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Genomic analysis of *Leuconostoc citreum* TR116 with metabolic reconstruction and the effects of fructose on gene expression for mannitol production

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Abstract

The species *Leuconostoc citreum* is often isolated from grain and vegetable fermentations such as sourdough, sauerkraut and kimchi. *Lc. citreum* has seen an increase in its use as a starter culture for various fermentations and food applications. The strain *Lc. citreum* TR116 has been applied previously in this laboratory aimed at sugar depletion through metabolism resulting in the reduction of fructose to mannitol, a polyol considered as a sweet carbohydrate. Besides reducing sugar, TR116 showed flavour modulating characteristics and contributes to the extension of microbial shelf life. In order to obtain a better understanding of this strain and to fully use its set of abilities, the genome of *Lc. citreum* TR116 was sequenced using the Illumina MiSeq, assembly with SPAdes and annotated by the Prokaryotic Genome Annotation Pipeline. Metabolic reconstruction was employed to elucidate carbohydrate, organic acid and amino acid metabolism in the strain. Of particular interest was the gene expression analysis ascertained the influence of fructose on the genes *mdh* and *manX* involved in the uptake of fructose and its conversion to mannitol. This investigation, the first in *Lc. citreum*, illustrates the metabolic processes involved in fermentation used by this strain and demonstrates that in the presence of fructose, expression of the genes *mdh* and *manX* is increased. The resulting transparency of the skill set of TR116 contributes highly to future functionalisation of food systems and food ingredients.

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1. Introduction

Leuconostoc citreum is a lactic acid bacterium species which was first described by Farrow, Facklam and Collins (1989). Not one of the original species of the genera *Leuconostoc*, it was classified as a new species through DNA homology with other *Leuconostoc* spp. strains. The reported isolation sources of *Lc. citreum* are predominantly in plant based food and beverage fermentations and include: sourdough (Laguerre et al., 2012; Sahin et al., 2019a), kimchi (Eom et al., 2007; Otgonbayar et al., 2011), sauerkraut (Yang et al., 2015) and fermented corn beverages (Olivares-Illana et al., 2002; Silva et al., 2017). *Leuconostoc* cells have an ellipsoidal shape and can be elongated, are Gram-positive, non-motile and asporogenous. They have an obligately heterofermentative metabolism and do not produce catalase (Björkroth et al., 2014). The species maintains the status 'qualified presumption of safety' (QPS) for use in foods (European Food Safety Authority, 2017) and hence is used in food production and the development of novel types of foods.

Use of *Lc. citreum* strains as starter cultures is increasingly reported; and favourable metabolic traits such as the production of mannitol, exopolysaccharide and antimicrobial compounds have led to its application in several different food products. One strain in particular received a lot of attention regarding functionalisation of foods in recent years: *Lc. citreum* TR116. Isolated from a yellow pea sourdough (Rice et al., 2020b), *Lc. citreum* TR116 showed its multifunctional properties in several studies which investigated its use to overcome quality loss in sugar-reduced foods and beverages. Due to an increasing trend of non-communicable diseases, such as cardiovascular disease, obesity and type-2 diabetes, there is a high demand for sugar-reduced/low sugar/no-added sugar food products. However, reducing the amount of sugar in food products leads to a loss in quality, since, besides sweetness, sugar contributes to texture, structure, microbial shelf life and flavour (Clemens et al., 2016; Sahin et al., 2017). TR116 expresses the enzyme mannitol-2-dehydrogenase which

reduces fructose to mannitol (Gänzle, 2015). Compared to other strains TR116 achieves fructose to mannitol reduction in a remarkably high yield (>80%) in different food matrices, which makes it a suitable strain to develop low-sugar food products. In quinoa-based milk substitutes, the single strain fermentation with TR116 resulted in a product with a higher nutritional value with 40% less sugar and a reduction in glycaemic response by 35% (Jeske et al., 2018). Rice, Sahin, Heitmann, *et al.* (2020) investigated the effect of TR116 in different worts (barley, oat and wheat) aiming for sugar reduction and flavour modulation. Besides reducing the sugar content, TR116 synthesised aroma compounds which are characterised as fruity, and brandy-like. *Lc citreum* TR116 was also used in several studying investigating its potential in a single strain sourdough fermentation to compensate quality loss in sugar reduced sweet bakery products. In burger buns a sugar reduction of 50% was achieved showing comparable characteristics to the full-sugar control (Sahin et al., 2019a); using sourdough technology with TR116 in cakes revealed no losses in sensory and techno-functional properties when sugar was reduced by 50% (Sahin et al., 2019b); and low-sugar biscuits with only 5% sugar were high in flavour (Sahin et al., 2019c). Besides for sugar reduction, *Lc citreum* TR116 was used to improve bread quality by the incorporation of fermented high protein faba bean flour (Hoehnel et al., 2020).

In accordance with this heightened interest in *Lc. citreum,* it is important and timely that the metabolic capabilities of selected strains are investigated in detail. Metabolic pathway reconstruction is a valuable tool to provide insights into important reactions during metabolism, which can be used to improve targeted functionalisation of foods and food ingredients. This has previously been applied to assess malolactic fermentation of wine by *Oenococcus oeni* (Mendoza et al., 2017). Further insights can be gained from genotypic and transcriptomic analysis. Recently, *Lc. mesenteroides* was analysed in depth through coupled pan-genomic and transcriptomic analyses which provided insights into its metabolic activity during kimchi fermentation (Chun et al., 2017). Other recent publications revealed the

genome analysis of several *Lc. citreum* strains, focusing on genes responsible for the synthesis of long-chain polysaccharides, such as levan (Han et al., 2021), starch (Wang et al., 2021), or other oligosaccharides (S. Kim et al., 2021; Münkel et al., 2019). Furthermore, a particular interest in the biosynthesis of certain enzymes, such as β-galactosidase (S. Y. Kim et al., 2021) and glucansucrases (Münkel et al., 2019), is on the rise. Gene expression analysis can also elucidate the influences of environmental variables on genes of interest, and this has been applied successfully in *Lb. reuteri* to examine the expression of the *mdh* gene in the presence and absence of fructose (Ortiz et al., 2017). This gen is of main interest in the investigation of the use of TR116 for sugar reduction.

This study investigates the genome of *Lc. citreum* TR116 to facilitate metabolic reconstruction which provides an insight into carbohydrate, organic acid and amino acid metabolism in this strain. The effect and the importance of the main metabolites in food applications is highlighted and discussed. Since TR116 was mainly used as a promising strain for realising sugar reduction in food products in previous studies, gene expression analysis of TR116 was conducted to explain the influence of fructose on the transcription of genes related to the technological attributes of the high yield mannitol production and hence a high success rate in sugar reduction. The results of this study provide essential information for the development of food products triggering specific abilities of this multifunctional lactic acid bacterium strain.

