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Lifestyle of *Lactobacillus hordei* isolated from water kefir based on genomic, proteomic and physiological characterization

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ABSTRACT

Water kefir is a traditional fermented beverage made from sucrose, water, kefir granules, dried or fresh fruits. In our water kefir granules, *Lactobacillus (L.) hordei* is one of the predominant lactic acid bacteria (LAB) species of this presumed symbiotic consortium. It faces abundant sucrose versus limitation of amino- and fatty acids in an acidic environment. Sequencing of the genome of *L. hordei* TMW 1.1822 revealed one chromosome plus three plasmids. The size of the chromosome was 2.42 Mbp with a GC content of 35% GC and 2461 predicted coding sequences. Furthermore, we identified 1474 proteins upon growth on water kefir medium. Metabolic prediction revealed all enzymes required for the glycolytic Embden-Meyerhof (EMP) and phosphoketolase (PKP) pathways. Genes encoding all enzymes involved in citrate, pyruvate and mannitol metabolism are present.

Moreover, it was confirmed that *L. hordei* is prototrophic for 11 amino acids and auxotrophic for 6 amino acids when combining putative biosynthesis pathways for amino acids with physiological characterization. Still, for glycine, serine and methionine no sure auxotype could be determined. The OppABCDF peptide transport system is complete, and 13 genes encoding peptidases are present. The arginine deiminase system, was predicted to be complete except for carbamate kinase, thus enabling neutralization reactions via ammonium formation but no additional energy generation. Taken together our findings enable prediction of the *L. hordei* lifestyle in water kefir: Abundant sucrose is consumed directly via parallel EMP and PK pathways and is also extracellularly converted to dextran and fructose by a glucansucrase, leaving fructose as additional carbon source. Essential amino acids (in the form of peptides) and citrate are acquired from fruits. In the lack of FabB unsaturated fatty acids are synthesized by predicted alternative enzymes. Formation of acetoin and diacetyl as well as arginine conversion reactions enable acidification limitation. Other members of the water kefir consortium (yeasts, acetic acid bacteria) likely facilitate or support growth of *L. hordei* by delivering gluconate, mannitol, amino- and fatty acids and vitamins.

1. Introduction

Water kefir is a traditional fermented beverage made from sucrose, water, kefir grains, and dried or fresh fruits (e.g. figs). The microbiota isolated from kefir grains comprise a stable presumably symbiotic multispecies consortium, which generally consists of lactic acid bacteria (LAB), acetic acid bacteria and yeasts (Gulitz et al., 2011; Irigoyen et al., 2005; Marsh et al., 2013; Vardjan et al., 2013). Strains of *L. hordei* are among the most commonly isolated LAB from kefir grains (Gulitz et al., 2011; Hsieh et al., 2012; Laureys and De Vuyst, 2017). Under a biochemical perspective, LAB are classified as homofermentative and heterofermentative based on their main products from carbohydrate fermentation. In general, homofermentative LAB including *L. hordei* convert carbohydrates mainly into lactic acid through the Embden-Meyerhof pathway (EMP), whereas heterofermentative LAB produce acetic acid, ethanol and carbon dioxide apart from lactic acid using the phosphoketolase pathway (PKP) (Årsköld et al., 2008; Kandler, 1983; Kleerebezem et al., 2003; Kleerebezem and Hugenholtz, 2003). However, along the species description *L. hordei* (isolated from barley (*Hordeum vulgare*)) is unable to ferment pentoses, but ferments a variety of hexoses and disaccharides, including sucrose and also mannitol to produce acids (Rouse et al., 2008). Therefore, the role of the PKP should be different from using pentoses in this organism.

Information on the metabolism of LAB from the water kefir

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consortium is restricted to a few studies. As sucrose is the main carbon source, the organisms either directly take it up and metabolize it intracellularly, or it is metabolized via extracellular or cell-bound bacterial glycosyltransferases producing glucans or fructans and leaving fructose or glucose as "secondary" carbon sources, respectively (Gulitz et al., 2011). Looking at the overall conversion reactions of the consortium it was found that a mixture of lactic and acetic acid as well as ethanol and glycerol are formed as can be expected from the composition of the microbial consortium of LAB, yeasts and acetic acid bacteria. In addition, mannitol formation has been observed (Laureys and De Vuyst, 2014). This suggests the use of fructose not only as carbon source but also as electron acceptor by some members of the consortium. Also, the consortium produces volatile compounds with sensory attributes including isoamyl acetate, isoamyl alcohol, ethyl acetate, 2-methyl-1-propanol, ethyl octanoate, ethyl decanoate, ethyl hexanoate (Laureys and De Vuyst, 2014). From this study, the specific contribution of groups or species remains speculative. In a more detailed study employing single isolates and mixtures, thereof the metabolic interaction of *L. nagelii* and *L. hordei* with *Zygotorulaspora (Z.) florentina* was examined (Stadie et al., 2013). This study suggests that *Z. florentina* appreciates the pH decrease caused by lactic acid fermentation, while a trophic interaction between *L. hordei* and this yeast is constituted by the release of amino acids and vitamin B_6. Co-cultivation of *L. nagelii* with *Z. florentina* apparently induced the release of arginine by the yeast, which was found to be essential for the growth of *L. nagelii*.

Despite the research on water kefir and its microbiome, the formation of the kefir granule is not yet understood. Previous research suggested *L. hilgardii* to be the main producer of the kefir granule polysaccharide (Fels et al., 2018; Pidoux, 1988; Waldherr et al., 2010). Still, it remains unclear, how other bacteria and yeasts become embedded into this polysaccharide network. In this context, recent results showed, that different *L. hordei* strains produce extracellular polysaccharides from sucrose, capable of inducing yeasts to form aggregates, which has not been observed for other lactic acid bacteria (Xu et al., 2018). This renders *L. hordei* as an interesting target for further investigations on its lifestyle and contribution to the formation of the water kefir consortium and granule.

Apart from the findings reported in the species description of *L. hordei* strains isolated from barley (Rouse et al., 2008), glycolytic pathways and pyruvate metabolism of *L. hordei* from water kefir as well as amino acid biosynthesis and conversion reactions are widely unexplored. Here, an isolate of *L. hordei* from water kefir was whole-genome sequenced for the first time and the putative metabolic pathways were reconstructed. Specific metabolic traits were also investigated in physiological tests. Together with a comprehensive proteomic analysis of cells growing in water kefir medium a basis is provided for understanding how *L. hordei* adapts to the water kefir environment and potentially interacts with other microbiota in water kefir.

