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# Label-free quantitative proteomic analysis reveals the lifestyle of *Lactobacillus hordei* in the presence of *Sacchromyces cerevisiae*

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ABSTRACT

Water kefir is a fermented beverage, which is traditionally prepared from sucrose, kefir grains, dried or fresh fruits, and water. *L. hordei* and *S. cerevisiae* are isolated as predominant and stable species of lactic acid bacteria and yeasts, respectively. In this study we demonstrate that label free quantitative proteomics is useful to study microbial interaction along the response of co-cultivated *L. hordei* TMW 1.1822 in the presence of *S. cerevisiae* TMW 3.221 as compared with their single cultures in a water kefir model. It is shown and *L. hordei* responds to *S. cerevisiae* in many respects revealing a mutualistic relationship. The data suggest that *L. hordei* responds to the presence of *S. cerevisiae* with adjustment of intracellular redox reactions controlled of proteins, which are part of Rex regulons and proteins involved in the glycolytic pathway and energy fermentation. An NADH, $H^+$-driven metabolic switch to preferential production of butanediol instead of acetate or lactate, and up-regulation of arginine deiminase, alleviated acid stress and concomitantly protected *S. cerevisiae* against an acidic environment, which *L. hordei* generated in single culture. Moreover, the data suggest that the presence of *S. cerevisiae* in the nitrogen and fatty acids limited environment of the water kefir facilitated and improved the growth of *L. hordei* by delivering gluconate, fructose, amino acids, fatty acids or substrates for their biosynthesis. Up-regulation of the OppABCDF peptide transport and enzymes involved in amino acid metabolism indicates enhanced peptide uptake, as well as cross-feeding of *L. hordei* by glutamine, glutamate, histidine, tryptophan, methionine, proline, tryptophan delivered by *S. cerevisiae*.

## 1. Introduction

Water kefir is a fermented beverage, which is traditionally prepared from sucrose, kefir grains, dried or fresh fruits, and water. After one or two days' fermentation, it results in an acidic and lightly alcoholic drink. It is reported that the microbial consortium isolated from water kefir grains generally consists of lactic acid bacteria (LAB), yeast, acetic acid bacteria (AAB) and bifidobacteria (Gulitz et al., 2011; Laureys and De Vuyst, 2014; Marsh et al., 2013). The most predominant genus isolated by Gulitz et al. (Gulitz et al., 2011) in water kefir was *Lactobacillus*, with abundant *Lactobacillus* (*L.*) *hordei* followed by *L. nagelii*, *Leuconostoc mesenteroides* and *L. casei* while the predominant yeast were respectively *Saccharomyces* (*S.*) *cerevisiae*, *Zygotorulaspora* (*Z.*) *florentina*, *Dekkera bruxellensis* etc. (Gulitz et al., 2011; Laureys and De Vuyst, 2014).

To date, there are rare studies exploring microbial consortium and their interaction in water kefir, so the specific contribution of groups or species such as LAB, AAB, yeast remains speculative. Stadie et al. (2013) delineate the metabolic interaction between LAB (*L. hordei* and *L. nagelii*) and yeasts (*S. cerevisiae* and *Z. florentina*) isolated from water kefir as mutualism based on the significant increase of cell yield. It was inferred that the growth of *L. hordei* TMW 1.1822 may be improved by the disposed nutrients produced by both yeast, such as release of several auxotrophic amino acids (isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine and valine) and vitamin B6 (Stadie et al., 2013). This study also suggests that *Z. florentina* appreciates the pH decrease caused by lactobacilli, whereas *S. cerevisiae* did not. Furthermore, arginine is released by *Z. florentina* due to the co-cultivation of L. *nagelii* and *Z. florentina*, which may support the growth of *L. nagelii*. Lactic acid and ethanol were found to be the main metabolites of water kefir consortium of LAB, yeasts and AAB (Laureys and De Vuyst, 2014). In addition, glycerol, acetic acid and mannitol formation has been observed in low concentrations (Laureys and De Vuyst, 2014).