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2. Materials and Methods

2.1. Bacterial strains and culture conditions

Lc. citreum TR116 isolated in previous work (Rice et al., 2020b) and deposited in the culture collection of the department of biological sciences in Munster Technological University,Ireland, was used in the current study. The strain was grown in MRS medium overnight at 30 °C to prepare cultures for DNA extraction. For gene expression experiments, the media used was based on the MRS5 media described by Meroth *et al.* (2003) with the sugar content modified to contain 50 g/L glucose (MRS5-G) or 50 g/L fructose (MRS5-F). Growth on MRS5-G was used as control and growth on MRS5-F represents the test condition to trigger *mdh* and *manX* gene expression. Single colony overnight cultures in MRS broth were washed in Ringer's solution and resuspended in the respective MRS5-G/F media. Measurement of OD_{600} of the cell suspension was used to calculate the volume required to inoculate fresh suspensions with an OD_{600} of 0.05. Cultures in MRS5-G and -F were grown at 30 °C and monitored until they reached an OD_{600} of 0.4 – 0.5 (mid-exponential growth phase) at which point they were used for RNA extraction. Growth in each media was conducted in three-independent experiments.

2.2. Genome sequencing, assembly, and annotation

Genomic DNA was extracted using the GenElute bacterial genomic DNA kit (Sigma Aldrich, St. Louis, United States), as per manufacturer's instructions. Library preparation and genome sequencing was conducted by microbes NG (University of Birmingham, UK). Genome sequencing was performed on the Illumina MiSeq platform using 250bp paired-end reads. Sequencing reads were trimmed using Trimmomatic (Bolger et al., 2014) and quality assessment, mapping and assembly were performed with Samtools (Li et al., 2009), Bedtools (Quinlan and Hall, 2010), BWA-mem (Li and Durbin, 2009) and SPAdes (Bankevich et al., 2012). All sequencing, quality assessment, mapping and assembly were conducted by

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MicronesNG. The assembled genome was annotated by the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016). Manual annotation of open reading frames was carried out using Basic Local Alignment Search Tool (BLAST; Altschul *et al.*, (1990)). Operon prediction was run on Operon-mapper (Taboada et al., 2018) and promoter region prediction on BPROM (Solovyev and Salamov, 2011). The TR116 genome was re-annotated using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) Automatic Annotation Server (KAAS) for ortholog assignment and pathway mapping (Moriya et al., 2007) to facilitate metabolic reconstructions.

2.3. Relative quantitation of *mdh* gene expression using real-time quantitative PCR

2.3.1. RNA isolation

Total RNA was isolated from 0.5 ml cultures grown to an OD_{600} of 0.4 containing < 7.5 x 10^8 cells. RNA was isolated using the RNeasy® Protect Bacteria Mini Kit RNeasy (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA was initially stabilised combining the culture with 2 volumes of RNAprotect-bacteria (Qiagen, Hilden, Germany). Protocol 5 was used for cell lysis. Briefly, cells were pelleted by centrifugation (5,000 × *g*, 10 min) and resuspended in 100 µl Tris-EDTA buffer (TE; 30 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing lysozyme (10 mg/ml) and proteinase K (10 µl/100µl) supplied by Qiagen (Hilden, Germany). The lysis suspension was incubated at room temperature for 10 min. Next, 700 µl of RLT buffer (provided in kit) containing 7 µl of β-mercaptoethanol was added and the sample was transferred to a 2 ml safe-lock tube containing 30 mg of acid washed glass beads (435-600 µm; Sigma-Aldrich, St. Louis, United States). Cells were disrupted in a MagNA Lyser (Roche, Basel, Switzerland) at top speed for 4 min and the beads and cell debris removed by centrifugation (10 s at 16,000 × g, 4 °C). Subsequently, 760 µl of supernatant was combined with 590 µl of 80% ethanol and transferred to a spin column, on which DNase treatment was performed using RNase-free DNase (Qiagen, Hilden, Germany). RNA purification was completed on the column and RNA was eluted in 30 µl of RNase free

water. An in-solution DNAase treatment was conducted on eluted RNA using the TURBO DNA-free kit (Thermo Fisher Scientific Inc., Rockford, USA) as per manufacturer's instructions to completely remove contaminating DNA. Following purification, RNA quantity and integrity were analysed on the Bioanalyzer 2100 (Agilent, Santa Clara, United States) instrument using the RNA 6000 Nano Kit (Agilent, Santa Clara, United States). A minimum RNA integrity number (RIN) of 7 was set as selective criteria for RNA samples.

2.3.2. cDNA synthesis

RNA was reverse transcribed using the Tetro cDNA Synthesis kit (Medical Supply Company, Dublin, Ireland). A 20 µl reaction volume contained 0.5 µg of total RNA, 1 µl of random hexamer primer, 1 µl of 10 mM dNTP mix, 4 µl of 5× RT reaction buffer, 1 µl of RiboSafe RNase Inhibitor and 1 µl of Tetro reverse transcriptase (200 U) made up to volume with DEPC- treated water. Each reaction set up also included a no reverse transcription control (NRTC) by replacing reverse transcriptase with water. Reaction mixtures were held at 25 °C for 10 min, then incubated at 45 °C for 30 min before reaction termination at 85 °C for 5 min. The resultant samples were stored at -20 °C prior to use.

2.3.3. Real-time quantitative PCR (RT-qPCR) assay design and validation

qPCR assay design was carried out using the Assay Design Centre (Roche Life Science; https://lifescience.roche.com/global_en/brands/universal-probe-library.html#assay-design-center). Specific primer pairs were designed for the reference genes *murC* (expression of UDP-N-acetylmuramate – L-alanine ligase), *pyrG* (expression of CTA synthase) and *gyrB* (expression of DNA-gyrase-subunit B) and the target genes *mdh* (expression of mannitol-dehydrogenase) and *manX* (expression of phosphotransferase system mannose-specific EIIAB component) from the annotated genome of *Lc. citreum* TR116 (whole genome sequence project: NTJC01). Primers were purchased from Eurofins MWG (Ebersberg, Germany). Product specific probes were selected from the Universal Probe Library (Roche Life Science, Basel, Switzerland). Probes are labelled at the 5' end with fluorescein (FAM)

and the 3' end with dark quencher dye. All oligonucleotides used in the study are presented in table 1. Primer specificity and the absence of primer dimers was confirmed by PCR using DNA as template and visualised on a 4% (w/v) agarose gel. Electrophoresis was run at 120 V for 60 min. Assays were optimised by performing qPCRs over a concentration range of 0.3 – 1.0 µM for primers and 0.05 – 0.20 µM for probes. PCR efficiency (E) for each primer set was calculated via the standard curve method using DNA as template. DNA concentration was standardised to 100 ng/ul, resembling 10^8 gene copies for a genome length of 1,831,463 bp as calculated using equation 1. Ten-fold dilutions of DNA to 10^2 copies were prepared in duplicate and used as template for amplification. The mean cycle threshold (CT) for each DNA standard was plotted against the log of copy number, and PCR efficiency was calculated by using the slope of the resulting linear regression plot according to equation 2. Primers were considered satisfactory if PCR efficiency was within the range 1.9 – 2.1 (90 % – 110 %).