2. Material and methods

2.1. Strain culture and DNA isolation

L. hordei TMW 1.1822, isolated from water kefir by Gulitz et al. (Gulitz et al., 2011), was spread directly on mMRS agar plates described by Stolz et al. (Stolz et al., 1995), from a −80 °C stock of our strain collection and incubated anaerobically at 30 °C for 2 d. A single colony was transferred into 15 mL liquid mMRS medium and cultured anaerobically at 30 °C for 20 h. Isolation of high molecular weight DNA was performed using the Genomic-tip 100/G kit (Qiagen, Venlo, Netherlands) according to the manufacturer. The isolation protocol was modified on the lysis time of proteinase K overnight. Quality and quantity of isolated DNA was checked by NanoDrop (Thermo Fisher Scientific) and agarose gel electrophoresis.

2.2. Sequencing strategy, assembly and annotation

About 15 µg of the isolated genomic DNA of *L. hordei* TMW 1.1822 at the concentration of 560 ng/uL was sent to and sequenced by GATC Biotech (Konstanz, Germany) via PacBio Single Molecule Real Time (SMRT) sequencing as described previously (Eid et al., 2009; Geißler et al., 2016a; McCarthy, 2010). Raw data were assembled according to SMRT Analysis (Version 2.2.0.p2), the hierarchical genome assembly process (HGAP) (Chin et al., 2013) and manual curation described by PacBio (https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/Finishing-Bacterial-Genomes). Upon assembly of chromosome and all plasmids separately, these contigs were circularized using minimus2 (AMOS, http://amos.sourceforge.net) to generate the whole genome. Finally, the complete whole genome sequence was submitted to NCBI GenBank. The genome was further annotated by the NCBI Prokaryotic Genome Annotation Pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/) and RAST, which is a SEED-based prokaryotic genome annotation service using default settings (Aziz et al., 2008; Overbeek et al., 2013).

2.3. Chromatographic analysis of sugars and organic acids, determination of auxotrophic and prototrophic amino acids

Consumption and production of sugars and organic acids of *L. hordei* grown in chemically defined medium (CDM, pH 6.5) after 24 h were quantified by a Dionex UltiMate 3000 HPLC system (Dionex, Idstein, Germany) with Rezex ROA-Organic Acid H^+ column (Phenomenex, Aschaffenburg, Germany) and RI-101 detector (Shodex, München, Germany) as described by Geissler et al. (Geißler et al., 2016b). The composition of CDM is listed in Table S1. The supernatants of each fermented sample were collected by centrifugation (14,000*g*, 10 min) and then treated with protein precipitation before injecting into HPLC system. Protein precipitation was carried out as follows. 1 mL of each sample in triplicate were added with 50 µL 70% perchloric acid (v/v) and vortex for mixing. After 24 h, standing at 4 °C, the mixture was centrifuged (14,000*g*, 10 min) again to collect the supernatant. The supernatant was diluted if necessary, filtered by 0.2 µm regenerated cellulose membrane (Phenomenex) and ready for chromatographic analysis. Analytes were separated at a constant flow rate of 0.7 mL/min with column temperature of 85 °C for 30 min. Sulfuric acid (Rotipuran, Roth, Karlsruhe, Germany) solution with a concentration of 5 mM was served as mobile phase.

The growth of *L. hordei* TMW 1.1822 was tested in full CDM in comparison to incomplete CDM, in which one amino acid was respectively omitted to reveal the auxotrophic or prototrophic for amino acids by Stadie et al. (Stadie, 2013). If *L. hordei* could grow, or could not grow when one amino acid was omitted, then *L. hordei* was considered as prototrophic or auxotrophic for this amino acid, respectively.

2.4. Genomic statistical analyses and visualization

A genomic atlas was generated using Artemis and DNA plotter (http://www.sanger.ac.uk/science/tools/artemis) (Carver et al., 2008) by importing the whole genome GenBank file. Subcellular localization of proteins was predicted utilizing the tool PSORTb (Version 3.0.2, http://www.psort.org/psortb/) (Gardy et al., 2004; Yu et al., 2010). Functional analysis was accomplished using SEED categorization based on RAST and SEED subsystem analysis (Subsystem and FIGfams Technology) (Aziz et al., 2008; Overbeek et al., 2013). The SEED subsystem analysis enables the assignment of predicted genes to a category, subcategory and subsystem. All the annotated EC and KO numbers, which were extracted from RAST fasta files (Table S2), could be directly imported into iPath 3.0 (https://pathways.embl.de/ipath3.cgi?map=metabolic) (Yamada et al., 2011) to obtain an overview of complete metabolic pathways and biosynthesis of other secondary metabolites customized in red color.

Based on the pathway of glycolysis, pentose phosphate and TCA cycle from the BioCyc Database Collection (https://biocyc.org/), all enzymes involved in each reaction step were subjected to manual check if they were present in translated open reading frames (ORFs) files annotated from NCBI and RAST. In detail, if one enzyme involved in a pathway was manually checked to be present in both files, its corresponding ORF was imported into NCBI Conserved Domain Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and Smart BLAST (https://blast.ncbi.nlm.nih.gov/smartblast/?LINK_LOC=BlastHomeLink) to manually double check the function of this enzyme. In this way, the enzyme was eventually confirmed to be present based on the functional genome prediction.

The presence of enzymes involved in pyruvate metabolism, the arginine deiminase (ADI) pathway and biosynthesis pathway of amino acids were verified in the similar workflow except the source of reference schematic pathway was different. The pathway of pyruvate metabolism was according to Geißler (Geißler, 2016) and Quintans et al. (Quintans et al., 2008), while ADI pathway based on Rimaux et al. and Tonon et al. (Rimaux et al., 2011; Tonon and Lonvaud-Funel, 2002), biosynthesis pathway of amino acids referred to KEGG mapper (http://www.genome.jp/kegg/pathway.html). The figure of the biosynthesis pathway of amino acids was generated using KEGG PATHWAY mapping tool (http://www.genome.jp/kegg/tool/map_pathway1.html) by importing EC numbers only involved in amino acids biosynthesis (Table S2).

The whole genome sequence of *L. hordei* DSM 19519 (Accession no. AZDX01000000), originally isolated from barley (Sun et al., 2015) was downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). Genomic differences to *L. hordei* TMW 1.1822 were identified by whole genome comparison using Blast Diagnostic Gene findEr (BADGE) (Behr et al., 2016) under default settings.

