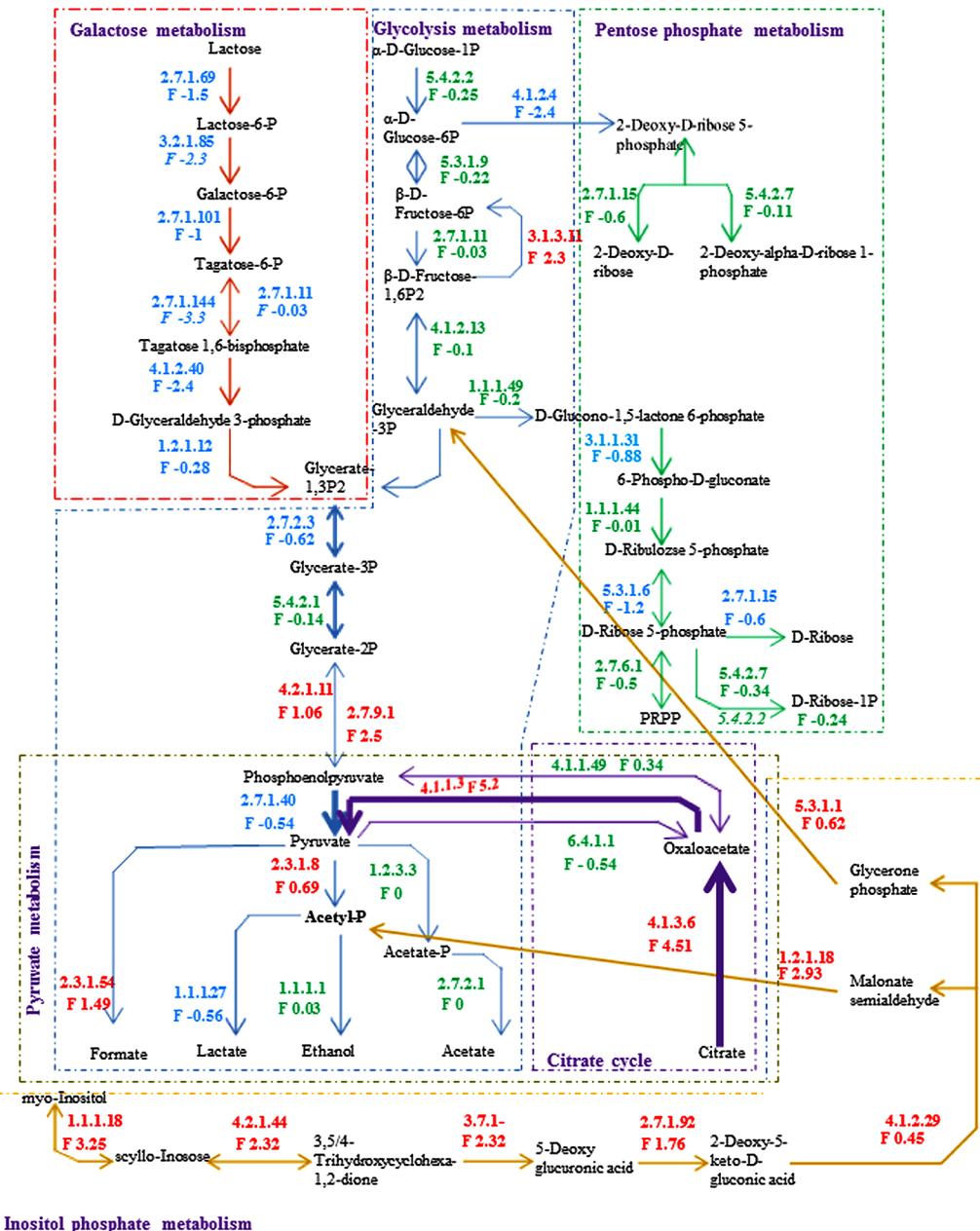
absence of utilizable carbohydrates and is to some extent being maintained or compensated given the lack of PEP that drives the process. The abundance of proteins in the glycolysis pathway and the Entner–Doudoroff pathway were otherwise overall unaffected, suggesting the fundamental importance of these pathways. We had previously shown that 0.2% lactose was growth-limiting, but this concentration was clearly sufficient to fully induce expression of lactose utilization genes.