Equation1: Copy number = Template (ng) × 6.0221×10^{23} (molecules/mol) genome length (bp) × 660 (g/mol) × 10^9 ng/g

Equation 2: $E = 10^{(-1/slope)}$

2.3.4. RT-qPCR assay

qPCR was performed on a LightCycler 96 instrument (Roche, Basel, Switzerland). Amplification was performed using the FastStart Essential DNA Probes Master kit (Roche, Basel, Switzerland). Each 20 µl reaction contained: 10 µl Master Mix 2X, 2 µl primer-probe mix (final concentrations: primer, 0.3 µM; probe, 0.2 µM), 6 µl of PCR grade water and 2 µl of cDNA or NRTC. The amplification program consisted of an initial step at 95 °C for 10 min followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C and 10 s at 72 °C. Amplification was detected by measuring the fluorescence of FAM (excitation: 492 nm, emission: 517 nm) released from TaqMan probes after annealing. RT-qPCR was performed in duplicate for each biological replicate.

2.3.5. Relative gene expression

The effect of experimental conditions (presence of glucose or presence of fructose) on the expression of candidate reference genes was determined using the 2^{-CT} method described by Schmittgen and Livak (2008). This was used to select the best candidate for normalisation of target gene expression. Assays were performed in duplicate on three independent biological replicates. The relative expression of the *mdh* gene in different conditions was estimated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Growth on MRS5-G was used as control and growth on MRS5-F was the test condition. Relative gene expression values reported for the *mdh* and *manX* gene are fold changes between control (glucose) and test (fructose) condition which were normalised using *gyrB* gene expression.

3. Results and discussion

3.1. General genome features of *Lc. citreum*

The genome assembly of *Lc. citreum* TR116 consists of 31 contigs with a total length of 1.83 Mb and a GC content of 38.8%. Genome annotation identified 1,853 open reading frames (ORFs) comprising 1,756 protein coding sequences, 40 pseudo genes and 57 RNAs. Non-coding RNA regions were identified as 51 tRNAs, 3 rRNAs; 1 5S, 1 16S, 1 23S, and 3 ncRNAs. A summary table of the general genome features of all available *Lc. citreum* genomes available at the time of analysis is provided in table 2. The mean species genome length was 1.85 (1.72 – 1.98) Mb, GC content was 39.1 (38.7 – 43.1) % and number of proteins 1754 (1601 – 1879). This represents a relatively narrow range in genome feature variability in *Lc. citreum* which may in part be due to the number of genomes. The species *Lc. pseudomesenteroides* with 27 available genomes, ranges in genome size from 1.81 – 3.24 Mb with the number of encoded proteins spanning 1688 – 2178. Even *Lc. lactis*, with a similar number of genomes (20) to *Lc. citreum* shows a range in genome length of 1.21 – 2.01 Mb. This portrays a paucity in genomic events such as horizontal gene transfer, gene loss and gene duplication which instil such variation.

3.2. Genomics of *Lc. citreum* TR116

3.2.1. Carbohydrate metabolism

Based on the predicted KEGG pathways and BLASTp analysis of genes related to carbohydrate fermentation, the metabolic pathways of *Lc. citreum* TR116 for carbohydrates were reconstructed (Figures 1 and 2). *Leuconostoc* spp. are heterofermentative lactic acid bacteria (LAB) and ferment sugars via the phosphoketolase pathway (Cogan and Jordan, 1994). In the case of hexose metabolism sugars will typically be converted to glucose as the substrate of the phosphoketolase pathway. Following the removal of CO_2 from glucose the resulting pentose is split by the phosphoketolase enzyme, yielding glycerol-3-phosphate (G-3-P) and acetyl- phosphate. G-3-P is reduced to lactate while acetyl-phosphate is reduced to ethanol. However, when additional electron acceptors are available to regenerate NAD^+ consumed in the oxidation phase, acetyl phosphate is converted to acetate with the formation of an addition ATP. This is the case when fructose is available to be converted to mannitol by mannitol-2- dehydrogenase. This pathway dominates the metabolism in TR116 resulting in high conversion yields reported in previous studies (Jeske et al., 2018; Rice et al., 2020a; Sahin et al., 2019a). During the metabolism of pentoses by the phosphoketolase pathway acetate will be formed in favour of ethanol as there is no NADH oxidation required to remove CO_2 from the hexose sugar and thus the pathway is balanced by the reduction of NAD^+ by lactate dehydrogenase (Cogan and Jordan, 1994). Acetate contributes to changes in flavour profile in food products and also contributes to prolonging microbial shelf life (Peyer et al., 2016; Sahin et al., 2019a), and a gene set encoding the enzymes that comprise this pathway were identified in *Lc. citreum* TR116. Prior to metabolism by cytosolic enzymes, sugars must be transported across the cell membrane. Sugar transport in LAB typically involves three types of transporters. The ATP binding cassette (ABC) transporters that use ATP as an energy source for translocation of molecules across the membrane. Secondary transporters which use the energy of electrochemical gradients across the membrane to transport

molecules and are predominantly of the major facilitator superfamily (MFS). These include uniporters, symporters, and antiporters.

Lastly, members of the phosphotransferase system (PTS) that transport sugars across the membrane with the addition of a phosphate group donated by phosphoenolpyruvate (PEP) (Zaunmüller and Unden, (2009). A number of gene loci encoding sugar transporters were annotated in the genome of *Lc. citreum* TR116 which are illustrated in figure 1. These included; an ATP transporter for glycerol-3-phosphate; PTS transporters of the Glc superfamily for β-glucoside, sucrose and cellobiose, and two PTS mannose/fructose/sorbose transporters which included the EIID transmembrane domain. Finally, two MFS transporters were present and based on being situated in operons with the respective sugars metabolic genes were given putative functions in xylose and maltose transport. Accordingly, the necessary enzymes for conversion of these sugars to enter the phosphoketolase pathway were present and the reactions are summarised in figure 1.