Although metabolic exchanges are ubiquitous in microbial

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communities, detecting metabolite cross-feedings constituting mutualism between species is difficult due to their intrinsically dynamic nature and the complexity of communities (Ponomarova and Patil, 2015). Also, such metabolites provided by an organism are immediately consumed by another one living in close proximity. Functional genomics approaches enable identification of metabolic pathways involved in adaptation of an organism to its specific habitat and also the presence of possible competitive or supportive metabolite formation (Durham et al., 2015; Maligoy et al., 2008; Ponomarova et al., 2017). Still, proteomic studies based on functional genomics have not yet widely been applied to study the adaptation or interaction of LAB and yeasts in a food consortium. An example for a respective transcriptome analysis is provided for the comparison of single and mixed cultivation of *Lactococcus lactis* IL-1403 in the absence or presence of *S. cerevisiae* CEN-PK905, respectively. A total of 158 genes was identified along determination of mRNA levels, which were significantly modified in their expression (particularly pyrimidine metabolism) (Maligoy et al., 2008). Another finding about the specific contribution of yeast (*S. cerevisiae* S90) to symbiotic LAB (*Lactococcus lactis* subsp. *lactis* IL1403 and *L. plantarum* WCFS1) through nitrogen overflow in grape juice was studied using mass spectrometry based on their genome-scale metabolic models (Ponomarova et al., 2017). It suggested that *S. cerevisiae* benefits *Lactococcus lactis* by secreting glutamine and threonine, and benefits *L. plantarum* by secreting glutamine, threonine, phenylalanine, tryptophan and serine. In addition, cross-feeding of 2-oxoglutarate to *L. plantarum* was also observed as predicted by metabolic modeling (Ponomarova et al., 2017). Still the knowledge of metabolic exchange between groups or species especially cellular regulation is limited to few communities with small number of exchanged metabolites. We used label-free quantitative proteomic analysis, whole-genome sequencing and reconstructed putative metabolic pathways to describe the lifestyle of *L. hordei* TMW 1.1822 in water kefir (Xu et al., 2019). It was demonstrated that 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.pre We postulated that 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. Based on these findings we explored the response of *L. hordei* to the presence of *S. cerevisiae* TMW 3.221 in this environment.

## 2. Material and methods

### 2.1. Strain culture and growth determination

*L. hordei* TMW 1.1822 and *S. cerevisiae* TMW 3.221 isolated from water kefir by Gulitz et al. (2011) were used to set up this co-cultivation experiment. Briefly, *L. hordei* TMW 1.1822 was pre-cultured anaerobically overnight at 30 °C in modified MRS (mMRS) medium (Stolz et al., 1995) as described previously (Xu et al., 2019), while *S. cerevisiae* from a − 80 °C stock was spread on YPG (Xu et al., 2018) agar plates and cultivated aerobically for 24 h at 30 °C, and a single colony was transferred into 10 ml YPG medium and pre-cultured anaerobically at 30 °C under the same growth condition as *L. hordei*. Then 1% (v/v) pre-cultured *L. hordei*, *S. cerevisiae* were separately inoculated into 40 ml water kefir medium (WKM) (Stadie et al., 2013) in replicate as single-cultivated samples, while 1% pre-cultured *L. hordei* and 1% *S. cerevisiae* were simultaneously inoculated into 40 ml WKM as co-cultivated samples. 100 µl aliquots were respectively taken out from each samples for plate counting at the fermentation time point of 0 h, 2 h, 4 h, 5 h, 6 h, 7 h, 8 h, 12 h, 24 h, 48 h. Single-cultivated *L. hordei* samples were plated in mMRS agar plates, and single-cultivated *S. cerevisiae* samples were plated in YPG agar plates. Meanwhile co-cultivated *L. hordei* and *S. cerevisiae* samples were plated in mMRS agar plates with added cycloheximide (final concertation at 0.1 g/l) to inhibit the growth of yeast for counting *L. hordei* cells, and also plated in YPG agar plates with added chloromycetin (final concertation at 0.1 g/l) to inhibit the growth of bacteria for counting *S. cerevisiae* cells.