#### Citrate Fermentation in the Presence of Low Carbohydrate Availability

Reduced lactose availability and lactose starvation resulted in an overall increased abundance of catabolic enzymes of alternative substrates; however, the stimulation was affected by carbohydrate availability as well as the presence of Tween 80. The six proteins (OadAB, CitXFED) that make up the citrate fermentation pathway resulting in the products pyruvate and CO<sub>2</sub> had the greatest abundance increases (2–33 fold) when in

the presence of Tween 80 (Figure 6 metabolic map). This was unsurprising because the mMRS growth medium contains 8.2 mM triammonium citrate; however, the influence of Tween 80 on the protein abundance levels was unsuspected. Utilization of citrate and conversion into end products such as diacetyl and acetaldehyde by *Lactobacillus* species has previously been reported and appears to be strongly subject to diauxy.<sup>35,36</sup>

Metabolism of pyruvate can yield in NSLAB different end products such as lactate, formate, acetate, ethanol, and compounds that contribute to the particular taste and aroma of cheese. The formation of formic acid in the presence of citric acid when under the limitation of other carbon sources such as galactose or lactose has been reported.<sup>37,38</sup> We observed that the pyruvate formate lyase complex in *L. casei* GCRL163 was more abundant under both starved and lactose-limiting growth conditions. The provision of acetyl-CoA to substrate level phosphorylation via acetate kinase (AckA) remained constant



**Figure 6.** Metabolic map of intermediary metabolism of *Lactobacillus casei* GCRL163 under starvation relative to 1% lactose control in the absence of Tween 80. The map was constructed from spectral abundance data and demonstrates relative abundance of pathway components during starvation and support data related to accumulated metabolic end-products. Pathway assignments of proteins are based on information from the KEGG database. Enzyme designations are given as their corresponding E.C. code. The width of the line is indicative of the relative abundance of the protein. Red arrows indicate proteins related to galactose metabolism, blue arrows indicate proteins related to the glycolysis pathway, green arrows indicate proteins related to the pentose phosphate pathway, purple arrows indicate proteins related to the citrate cycle, and yellow arrows indicate proteins related to inositol phosphate metabolism. Numerical values underneath the E.C. codes indicate the average log ratio of the fold change in protein abundance with red, green, or blue colors, denoting significant increases, decreases, or insignificant changes in protein abundance.

regardless of the growth conditions; AckA is an important enzyme that helps to convert pyruvate to different end product and yielding ATP<sup>39</sup>.

#### Lactose Starvation Forces Scavenging of Alternative Carbon Sources

There was also an observably large increase in abundance for most enzymes of the *myo*-inositol catabolic pathway (IolAC-DEG1G2). This change in abundance occurred only in the absence of Tween 80 (3–17 fold increase), while under other conditions these enzymes were not detectable. An analogous

response was also observed for glycerol catabolic enzymes (GlpO, GlpK, GpsA), with Tween 80 also interfering with this response. The effect of Tween 80 potentially relates to its perturbation of lipid metabolism (discussed further below). Glycogen,  $\beta$ -glucoside, galactose, maltose, trehalose, and ribose catabolic enzymes were also stimulated to a lesser degrees under carbohydrate-limited conditions, with catabolic enzymes of the latter three substrate more abundant during complete absence of lactose (Figure 5 heat map, Figure 6 metabolic map).