Further, α- β- and 6-phospho-β- glucosidases were identified, supporting the association of *Lc. citreum* with plant material through the capacity to release glucose from glycosylated plant metabolites and disaccharides (Michlmayr and Kneifel, 2014). An *mdh* gene was not described in the TR116 genome following initial automated annotation. To identify a candidate ORF encoding MDH, an alignment of the *mdh* gene from the prototypical mannitol-2-dehydrogenase producer, *Leuconostoc mesenteroides* ATCC 9135 (accession: AY090766), with the assembled genome of TR116 using BLAST was performed. This search produced an alignment with 85.1 % identity corresponding to an ORF in contig 10, annotated to encode a threonine dehydrogenase. A BLASTp alignment of the translated amino acid sequence of this ORF with that of a *Lc. citreum* MDH (accession: CDX65950) produced a 100% identity alignment with 100% query coverage. The ORF was selected as the *mdh* of TR116. This is an example of erroneous annotation of a gene by predictive software, an outcome that should be kept in mind when working with such data. This occurrence for the

mdh gene is further evident by the paucity of mannitol-2-dehydrogenase nucleotide and protein items in the NCBI databases compared to number of genome assemblies for heterofermentative LAB.

3.2.2. Organic acid metabolism

Organic acid metabolism was also evaluated in *Lc. citreum* TR116 and illustrated in figures 1 and 2. Organic acids are of high interest in food applications, since they act as natural preservatives prolonging microbial shelf life, and contribute to flavour formation and modulation (Gurtler and Mai, 2014). Technological importance of organic acid metabolism by LAB includes wine (malolactic) fermentation for acidity regulation and dairy (citrate) fermentation for flavour compound formation (Hugenholtz, 1993; Versari et al., 1999). *Leuconostoc s*pp. have an incomplete tricarboxylic acid pathway. Putative loci for malate, citrate and fumarate were identified and included secondary transporters and metabolic enzymes. Both citrate and malate loci had a secondary transporter annotated as a citrate:sodium symporter. Characterisation of the citrate carrier CitP in *Lc. mesenteroides* found that the transporter was also involved in malate uptake (Marty-teysset et al., 1995). This is possibly the case for the citrate transporters of *Lc. citreum* TR116. Regarding citrate metabolism, genes encoding the citrate lyase complex and an NADP-dependant malic enzyme (oxaloacetate-decarboxylating) completed the locus. For malate, the locus also held a gene for an NAD-dependant malic enzyme for conversion of malate to pyruvate. This constitutes the capacity for malolactic fermentation in conjunction with lactate dehydrogenase (LDH). Malolactic fermentation can play an important role in the development of novel beverages to achieve a rather soft acidic flavour profile than sharp acidity which can be perceived as overpowering.

At the fumarate locus a putative anion permease preceded fumarate reductase and fumarate hydratase genes. The metabolism of organic acids does not directly provide cellular energy but influences growth and metabolite formation during co- metabolism with sugars (Cogan,

1987). However, it is involved in the generation of membrane potential, contributing to proton motive force (Marty-teysset et al., 1995).

3.2.3. Pyruvate metabolism

The metabolism of pyruvate by *Lc. citreum* is also an important consideration as it is the main electron acceptor in LAB. The genome of *Lc. citreum* TR116 contains multiple copies of both D- and L-LDHs, highlighting their importance in NAD^+ regeneration. Alternative pathways of pyruvate metabolism are also possible. TR116 has the genes of the pyruvate dehydrogenase complex, encoding the enzymes responsible for fermentation of pyruvate through its conversion to acetyl-CoA, which is further converted to acetate, yielding ATP (Bleckwedel et al., 2020). This activity was described in *Leuconostoc mesenteroides* with pyruvate as the carbon source by Wagner *et al.* (2005). In the same study, pyruvate oxidase activity was also reported in the presence of oxygen. A gene encoding pyruvate oxidase was not present in *Lc. citreum* TR116. Hence, acetate is produced via the synthesis of Acetyl-CoA. Another outcome of pyruvate metabolism is the formation of α-acetolactate by the reaction combining two pyruvate molecules and release of CO_2, catalysed by acetolactate synthase. α-Acetolactate is subsequently either converted to acetoin or non-enzymatically and spontaneously transformed into diacetyl, which may also be reduced to acetoin. Diacetyl is considered as an off-flavour if present in amounts higher than 0.15 mg/L (Tan and Siebert, 2004), which should be considered when using TR116 for food and beverage fermentations. Further reduction of acetoin yields 2, 3 butanediol. The genes of this pathway are also present in *Lc. citreum* TR116. Therefore, the possible outcomes of pyruvate metabolism are lactate, diacetyl, acetoin and 2, 3 butanediol. These pathways are outlined in figure 2.

3.2.4. Amino acid metabolism

The metabolism of amino acids by *Lc. citreum* TR116 was elucidated from the KEGG pathway map of amino acid biosynthesis generated using KAAS (figure 3). While interconversion of amino acids was apparent, the synthesis of amino acids from primary

metabolites appeared to be absent. Overall, glutamine, proline and arginine could be formed from glutamate; threonine could be formed from aspartate; and leucine formed from valine. While TR116 lacked the ability to synthesise alanine and asparagine. Interestingly, the pathway for lysine biosynthesis from aspartate is incomplete by the absence of only one enzyme: diaminopimelate epimerase. This may be an example of gene loss due to adaptation to protein rich environments as this gene is also absent in the KEGG pathway map of the reference strain KM20. Complete ABC transporters for cysteine, aspartate/glutamate/glutamine, arginine/lysine/histidine, methionine and leucine/isoleucine/valine were all detected in addition to multiple generic amino acid transporters. Previous assessment of the amino acid requirements of *Leuconostoc* spp. has found substantial variation at the strain and species level. However, in a study by Garvie (1967) commonality was found in the requirement of valine and glutamine by all strains and no requirement for alanine, implying an inherent ability to form alanine in *Leuconostoc* spp. Multiple aminotransferase genes were present in *Lc. citreum* and possibly one of these catalyses the conversion of pyruvate to alanine even though this was not evident through pathway mapping.

For peptide uptake an oligopeptide ABC transporter (Opp) was present in *Lc. citreum* TR116. An array of putative peptidases was identified including five aminopeptidases, three dipeptidases and sixteen other peptidases with endopeptidase, metallo- peptidase and carboxypeptidase activities. Aminopeptidase annotation suggested specific glutamyl- prolyl- and methionyl-group activities. Notably, one dipeptidase and one carboxypeptidase were specific for D-alanyl-d-alanine sites. This large complement of peptidases is consistent with auxotrophy for multiple amino acids. Since TR116 is well equipped with genes to express different types of peptidases, this strain would be a suitable for the functionalisation of protein rich ingredients, such as plant protein rich flours (Hoehnel et al., 2020). Phenotypical

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study of the strain would allow for comparison with genotypic findings, such as has been performed previously for *L. helveticus* (Christiansen et al., 2008).