2.5. Sample preparation for proteomic analysis, liquid chromatography and mass spectrometry

1% (v/v) pre-cultured *L. hordei* were inoculated into 40 mL WKM in triplicate and cultured anaerobically at 30 °C for 10 h. Afterwards, trichloracetic (TCA) was added to those samples to a final concentration of 6.25% *w/v* (stock concentration TCA 100% w/v). Subsequently samples were stored on ice for 10 min. The bacterial pellets were collected by centrifugation (5000 rpm, 5 min) at 4 °C, washed twice with acetone, reconstituted in lysis buffer (8 M urea, 5 mM EDTA di-sodium salt, 100 mM $(NH)_4HCO_3$, 1 mM Dithiothreitol (DDT), pH 8.0) and mechanically disrupted with acid-washed glass beads (G8772, 425–600 µm, Sigma, Germany). Total protein concentration of the lysate was determined using the Bradford method (Bio-Rad Protein Assay, Bio-Rad Laboratories GmbH, Munich, Germany). 100 µg protein extract was used per sample for in-solution digestion. Proteins were reduced with 10 mM DTT at 30 °C for 30 min, and subsequently carbamidomethylated with 55 mM chloroacetamide in the dark at room temperature for 60 min. Subsequently proteins were digested by addition of 1 µg trypsin (1:100 trypsin:protein) for 3 h at 37 °C and another 1 µg of trypsin overnight at 37 °C. Digested peptide samples were desalted according to the manufacturer's instructions by C18 solid phase extraction using Sep-Pak columns (Waters, WAT054960). Purified peptide samples were dried in a SpeedVac and resuspended in 2% acetonitrile, 98% H_2O, 0.1% formic acid to a final concentration of 0.25 µg/µl as determined by Nanodrop measurement.

Generated peptides were analyzed on a Dionex Ultimate 3000 nano LC system coupled to a Q-Exactive HF mass spectrometer (Thermo Scientific, Bremen, Germany). Peptides were delivered to a trap column (75 µm × 2 cm, self-packed with Reprosil-Pur C18 ODS-3 5 µm resin, Dr. Maisch, Ammerbuch, Germany) at a flow rate of 5 µL/min in solvent A_0 (0.1% formic acid in water). Peptides were separated on an analytical column (75 µm × 40 cm, self-packed with Reprosil-Gold C18, 3 µm resin, Dr. Maisch, Ammerbuch, Germany) using a 120 min linear gradient from 4 to 32% solvent B (0.1% formic acid, 5% DMSO in acetonitrile) and solvent A_1 (0.1% formic acid, 5% DMSO in water) at a flow rate of 300 nL/min. The mass spectrometer was operated in data dependent mode, automatically switching between MS1 and MS2 spectra. MS1 spectra were acquired over a mass-to-charge (m/z) range of 360–1300 m/z at a resolution of 60,000 (at m/z 200) using a maximum injection time of 50 ms and an AGC target value of 3e6. Up to 20 peptide precursors were isolated (isolation window 1.7 m/z, maximum injection time 25 ms, AGC value 1e5), fragmented by higher-energy collisional dissociation (HCD) using 25% normalized collision energy (NCE) and analyzed at a resolution of 15,000 with a scan range from 200 to 2000 m/z. Precursor ions that were singly-charged, unassigned or with charge states > 6+ were excluded. The dynamic exclusion duration of fragmented precursor ions was 20 s.

2.6. Peptide and protein identification and quantification

Peptide and protein identification plus quantification were performed with MaxQuant (version 1.5.7.4) (Cox and Mann, 2008) by searching the MS2 data against all protein sequences from *L. hordei* (cf. Section 3.1, GenBank CP018176 - CP018179) using the embedded search engine Andromeda (Cox et al., 2011). Carbamidomethylated cysteine was a fixed modification; oxidation of methionine, and N-terminal protein acetylation were variable modifications. Trypsin/P was specified as the proteolytic enzyme and up to 2 missed cleavage sites were allowed. Precursor and fragment ion tolerances were 10 ppm and 20 ppm, respectively. Label-free quantification (Cox et al., 2014) and data matching between consecutive analyses were enabled within MaxQuant. Search results were filtered for a minimum peptide length of 7 amino acids, 1% peptide and protein false discovery rate (FDR) plus common contaminants and reverse identifications. MaxQuant output files (proteinGroups.txt) were further performed using Perseus (version 1.5.6.0) (Tyanova et al., 2016). NCBI annotation, Psortb subcellular localization, SEED category (subcategory and subsystem) as previously annotated (cf. Sections 2.2 and 2.4) were added to the matrix through matching rows by Protein IDs and Locus.

3. Results and discussions

3.1. General genomic features and prediction of metabolic pathways

L. hordei is a predominant and stable fermentation species isolated from water kefir (Gulitz et al., 2011). The complete genome of *L. hordei* TMW 1.1822 in this study was sequenced by PacBio RS II SMRT to get insight into its niche adaptation to the water kefir system and provide the basis for consecutive analyses. It was submitted to GenBank designated as BioSample SAMN06052353, which was part of the BioProject PRJNA343197. Referred to as accession numbers CP018176 to CP018179, the four resulting contigs were identified as one chromosome plus three plasmids. Consisting of 2.42 Mbp in size, the chromosome was found to fit in the typical range of some other lactobacilli (Vogel et al., 2011), and exhibits a GC content of 35%. The 3 plasmids exhibited a GC content of 37.37%, 39.27%, 40.12%. Plasmid sizes ranged from 38, 056 bp, 60, 901 bp to 68, 542 bp, resulting in an overall genome size of 2.59 Mbp. The number of coding sequences (CDS) according to NCBI were 2461 and the coding density is 86.27%, which is visualized as a circular genome in Fig. 1. There were 105 pseudogenes, 61 tRNA and 6 rRNA found randomly distributed in the chromosome. The GC skew graph observed in circular chromosome was clarifying the shift point, which was reported, correlated with the loci of *ori* and *ter* (Frank and Lobry, 1999). The genome of *L. hordei* TMW 1.1822 isolated from water kefir is the second whole genome sequence besides *L. hordei* DSM 19519, which was isolated from malted barley in 2015 (Sun et al., 2015). The availability of the genome sequence provided the basis for consecutive proteomic analysis, with the objective to derive the basic metabolism and lifestyle of *L. hordei* in water kefir. An