In general, lactose starvation increased the abundance of pyruvate-related metabolic enzymes that would suggest a slight

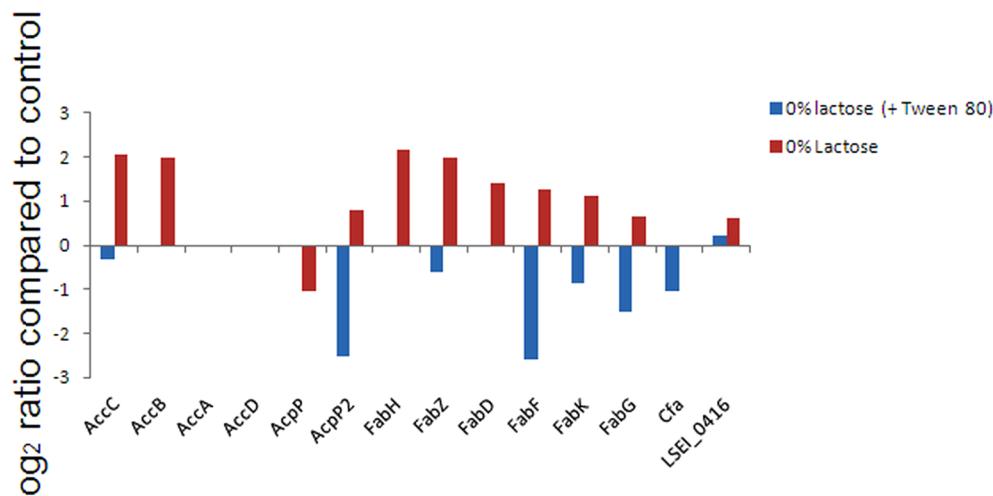


Figure 7. Fatty acid proteins with and without Tween 80.

promotion of mixed acid fermentation likely related to citrate fermentation. A five- to six-fold increased abundance of pyruvate orthophosphate dikinase, suggesting gluconeogenesis while at the same time provision of acetyl-CoA, reductant supply, and substrate-level phosphorylation occurs via acetate kinase (Figure 6 metabolic map). Reduced carbohydrate availability also induced greater abundance of a certain peptidases, including dipeptidase A (PepD), Xaa-Pro aminopeptidase (PepQ), and a dipeptidyl aminopeptidase/acylaminocycl dipeptidase-like protein (Table S1 in the Supporting Information). Besides these proteins, amino acid metabolism was largely unchanged or slightly suppressed (AspB, IlvE, MetA, CysK, PepT, PepN) during starvation.

#### Tween 80 Represses Fatty Acid Biosynthesis-Associated Enzyme Abundance during Carbohydrate Starvation

Protein abundance estimated in the present study suggest that in general carbohydrate starvation appears to broadly repress a wide range of cellular functions in GCRL163, including DNA synthesis, replication, repair, and uptake; tRNA charging, protein synthesis, and subsequent folding and turnover; and proton-motive-force-driven ATP synthesis. Collectively, this repression, though not resulting in a substantially slower growth rate, manifests in lower growth yields when citrate serves as the main source of carbon and energy; this is also coupled to slightly greater cell viability over time (Figure 1).

On the basis of our proteomic data, the presence of Tween 80 leads to reduced abundance of enzymes involved in fatty acid biosynthesis (Figures 5 and 7), while in the absence of Tween 80 they are instead promoted relative to the 1% lactose-grown control cultures. The abundance of other functional protein sets, including cell wall biogenesis, RNA degradation, and ribosomal proteins, are also suppressed to some extent in the presence of Tween 80 during carbon starvation. The reason for the apparent effect of Tween 80 could be due to release of oleic acid via nonspecific cytosolic esterases. It has been observed that the oleic acid moiety of Tween 80 can be incorporated into the cell membranes of lactic acid bacteria directly.<sup>18</sup> This process potentially diminishes the need for the de novo synthesis of fatty acids. The mechanistic basis of the effect Tween 80 has on other aspects of cellular physiology is somewhat unclear and requires further investigation.