3.3. Relative gene expression of *mdh* and *manX* genes

3.3.1. Assay validation

Agarose gel electrophoresis of the amplification products formed using each primer pair resulted in a single product of expected product size for all primers (figure 4). PCR amplification efficiencies of primers for candidate reference and target genes were all within the acceptable range (1.9 – 2.1) and were also within 10% of each other as suggested by Schmittgen and Livak (2008) to meet the assumption of similar amplification efficiency (table 3). Standard curves for efficiency determination all had R^2 values greater than 0.998.

3.3.2. Relative gene expression

Relative expression of the genes of interest, *mdh* and *manX* in *Lc. citreum* TR116 was assessed during log phase growth in media containing fructose (test condition) or glucose (control condition). The candidate reference genes for normalisation of target gene expression included three housekeeping genes; *pyrG*, encoding cytidine triphosphate (CTP) synthase, involved in pyramidine synthesis, *gyrB*, encoding subunit B of DNA gyrase which is involved in negative super-coiling of DNA during transcription and replication processes; and *murC*, encoding UDP-N-acetylmuramate– L-alanine ligase involved in cell wall synthesis. These genes were selected having no expectation of differential expression between conditions and being involved in different cellular activities. To assess expression stability between the control and test condition, the ratio of log transformed CT values (2^{-CT}) for each candidate reference gene was determined as is suggested by Schmittgen and Livak (2008) and is presented in table 4. Taking a ratio of 1 as the ideal value, representing no change in expression, the most stable reference gene was *gyrB* with a 2^{-CT} ratio of 0.089. This equated to a fold change in expression of 1.13 between control and test conditions. *gyrB* was selected for as the reference gene for normalisation of target gene expression. Relative gene

expression was determined using the $2^{-\Delta\Delta CT}$ method according to Livak and Schmittgen (2001). Expression of the genes *mdh* and *manX* exhibited fold increases of 7.3 ± 1.2 and 3.4 ± 0.6 in the presence of fructose respectively (figure 5). This indicates an induction effect of fructose on the expression of these genes which is more prominent for *mdh*. Annotation of the *mdh* gene predicted it to be part of an operonic gene pair a gene encoding a putative fructose permease. This is consistent with a report of co-transcription of *mdh* with a downstream gene, in *Lc. mesenteroides* by Aarnikunnas, Rönnholm and Palva (2002). Promoter prediction at this site identified a promoter with -10 and -35 motifs of GTTTAACAT and TTGACA, respectively. This supports the assumption that the genes are expressed simultaneously and therefor a similar fold increase in the expression of this putative fructose permease would be expected in the presence of fructose. In a study of *mdh* expression in *L. reuteri* CRL 1101, under similar experimental conditions, Ortiz *et al.* (2017) reported a 42- fold increase in *mdh* expression in cells grown for 8 h (log phase) and showed constitutive expression of the gene in the absence of fructose. Ortiz *et al.* (2017) also highlighted the influence of culture media on gene expression, emphasising the importance of the compounds in the medium. Seemingly, the absence of interfering compounds which are present in complex media, such as MRS, enhances gene expression. This represents an important information for further studies. While also representing an induction effect of fructose, the lower fold change in *mdh* gene expression determined in this study (7-fold) may be due to many reasons such as, a higher level of constitutive expression of the *mdh* gene in *Lc. citreum*, a less prominent regulatory effect of fructose on expression of the gene or procedural variations. However, an absolute quantitative gene expression analysis would be required to inform the underlying differences in gene copy numbers. To the authors' knowledge there are no other examples of gene expression analysis for the *mdh* gene in LAB for further comparison. Bacterial PTS transporters are involved in the uptake of monosaccharides and disaccharides in many LAB. Phosphate is transferred from the donor, phophoenolpyruvate (PEP) by the universal PTS

enzymes, EI and phospho-carrier protein (HPr), to the sugar-specific membrane associated proteins. This sugar specific component of a PTS transporter consists of three or four proteins EIIABC(D), which may be fused in different combinations. The membrane associated complex facilitates the transfer of phosphate to the sugar substrate unit and its translocation across the membrane (Zaunmüller and Unden, 2009). The second GOI, *manX* encodes the EIIA subunit of a mannose-fructose type PTS transporter. Gene expression of *manX* was also upregulated with a 3-fold increase in expression recorded during growth in the presence of fructose. Increased expression of PTS genes in the presence of substrate sugars has previously been demonstrated in *L. acidophilus* using cDNA microarrays, which revealed that fructose, sucrose and trehalose were transported by PTS transporters and that the gene loci were induced by the presence of their respective sugar substrates (Barrangou et al., 2006). Further, the gene loci containing the PTS genes also encoded catabolic enzymes for the respective sugars. The energisation of sugar molecules as they pass through PTS transporters through the addition of a phosphate group primes the sugars for catabolic pathways. It is pertinent that the transporter co-transcribed with the mannitol dehydrogenase gene encodes a fructose permease which transport fructose into the cell unmodified and can be utilised as an electron acceptor during the oxidation of NADH by MDH. Further, the induction of *mdh* expression by fructose was greater than that of *manX*. This is consistent with the description of fructose metabolism in heterofermentative LAB by Wisselink *et al.* (2002) when only fructose is available as a carbohydrate source. Mannitol is formed in a 2:1 molar ratio to fermentation end products (lactate, acetate, CO_2) as 2 mol of fructose are reduced to mannitol for every 1 mol that is fermented. This of course does not consider post-transcriptional factors such as mRNA stability, translation efficiency or the relative kinetics of these transporters.

4. Conclusion

This study reveals the genome of *Lc. citreum* TR116, a lactic acid bacteria strain used in several studies to reduce sugar in bakery products and beverages. General genome features were consistent with those of the species, with a genome length of 1.83 Mb, a G+C content of 38.8%, and with 1,853 open reading frames. Mapping of the annotated genome to KEGG pathways revealed the metabolic capacity for carbohydrates including glucose, fructose, xylose, ribose, trehalose, maltose, sucrose and organic acids, citrate, malate and fumarate. A lack of amino acid biosynthetic capacity was compensated for with multiple amino acid transporters and an extensive proteolytic system. Operon prediction proposed the co-transcription of the *mdh* gene with a fructose permease encoding gene. Further in-depth genomic analysis, such as that possible with pan-genomics, would be valuable to elucidate the genetic diversity of the *Lc. citreum* species and help to identify other strains with potential technological value which can be used in future food applications. Relative gene expression analysis demonstrated the inducing effect of fructose on the *mdh* and *manX* gene, which suggests a role for both secondary transporters and PTS in the metabolism of fructose in *Lc. citreum*. This complements previous phenotypical studies of the strain TR116, which showed a dosage response in mannitol formation from fructose. The influence of fructose concentration on relative gene expression of this gene set would be of interest for future interrogations to consolidate these findings. In summary, TR116 is a multifunctional strain which possesses a set of genes to synthesise a) organic acids and flavour compounds which can contribute to prolonged microbial shelf life and modulate off-flavours, b) different types of proteases which putatively can be used for protein functionalisation, especially plant-based proteins, and c) to reduce fructose to mannitol in a high yield which provides a strategy to reduce sugar in different food matrices. Hence TR116 can be used as a cell factory targeting different goals in the development of foods and beverages.