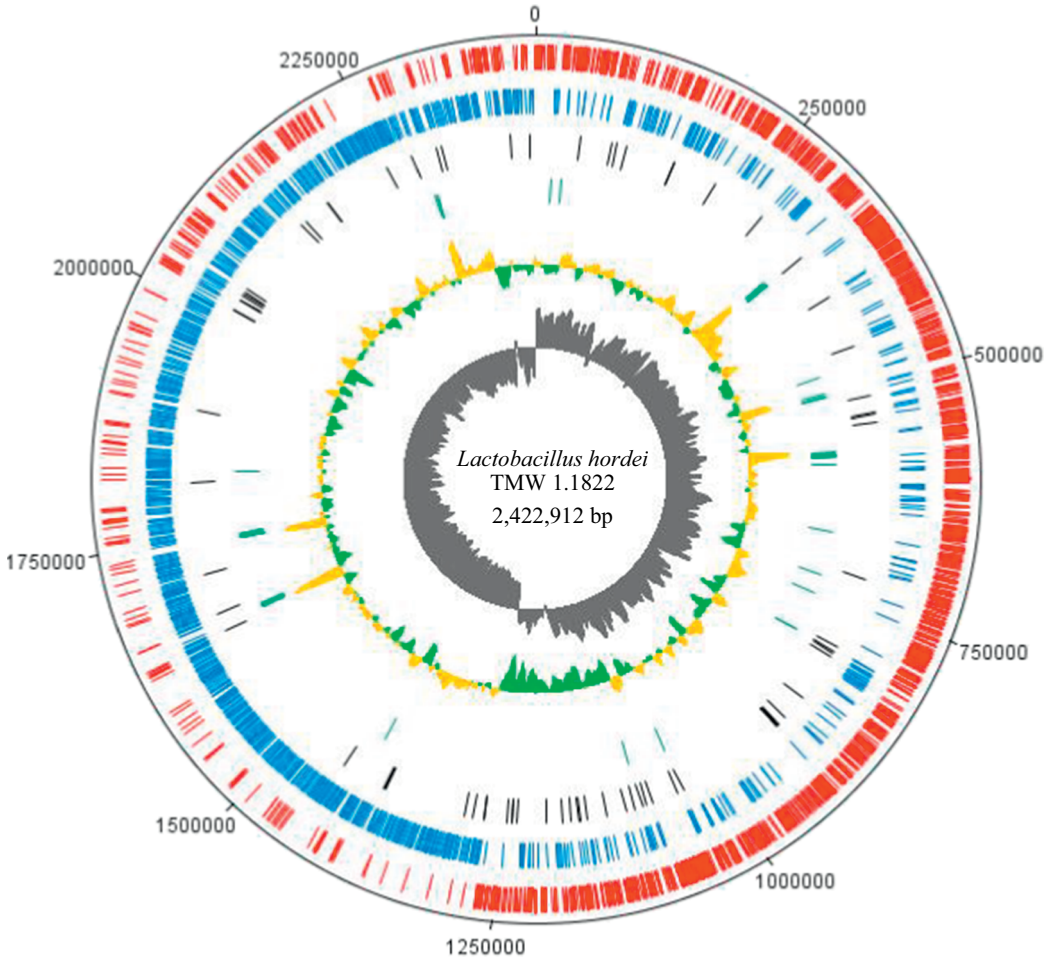


Fig. 1. Genomic atlas of *L. hordei* TMW 1.1822. From the outer circle to inner circle are as below. Forward CDS (red), Reverse CDS (blue), Pseudogenes on both strands (black), tRNA and rRNA (dark green), %GC plot (green, low GC spike and yellow, high GC spike), GC skew [(G − C) / (G + C)] (grey). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

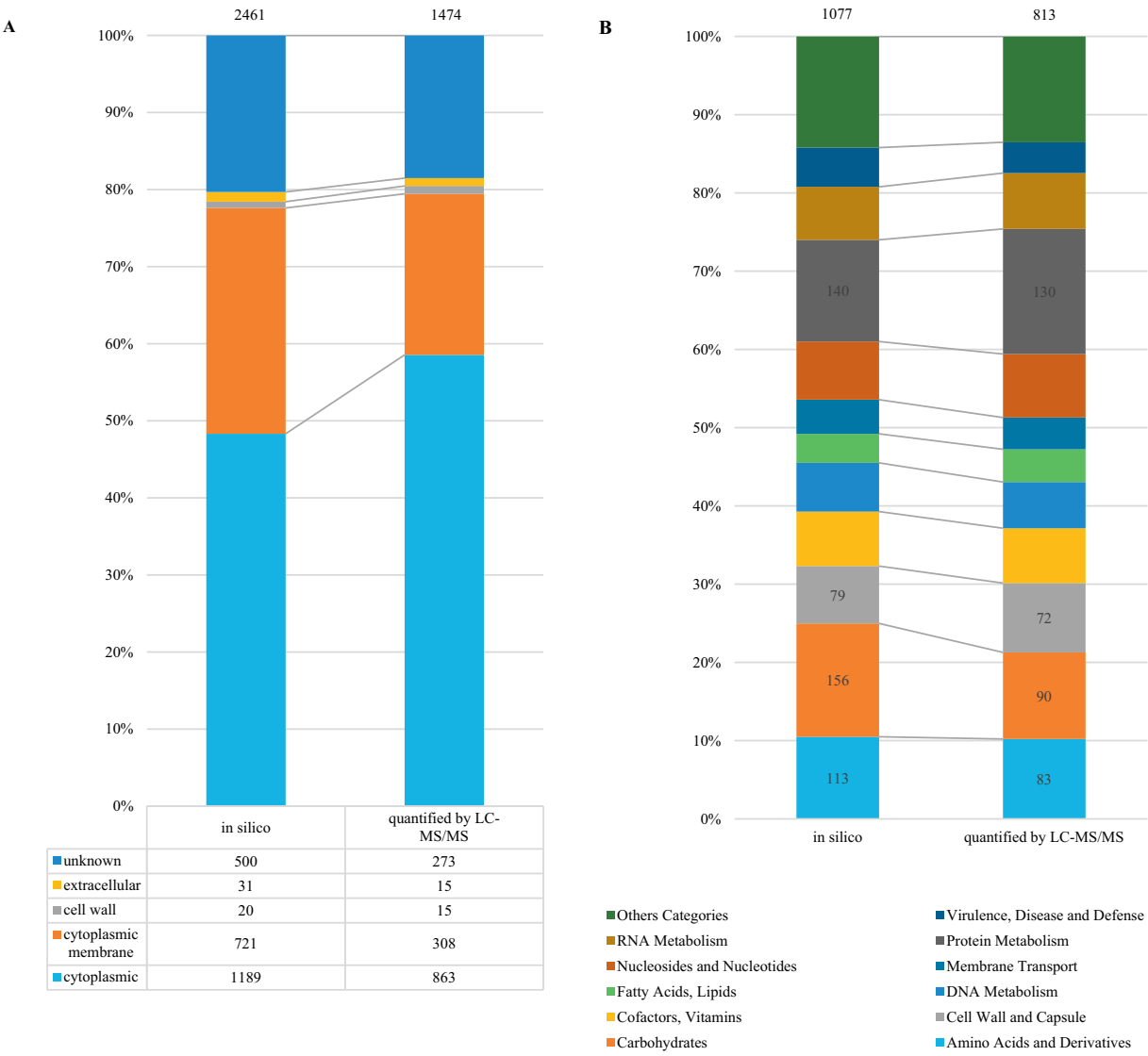
overview of the metabolic pathways was generated as a basis for subsequent, detailed analyses (shown in Fig. S1).