#### Starvation Leads to Repressed Nucleotide Synthesis

Proteins of the purine and pyrimidine biosynthetic pathways were repressed in response to carbohydrate starvation (Table S1 in the Supporting Information), which likely associates with the resultant slower growth rate. This is likely due to reduced levels of 5-phosphoribosyl- $\alpha$ -1-pyrophosphate being availability. The reduced abundance of these pathways is consistent with previous studies in acid- and heat-stressed Gram-positive bacteria.<sup>40,41</sup>

Protein production not only depends on specific regulatory mechanisms but also changes with growth rate, as dictated by environmental conditions. The optimal level of RNA polymerases and ribosomes necessary or required by cells is tightly regulated.<sup>42,43</sup> In the present study, differences in the growth rate of *L. casei* GCRL163 under each of the growth conditions were observed. The proteomic analysis revealed a significant decrease in the production of tRNA amino-acyl synthetases in starved *L. casei* GCRL163 (0% lactose with and without Tween 80). The data also indicated that the overall abundance of ribosomal proteins was also significantly reduced under starvation condition (0% lactose, with and without Tween 80). A decrease in cell growth was also reflected in the down-regulation of the cell division proteins FtsX in 0 and 0.2% lactose (with Tween 80) and FtsA in 0% lactose (without Tween 80). Decrease in cell-division proteins has been reported by Wang,<sup>44</sup> who show that acid-stressed *L. casei* Zhang also show decreased levels of various cell division proteins.

#### Stress-Associated Proteins Are Induced during Starvation

Starvation has been shown to induce enhanced physicochemical stress tolerance in *Lactococcus lactis*.<sup>45</sup> Little equivalent data is available for lactobacilli. Here we can show that starvation leads to increased abundance (2–4 fold) of organic hydroperoxide resistance protein OhrA and glycine betaine/carnitine/choline ABC-type transporter OpuA. In the absence of Tween 80, there is also a large increase in the protein equivalent to LSEI\_0285, annotated as a NADH oxidase in most *L. casei* strain genomes. On the basis of an examination of conserved domains, however, this enzyme appears to be more similar to CoA-disulfide reductases and thus may fill a thiol-protective role,<sup>46</sup> in addition to that provided by glutathione. Overall, the data may suggest starvation results in some level of oxidative stress cross-protection in *L. casei* GCL163 as well as possible protection via compatible solute uptake above and beyond that provided by the

general stress response. In addition to this, it was observed that starvation also increased the abundance of a peptidoglycan-bound protein homologous (44% identity) to BacA of *Enterococcus faecium*. BacA is a cell-wall lysin that is also a bacteriocin activator.<sup>47</sup> These responses overall potentially favor the survival of *L. casei* GCRL163 during carbohydrate limitation.

## CONCLUSIONS

The study provides basic information about *L. casei* GCRL163 responses under different carbohydrate-limiting conditions. The data suggest that starved *L. casei* GCRL163 induces several pathways targeted at cellular energy production. This primarily includes activation of citrate fermentation pathway as well as pathways for the catabolism of polyols including inositol, glycerol, as well as other sugars, likely present in the medium at low levels or due to triglyceride turnover. It was observed the presence of Tween 80 affected the abundance of these proteins markedly, with citrate fermentation enzymes strongly promoted while carbohydrate scavenging-related proteins were only promoted in the absence of Tween 80. Because octanoic acid and caproic acid were detected as end products, this suggests that this strain has the ability to weakly degrade Tween 80 with resultant metabolites potentially influencing cell physiology, including suppressing fatty acid biosynthesis. Further research is needed to understand the underlying phenomena resulting in this physiological response. The data shown here contribute to explaining the adaptations of *L. casei* GCRL163 undergoes following cessation of primary carbohydrate fermentation.

## ASSOCIATED CONTENT

### Supporting Information

Dry weight of *Lactobacillus casei* GCRL163 in modified buffered MRS broth. Distribution of identified protein groups associated with their functional groups of *Lactobacillus casei* GCRL163 identified in the study. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## AUTHOR INFORMATION

### Corresponding Author

\*Phone: +61362268511. E-mail: ali.alnaseri@utas.edu.au.

### Notes

The authors declare no competing financial interest.

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