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6. Declaration of interest

None.

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7. References

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Tables

Table 1. Primers and probes used in the gene expression study.

Gene	Forward primer	Reverse primer	Amplicon size (bp)	Probe sequence (UPL no.*)	Locus tag
murC	CTGCTCGCCAAAAGTATCCTA	AATTCATCCTTATAAGCAATTACACG	88	CAGCCACA (5)	CMW49_05310
pyrG	TGCACCAAAAGCTGATATGG	ACTGTCTTCTTTGGATGCTTGAT	69	TGGTGGAA (31)	CMW49_03370
gyrB	GATGATGAAGAGATTCGCAGTG	TTGAATTTGATCGGCATTGTA	72	TGCTGGAG (67)	CMW49_08005
mdh	GTATGCTGGTTTGCCAGGTT	TTTTCGTGTCCCAAAACGAT	60	CTCCAGCC (19)	CMW49_07790
manX	GGGATTAAGAGATGCTTTGAGC	CACCCGATTTTAATCCAATAGC	78	CTTCCCCA (9)	CMW49_00300

*number of the probe in the Universal Probe Library (ROCHE).

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Strain	Genome assession number	Size (Mb)	GC%	Scaffolds	Genes	Proteins
KM20	DQ489740	1.90	38.9	5	1915	1776
EFEL 2700	CP024928.1	1.92	39.0	5	1962	1805
Citreum 1[a]	LM993654	1.72	39.2	1	1719	1601
Citreum 2[a]	LN589840	1.75	39.0	1	1753	1646
LBAE C10	CAGE00000000	1.93	38.8	76	1939	1826
LBAE C11	CAGF00000000	1.97	38.7	83	1976	1827
LBAE E16	CAGG00000000	1.80	38.9	45	1806	1718
DmW_111	NDXG00000000	1.83	38.8	19	1847	1756
CW28	MWJP00000000	1.98	38.7	9	2028	1879
ATCC 49370	AB494725	1.84	39.0	10	1859	1748
TR116	MN135986	1.83	38.8	31	1842	1746
G4	QFDE01000000	1.84	38.8	4	1861	1765
TR153	QFDI01000000	1.90	38.7	39	1943	1846
1300_LCIT	GCA_001062635	1.81	43.1	58	1826	1677
1301_LGAS	JVUU01000000	1.82	39.0	37	1784	1686
UBA3877[b]	DGGJ01000000	1.73	38.7	115	-	-
UBA11316	DPZH01000000	1.84	38.9	34	1837	1760
Mean		1.85	39.1	34	1869	1754
Max		1.98	43.1	115	2028	1879
Min		1.72	38.7	1	1719	1601

[a] Entries have no strain designation.

[b] Annotation limited to general features.

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Table 3. Summary of standard curves used to determine amplification efficiency of qPCR assays.

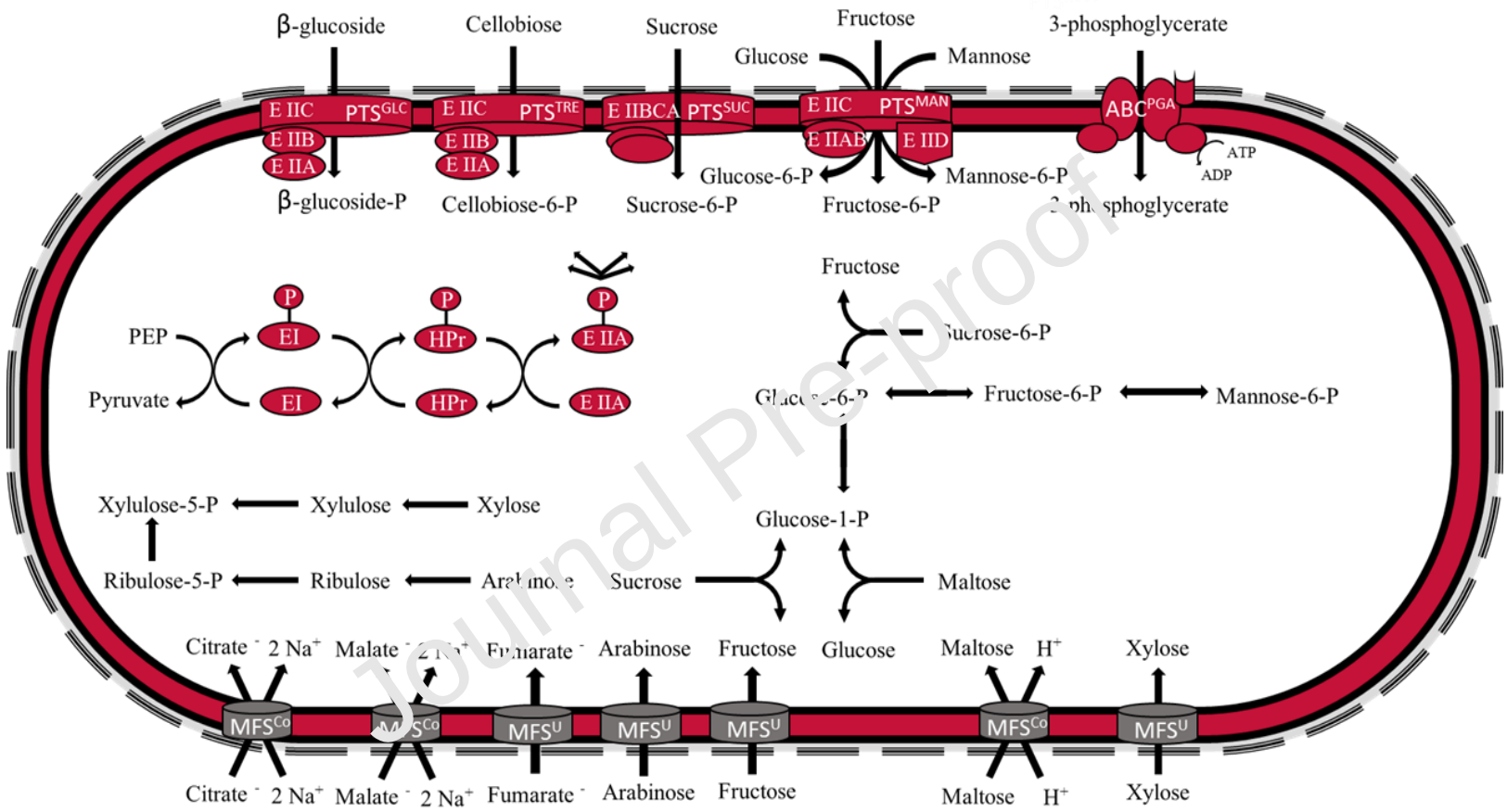
Gene	*murC*	*pyrG*	*gyrB*	*mdh*	*manX*
Slope	-3.407	-3.346	-3.391	-3.486	-3.407
Efficiency	1.97	1.99	1.97	1.94	1.97
R^2	0.999	0.999	0.999	1.000	0.999

Table 4. Assessment of expression variation in candidate reference genes.