3.2. Proteomic analysis

A total of 1474 proteins were identified in triplicate proteomic analyses from *L. hordei* growing in water kefir medium, representing nearly 60% of the genes predicted from the genome. The in silico predicted and identified proteins were thoroughly annotated in terms of subcellular localization and SEED category prediction. Fig. 2A shows the absolute number of respective proteins in cytoplasmic, cytoplasmic membrane, cell wall, extracellular space and unknown location in the table below and their proportions with respect to the total number of proteins (above the histogram) by the bar chart. As shown in Fig. 2B, most abundant proteins were assigned to protein metabolism (16%) and carbohydrates (11%), followed by amino acids and derivatives (10%) and cell wall and capsule (9%). Taken together, it was in agreement with the fact that LAB express a relatively simple, limited in metabolism and generally need nutrient-rich environments where carbohydrates, amino acid sources and other nutrients are abundant. Since water kefir medium is characterized by rich sucrose supply at the concentration of 80 g/L and is limited in the supply of amino acids and lipids, we focused our analysis on respective metabolic and biosynthetic functions to depict the lifestyle of *L. hordei* in water kefir.

3.3. Sugar transport and central carbohydrate metabolism

An overview on the key reactions involved in sucrose metabolism of *L. hordei* is provided in Fig. 3. Generally, sucrose can either be taken up and metabolized or converted by a glycosyltransferase (glucansucrase) into a glucan and fructose, which is subsequently metabolized. The structure and ecological function of *L. hordei* glucan have been previously characterized (Xu et al., 2018). It has been demonstrated that a specific glucan structure is produced, which induces network formation of *Saccharomyces cerevisiae* sharing the water kefir consortium and thus ensures close proximity of this yeast. A total of 10 PTS sugar transport systems were predicted from the genome, which catalyze the phosphorylation of incoming sugar and transport them into the cell. According to the annotation, these PTS should enable for the transport of sucrose, glucose, fructose, mannose, sorbose, mannitol, β-glucoside, cellobiose, galactitol and galactosamine. These PTS deliver phosphorylated sugar derivatives, which feed the central carbon metabolism. Analysis of the annotated whole genome of *L. hordei* confirmed the presence of the genes encoding all enzymes required for the EMP and PKP pathways. Detailed information on these enzymes is given in Table S3, which includes locus tags of NCBI and IDs of RAST. The proteins encoded in those genes were also identified as being expressed when *L. hordei* was grown in WKM after 10 h. Thus, *L. hordei* TMW 1.1822 could be considered as facultatively homofermentative with regard to the discrimination of LAB into three physiological groups: (i) the facultatively homofermentative LAB, possessing both EMP and PKP, but



	in silico	quantified by LC-MS/MS
unknown	500	273
extracellular	31	15
cell wall	20	15
cytoplasmic membrane	721	308
cytoplasmic	1189	863

Fig. 2. Subcellular localization of proteins (in silico, identified by LC-MS/MS) which were predicted by PsortB (A). The proportion of proteins assigned to each respective subcellular compartment and the group "unknown" with respect to the total number of proteins is shown by the bar chart. The table below shows the respective absolute numbers, and the number above is the total number of proteins. SEED categories of proteins (in silico, identified by LC-MS/MS) which were predicted by SEED (B). The proportion of proteins assigned to each respective categories of metabolism and the group "other categories" which is the sum of several small categories with respect to the total number of proteins is shown by the bar chart. Above each column is the total number of predicted SEED categories.

preferably degrading glucose via EMP and potentially use the PKP for the degradation of pentoses such as *L. plantarum* WCFS1, *Lactococcus lactis* (Kleerebezem et al., 2003; Kleerebezem and Hugenholtz, 2003), (ii) the obligately homofermentative LAB, lacking both glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase (*Thermobacterium*), and (iii) the heterofermentative LAB, lacking fructose-bisphosphate aldolase (*Betabacterium*) (Ljungh and Wadström, 2009) or 6-phosphofructokinase (Cibrario et al., 2016) The expression of the PKP in *L. hordei* could enable production but also utilization of gluconate. The gluconate may simply be an intermediate of the PKP but could also result from the metabolism of acetic acid bacteria in this water-kefir consortium, and thus gluconate utilization appears to be a decisive trait reflecting adaptation to live in the water kefir consortium. Phosphoketolase as key enzyme of PKP can catalyze xylulose-5-phosphate into acetylphosphate (acetyl-P) and glyceradehyde-3-phosphate (GAP). On one hand, acetyl-P could be converted into ethanol or (preferably) acetate enabling additional ATP generation in the acetate kinase reaction. This reaction depends on the presence and utilization of external electron acceptors, which would be needed to enable maintenance of the redox balance. In fact, fructose is generated by the activity of the glycosyltransferase, which produces a glucan from sucrose. However, *L. hordei* does not possess a known homolog for mannitol dehydrogenase. Still, mannitol could be generated by other members of the consortium, e.g. *S. cerevisiae*, which is held in proximity by the glucan-induced network formation (Xu et al., 2018), and serve as a substrate for *L. hordei*. Indeed, a predicted PTS system for mannitol transport and phosphorylation as well as mannitol-P-5 dehydrogenase delivering fructose-6-phosphate are expressed, which enable the growth on mannitol even as sole carbon source upon entering the EMP. Indeed, *L. hordei* can grow with mannitol and also gluconate as sole carbon sources, which matches the prediction from the genome.