Reference gene	Mean 2^{-CT}		2^{-CT} ratio	Fold change
	Control	Test		
Slope	2.48E-8	1.95E-8	0.78	1.28
Efficiency	7.11E-8	8.25E-8	1.16	1.16
R^2	8.70E-8	7.72E-8	0.89	1.13

Control represents the results of *Lc. citreum* TR116 grown on MRS5-glucose; Test represents results of *Lc. citreum* TR116 grown on MRS5-fructose.

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Figures

Figure 1. Carbohydrate and organic acid transport and sugar interconversions in *Lc. citreum* TR116.

Figure 2

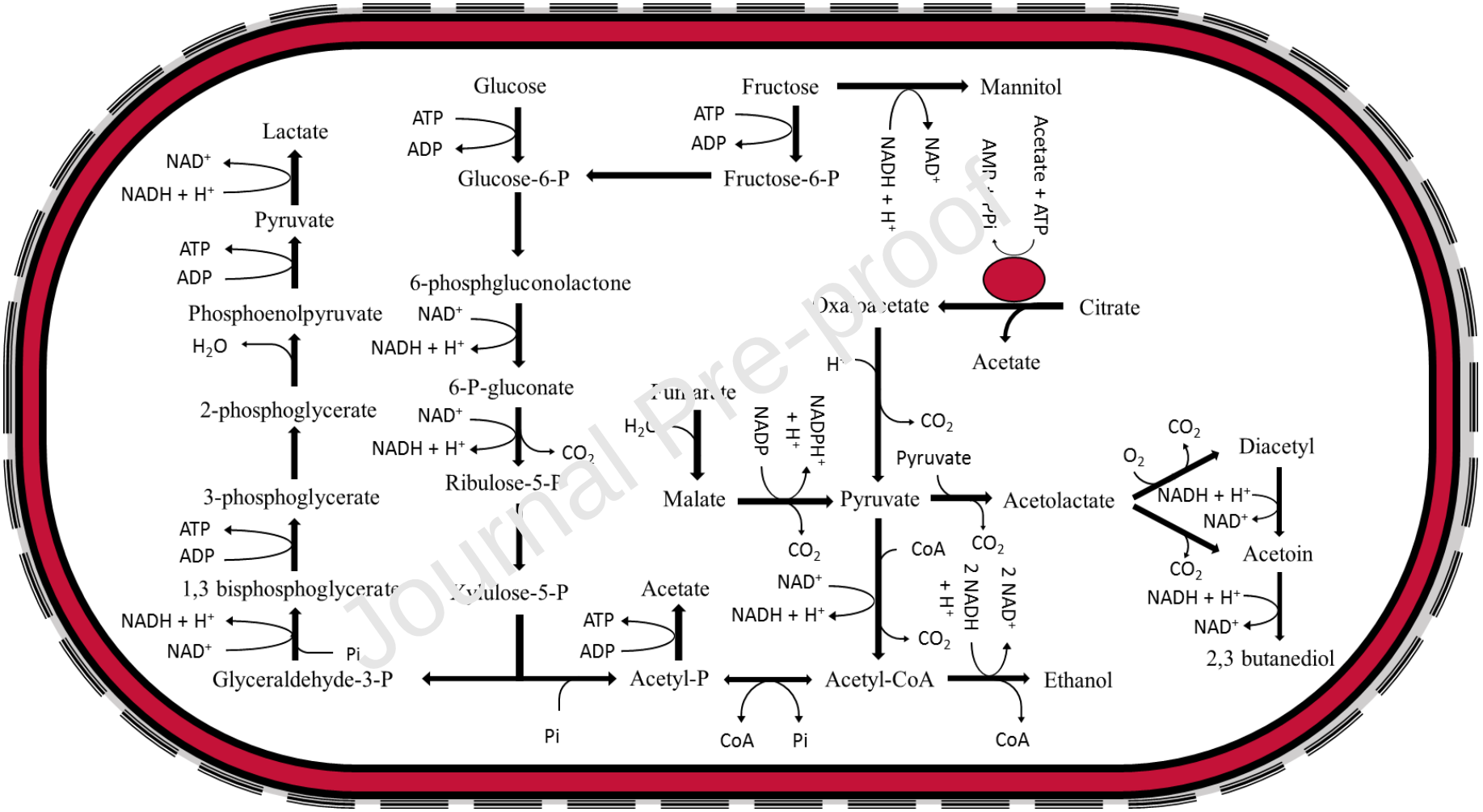


Figure 2. Carbohydrate, organic acid and pyruvate metabolism in *Lc. citreum* TR116.