Furthermore, *L. hordei* produced 19.8 mM lactic acid and 6.1 mM acetic acid, while no ethanol was detected by HPLC after 24 h fermentation (Fig. 4). In the presence of EMP and PKP pathways *L. hordei* could have two alternatives for acetate formation. Acetate could result from the PKP as an alternative to ethanol, or via pyruvate decarboxylase from pyruvate stemming from either EMP or PKP. With a lactate/acetate ratio of 3.2 *L. hordei* cannot be considered an obligate

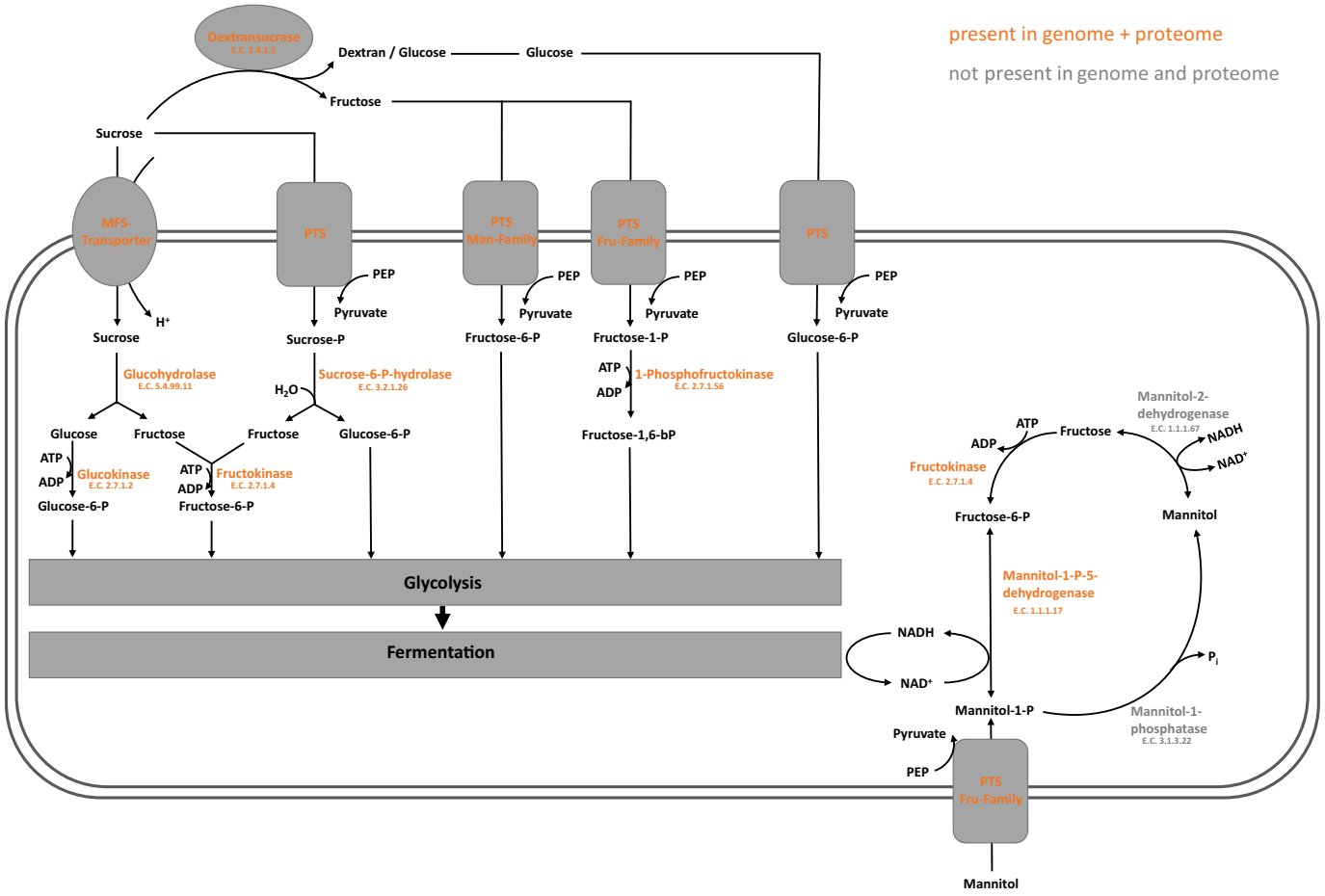


Fig. 3. Overview on the key reactions involved in sucrose metabolism of *L. hordei* TMW 1.1822.

homofermenter anyway. On the other hand, maintenance of a redox balance in the case of acetate formation requires recycling of NAD for these pathways, which may be enabled by utilization of gluconate as electron acceptor and/or the formation of butandiol as an alternative to ethanol. Both assumptions would fit the co-existence with acetic acid bacteria and yeasts in the water kefir, which provide gluconate and limit ethanol production by *L. hordei* by their own ethanol production, respectively.

The pentose phosphate pathway is incomplete by the lack of transaldolase. Actually, there are five enzymes converting glucose into ribulose-5-phosphate, which are the same as those involved in pentose phosphate pathway and PKP. So the specific part of the pentose phosphate pathway is represented only by two genes encoding ribose-5-phosphate isomerase and transketolase. Still, the absence of transaldolase in the last step only interrupts the link to glycolysis but does not influence the generation of NADPH and formation of pentoses including ribose, which is an essential component for the synthesis of nucleotides.

Since water kefir is an environment requiring its microbiota to manage high sucrose concentrations as main carbon source, it was not a surprising result, that *L. hordei* TMW 1.1822 displayed the adaption in its genome and also proteome, as discussed above. *L. hordei* DSM 19519 was isolated from barley, which offers a different spectrum of carbohydrate substrates, namely starch (maltose) or arabino-xylans. In contrast to the water kefir isolate, there were no sucrose specific PTS and

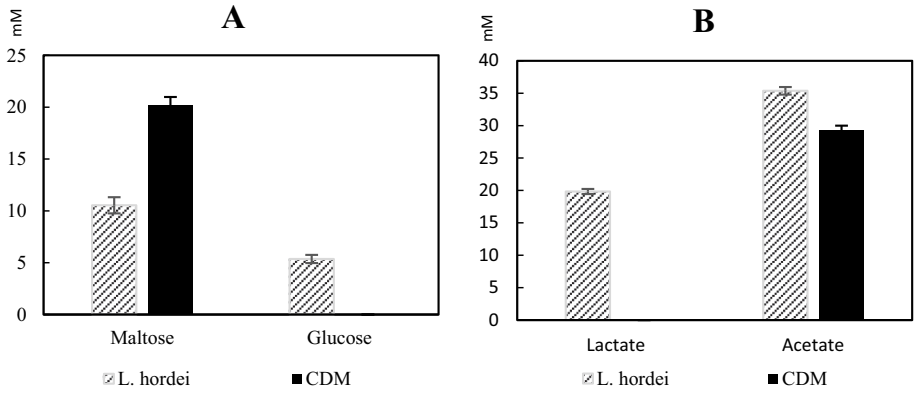


Fig. 4. Consumption and production of sugar (A) and organic acids (B) of *L. hordei* TMW 1.1822 isolated from water kefir grown in CDM after 24 h. Black histogram represented the negative control CDM, slash histogram represented detectable consumption of CDM after incubation with *L. hordei* TMW 1.1822 for 24 h.

extracellular glycosyltransferases found in the genome. Also, transporters involved in fructose import were less represented than in *L. hordei* TMW 1.1822 from water kefir. These findings provide evidence for the adaption of *L. hordei* TMW 1.1822 to the sucrose-rich water kefir environment as a stable part of the water kefir consortium.

3.4. Citrate and pyruvate metabolism, TCA cycle

Since water kefir contains lemon slices, metabolic conversion of citrate is a common trait of associated lactobacilli (Hugenholtz, 1993). Therefore, the putative functional genome of *L. hordei* was searched for the presence of genes involved in citrate uptake and metabolism.

Besides undirected transfer of citrate into the cytoplasm at low pH, the organism should be capable of directed import via a citrate/sodium symporter, which was also detected upon proteomic analysis. Also found in genome and proteome of *L. hordei*, the enzymes citrate lyase and oxaloacetate decarboxylase contribute to pyruvate generation by conversion of the imported citrate, requiring no NAD^+ recycling upon acetate formation. Completing these results, also the presence and expression in the proteome of the citrate lyase transcriptional regulator (*citI*) was confirmed, which was reported as a citrate activated switch, allowing the cell to optimize generation of metabolic energy (Martin et al., 2005). Thus, the pyruvate pool is filled from both the glycolytic pathway and citrate metabolism. Depicted in Fig. 5, further genomic and proteomic analysis revealed the presence of enzymes involved in different strategies for downstream pyruvate conversion. *L. hordei* should not only be capable of ATP generation upon acetate formation by an acetate kinase, but also of maintenance of redox balance by L/D-lactate (No. 11, shown in Table S3) and ethanol release. Since ethanol was not detectable by HPLC analysis, other strategies for NAD^+ recycling appeared to be more favorable, also including transformation of pyruvate to diacetyl, acetoin and 2,3-butanediol. Catalyzing the reaction of pyruvate to α-acetolactate, acetolactate synthase represents the key enzyme in the synthesis of these compounds. Once synthesized, α-acetolactate is unstable and is either non-enzymatically decarboxylated to diacetyl or converted to acetoin by α-acetolactate decarboxylase. Eventually, a diacetyl/acetoin reductase transforms diacetyl to acetoin, which is subsequently converted to 2,3-butanediol upon simultaneous NAD^+ recycling. Since *L. hordei* was found to express all relevant enzymes in its proteome for the synthesis of these C_4 compounds, it may also contribute to the sensory characteristics of water kefir.

Further genomic analysis revealed the lack of citrate synthase, 2-oxoglutarate dehydrogenase, succinyl-CoA synthetase, succinate dehydrogenase, malate dehydrogenase and malate oxidoreductase, impeding the organism from running a complete TCA cycle. This was confirmed by proteomic analysis and is consistent with other LAB, including *Lactococcus lactis* IL1403 and *L. plantarum* WCFS1 (Bolotin et al., 2001; Kleerebezem et al., 2003; Kleerebezem and Hugenholtz, 2003), which are the most extensively studied LAB in food fermentation.

3.5. Proteolytic system, amino acids biosynthesis, metabolism and transport

Many LAB, namely those adapted to environments lacking intrinsic protease activity (e.g. milk in contrast to meat or plant-derived environments) are equipped with a protein-degradation machinery, which enables the ability to obtain peptides and amino acids from proteins (Lopez-Kleine and Monnet, 2011). However, our in silico analyses of the genome of *L. hordei* isolated from water kefir did not reveal homologs to a protease (*prt*) gene. Still, it encoded and expressed on proteome level the complete uptake system for peptides, which originate from the fruits in the water kefir directly of via the proteolytic activity of plant enzymes or other microbes of the consortium. There were OppABCDF consisting of an oligopeptide-binding protein (OppA), two integral membrane proteins (OppB and OppC), and two nucleotide-binding proteins (OppD and OppF) which transport di-, tripeptides, and oligopeptides (Detmers et al., 1998; Tynkkynen et al., 1993). Upon uptake, these peptides can be degraded by a variety of peptidases (Christensen et al., 1999; Kunji et al., 1996). The *L. hordei* genome contains 13 genes encoding peptidases such as aminopeptidases, oligo-/di-/tri-peptidases, endopeptidases and uncharacterized peptidases listed in Table S4. The predicted peptidases of *L. hordei* were shown to be more closely related to those of *L. sanfranciscensis* TMW 1.1304 and *L. plantarum* WCFS1, but more distant to intestinal *L. johnsonii* NCC 533, which were shown to have > 25 peptidases (Kleerebezem et al., 2003; Pridmore et al., 2004; Vogel et al., 2011). Possibly this reflects their relation to plant proteins versus milk proteins.

The analysis of the *L. hordei* genome revealed biosynthesis pathways for 12 amino acids and incomplete pathways for 8 amino acids (shown in Fig. S2). Therefore, prototrophy was predicted of *L. hordei* for 12 amino acids and auxotrophy for 8 amino acids (Table S5). This result was not further proven by identified proteins, because there were only 83 proteins identified by LC-MS/MS compared to 113 proteins predicted in silico involved in amino acids and derivatives (Fig. 2B). Due to the absence of branched-chain amino acid aminotransferase, biosynthesis pathways of branched-chain amino acids (leucine, isoleucine and valine) were incomplete. The biosynthesis pathways of phenylalanine, tryptophan and tyrosine were incomplete either, due to the absence of 3-deoxy-7-phosphoheptulonate synthase and other enzymes. Auxotrophy for serine and glycine was predicted from the absence of

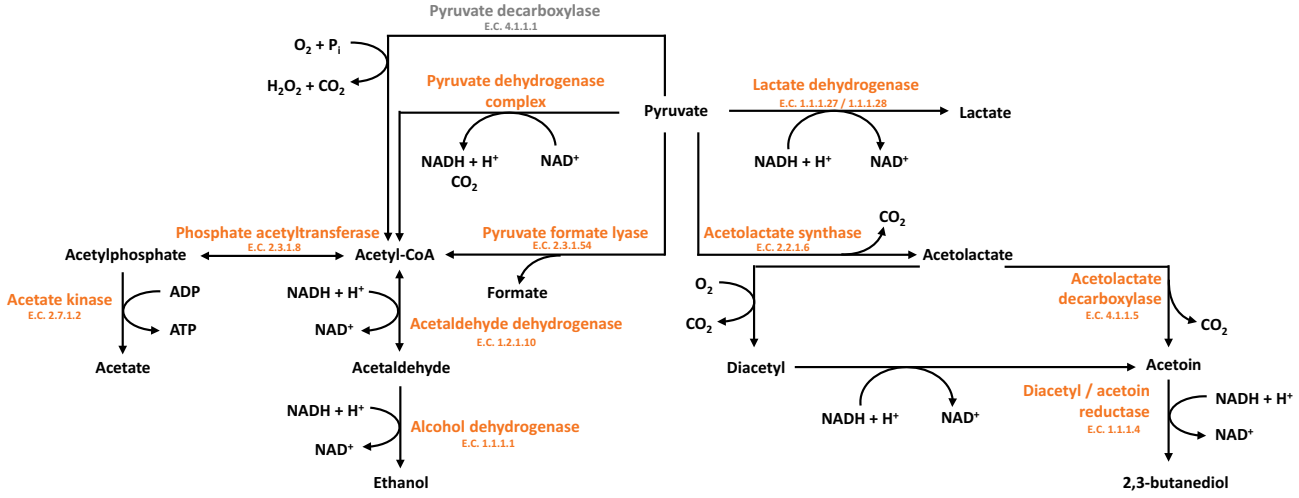


Fig. 5. Pyruvate metabolism of *L. hordei* TMW 1.1822.