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Figure 3

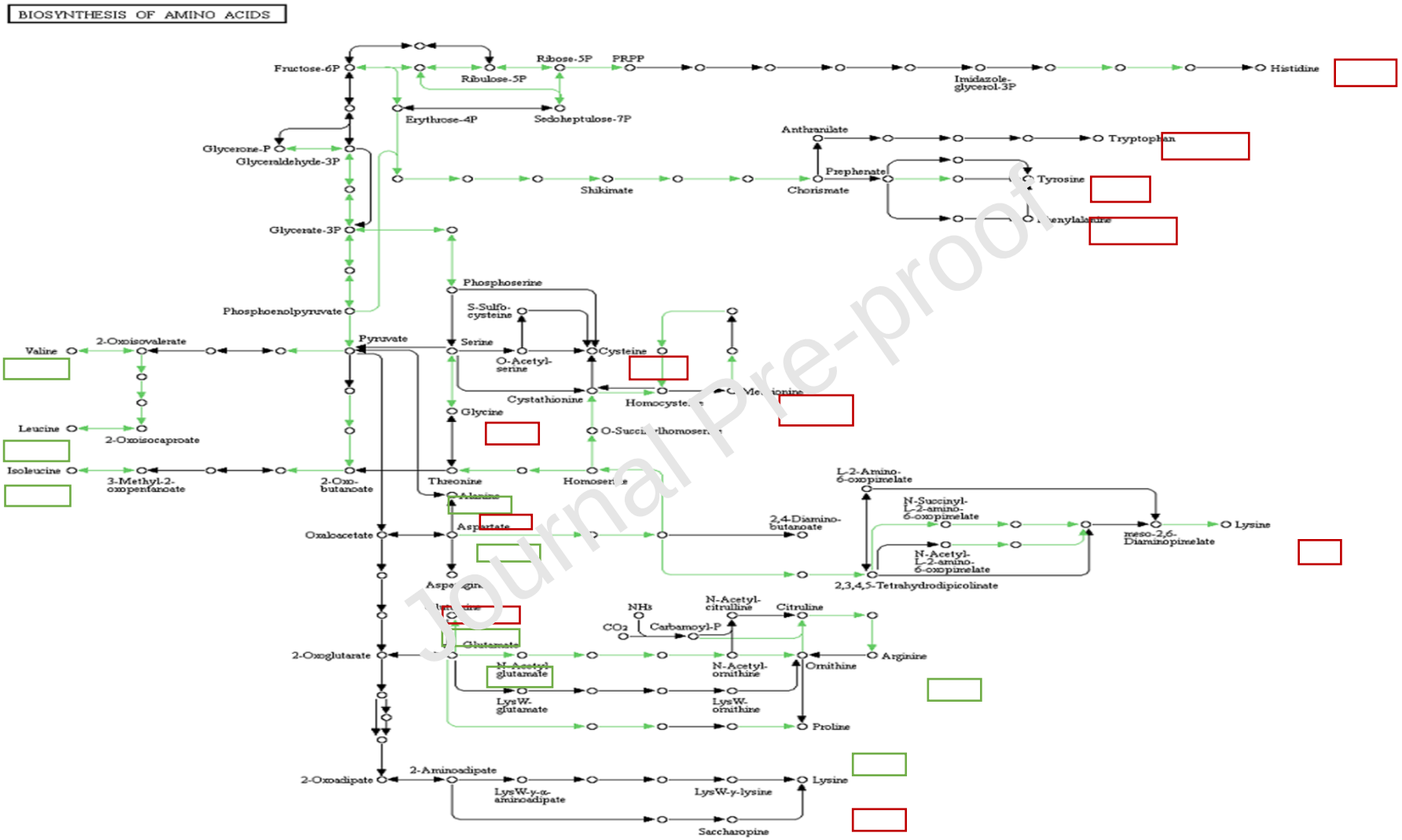


Figure 3. KEGG pathway of amino acid biosynthesis for *Lc. citreum* TR116 produced by KAAS; green arrows represent present reactions and amino acids framed in green are the amino acids being synthesised while a red frame indicates no synthesis of those amino acids due to missing reactions.

Figure 4

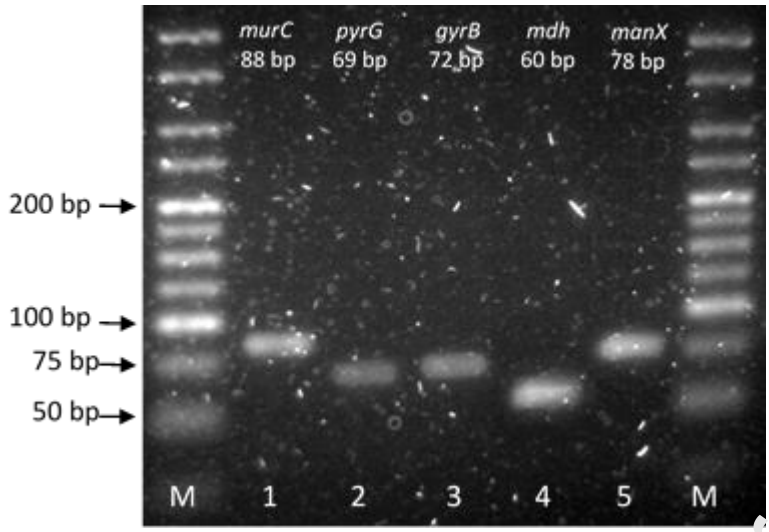


Figure 4. Agarose gel electrophoresis of qPCR primer pair amplification products. M, Hyper-ladder V MW marker; lanes 1-5 are labelled with the corresponding amplification products *murC, pyrG, gyrB, mdh, manX*.

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Figure 5

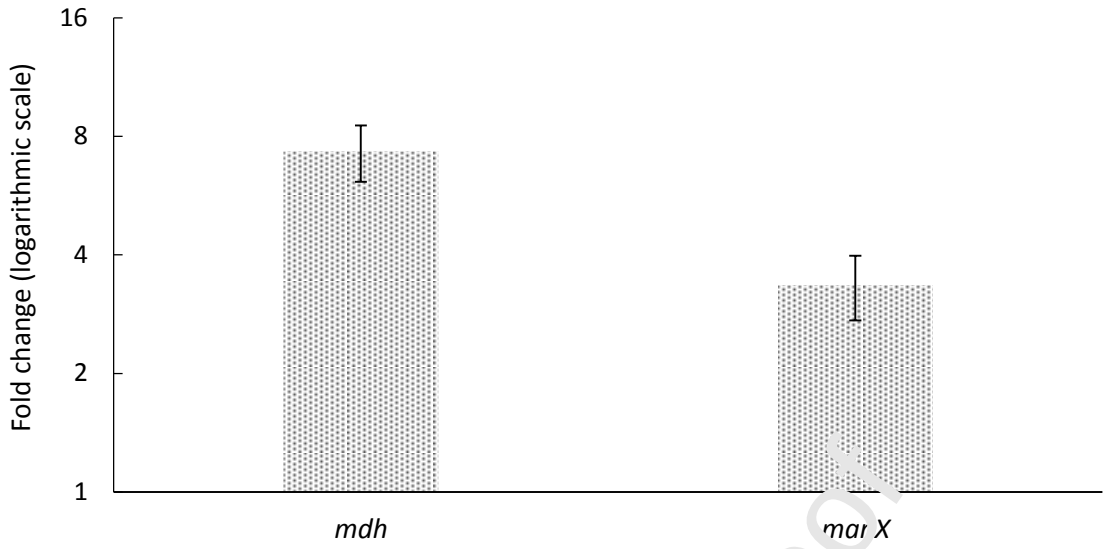


Figure 5. Relative expression levels of *mdh* and *manX*. Expression levels are fold changes in the expression of each gene for cultures grown in MRS5-F (test) relative to cultures grown in MRS5-G (control), where the fold change in the control is standardised to a value of 1. Expression of the target genes was normalised by the expression of the reference gene *gyrB*.

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Conflict of interest

Dear Editor,

The authors wish to confirm that there are no actual or potential conflicts of interest including any financial, personal, or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, this work.

Sincerely,

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Highlights

- Genome sequence of a characterised high mannitol producing *Lc. citreum* strain.
- Metabolic modelling of carbohydrates, organic acids and pyruvate metabolism.
- Mapping of amino acid metabolism and description of proteolytic complement.
- Effect of fructose on expression of mannitol dehydrogenase and fructose transporter genes.

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