phosphoserine phosphatase and other enzymes (see Table S5 for details). What is more, auxotrophy for serine, glycine, leucine, isoleucine and valine is consistent with the presence of serine/alanine/glycine transporter (CycA) and the branched-chain amino acid transporter (AzlC). It suggests the capability to uptake these amino acids directly from the secretion of other symbiotic microbes such as yeast or the degradation of peptides from fruits or yeast. A gene encoding a putative arginine transporter was predicted to be present, which could import any arginine produced by members of the consortium.

In addition, the growth of *L. hordei* was examined in incomplete CDM compared to full CDM to reveal amino acids auxotrophies. The results were almost identical with biosynthesis pathways of amino acids predicted from the genome, but different in the deductive auxotype of glycine, serine and methionine (Table S5). For instance, *L. hordei* was considered as auxotroph for serine from genomic prediction, but inferred to be prototroph with respect to serine upon physiological characterization. This suggests an alternative pathway for the biosynthesis of serine or a mistake in genome sequencing or annotation. Hence, it was confirmed that *L. hordei* was surely prototrophic for 11 amino acids and auxotrophic for 6 amino acids when combining genomic prediction with physiological analysis. For comparison, the genome of *L. plantarum* WCFS1 predicts prototrophy for 17 amino acids except for leucine, isoleucine and valine (Kleerebezem et al., 2003). In contrast, *L. sanfranciscensis* TMW 1.1304 is predicted to be auxotrophic for 12 amino acids (Vogel et al., 2011), and *L. acidophilus* NCFM for 14 amino acids (Altermann et al., 2005). So the capability of de novo synthesis of amino acids of *L. hordei* is intermediate, which suggests that in the water kefir there are still enough amino acids available from (dried) fruit or symbionts to enable prominent *L. hordei* growth.

3.6. Acid tolerance via arginine deiminase system

Degradation of arginine via the arginine deiminase (ADI) system is widely distributed in LAB genera such as *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Weissella*. Functionally, it enables enhanced acid tolerance and energy provision (Fernández and Zúñiga, 2006; Rimaux et al., 2011; Tonon and Lonvaud-Funel, 2002). The ADI system comprises three reactions catalyzed by arginine deiminase (ADI), ornithine transcarbamoylase (OTC), carbamate kinase (CK) and a transmembrane arginine/ornithine antiporter, catalyzing the exchange between extracellular arginine and intracellular ornithine. According to genomic and proteomic analysis no CK could be found in *L. hordei*. Therefore, ADI can convert arginine to citrulline and ammonia, resulting in the alkalization of the cytoplasm and protection against an acidic external pH (Cotter and Hill, 2003). Secondly, citrulline could be converted by OTC into carbamoyl phosphate (carbamoyl-P), which is essential for de novo pyrimidine biosynthesis (Kilstrup et al., 2005). Nevertheless, carbamoyl-P cannot not be converted in the lack of CK, which generates additional ATP in other organisms harboring the full ADI pathway. This does not appear to be a disadvantage/not necessary in a high energy containing environment as water kefir. The fate of carbamoyl-P therefore remains unclear. *L. hordei* did not encode all the enzymes involved in alternative systems, which are corresponding to acid tolerance such as the agmatine deiminase (AGDI) system and glutamate decarboxylase (GAD) system. To date, few LAB producing putrescine by AGDI system had been detected, such as *L. brevis*, *L. sakei* (Griswold et al., 2004; Lucas et al., 2007). The GAD system, which catalyzes the decarboxylation of L-glutamate to γ-aminobutyric acid (GABA), had been studied in *L. paracasei* NFRI 7415, *L. plantarum* DSM 19463 (Di Cagno et al., 2010; Komatsuzaki et al., 2008) and related to their acid tolerance. Therefore, above neutralization via ammonia from the partial arginine conversion, acidification appears mainly limited by the switch from lactic and acetic acid to butanediol formation.

3.7. Fatty acid biosynthesis

In addition to the limited availability of amino acids, *L. hordei* suffers from limits of fatty acids in the water kefir. On first glance, it appears that *L. hordei* should not be able to synthesize any unsaturated fatty acids by itself in the absence of *fabB*. However, it grew to high cell densities in water kefir medium without any fatty acids added. An answer to these conflicting results may be from in the study of Wang and Cronan (Wang and Cronan, 2004). These authors demonstrate that FabF from *Enterococcus faecalis* can functionally replace FabB in *E. coli*. Furthermore *E. faecalis* FabZ can replace *E. coli* FabA functions, despite different sequence alignments. They conclude that bacterial fatty acid biosynthetic pathways cannot be deduced solely by sequence comparisons. While this may be true also for other predictions from genomics, it is clear from growth experiments that in such alternative functions of bifunctional enzymes must exist for the biosynthesis of unsaturated fatty acids enabling growth of *L. hordei* under strict limitation of external fatty acids. In the lack of *fabB* and other sequence homologs annotated for functions introducing double bonds in fatty acids this gap is likely closed by the present functional homologs residing in FabA and FabZ, which may replace respective functions of the missing FabB and FabF in *L. hordei* (Table S6).

4. Conclusions

The putative functional genome and protein identification of *L. hordei* TMW 1.1822 revealed adaptation to the water kefir environment and suggests links to other members of the consortia, which likely reside in mutualistic (related to compounds) but also environmental (related to pH and redox maintenance) relationships. This way, it can handle sucrose in various effective ways including EMP, PKP and glucansucrase reactions, use gluconate and mannitol as additional carbon sources and for redox maintenance and limit acid stress by formation of butanediol instead of lactate or ammonia formation upon arginine conversion. The limits in amino- and fatty acids are handled by the uptake of peptides, synthesis of essential amino acids and use of alternative enzyme functions for the biosynthesis of unsaturated fatty acids. This work also shows, that genomic analysis greatly wins upon its support by proteomics and targeted physiological testing for traits in question from prediction.

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