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

1% (v/v) pre-cultured *L. hordei*, *S. cerevisiae* were separately inoculated into 40 ml WKM in triplicate and cultured anaerobically at 30 °C for 10 h to prepare single-culture samples, while 1% *L. hordei* and 1% *S. cerevisiae* were simultaneously inoculated into 40 ml WKM in triplicate and cultured in the same growth condition to prepare co-cultivation samples. As previously described (Xu et al., 2019), trichloroacetic (TCA) were added to those single-culture and co-cultivation samples to a final concentration of 6.25% (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 and mechanically disrupted with 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 for 60 min. Subsequently proteins were digested by addition 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.

Generated peptides were analyzed on a Dionex Ultimate 3000 nano LC system coupled to an 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) 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) 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 $m/z$ 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.

### 2.3. Peptide and protein identification and quantification, statistical analysis

Peptide and protein identification plus quantification were performed with MaxQuant (version 1.5.7.4) by searching the MS2 data against all protein sequences obtained from UniProt - Reference proteome *S. cerevisiae* S288c (6724 entries, downloaded 13.03.2017) and all protein sequences from *L. hordei* using the embedded search engine Andromeda (Cox et al., 2011) as previously described (Xu et al., 2019). MaxQuant output files (proteinGroups.txt) were further analyzed using Perseus (version 1.5.6.0) (Tyanova et al., 2016). Protein groups were filtered for entries “only identified by site”, reverse identifications and

contaminants. Protein groups from each investigated species were moved to different data matrices. iBAQ intensities were $\log_2$-transformed and used for further statistical analysis. NCBI annotation, PSORTb subcellular localization, SEED category (subcategory and subsystem) as previously annotated (Xu et al., 2019) were added to the matrix through identifier matching. For the comparison between two groups, *t*-tests were performed. Log2 fold change $\geq 2$ or $\leq -2$ and -Log10 *P*-value $\geq 2$ ($P$ value $\leq 0.05$) were considered to be significantly differentially expressed proteins of *L. hordei* TMW 1.1822 in response to co-cultivation with *S. cerevisiae* TMW 3.221.

### 2.4. Chromatographic analysis of amino acids, pH measurement

1% pre-cultured *L. hordei* TMW 1.1822 and *S. cerevisiae* TMW 3.221 were separately inoculated into chemically defined medium (CDM, pH 6.5) (Xu et al., 2019) in triplicate. After 24 h cultivation at 30 °C, 1 ml *L. hordei*, *S. cerevisiae* cultures and CDM were respectively mixed with 50 µl of 70% (v/v) perchloric acid (Sigma-Aldrich, St. Louis, USA) standing overnight at 4 °C for protein precipitation. After centrifugation (12,000 rpm, 10 min), the supernatant was collected and diluted 1:5 in 0.1 M HCl. Samples were filtered through Phenex™ Nylon Filter Membrane (0.2 µm, Phenomenex, Germany). Amino acids were analyzed on a Dionex Ultimate 3000 HPLC system (Dionex, Idstein, Germany) using a Gemini C18 column (Phenomenex, Aschaffenburg, Germany) with UV detection at 338 and 269 nm. Before separation, samples were performed to pre-column derivatisation with o-phthalaldehyde-3-mercaptopropionic acid (OPA) and 9-fluorenylmethyl chloroformate (FMOC) following Bartóak et al. (1994). A gradient was as described by Schurr et al. (2013) with a flow rate of 0.8 ml/min. Quantification was executed employing calibration adjustment by external HPLC grade standards and the Chromeleon software version 6.80 (Dionex, Idstein, Germany). After 72 h fermentation, single-cultivated *L. hordei* TMW 1.1822, single-cultivated *S. cerevisiae* TMW 3.221, co-cultivated *L. hordei* and *S. cerevisiae* samples in triplicate were respectively performed pH measurement (761 pH-meter Calimatic, Knick, Germany).

### 2.5. Statistical analysis

All the annotated EC and KO numbers, which were extracted from RAST fasta files were performed into iPath 3.0 (http://pathways.embl.de/index.html) (Yamada et al., 2011) to obtain an overview of complete metabolic pathways and biosynthesis of other secondary metabolites customized in thin red color as previously described (Xu et al., 2019). While up-regulated enzymes were customized in bold red color, down-regulated enzymes were customized in bold blue color. Other data visualization and analysis (cell counts, subcellular localization, SEED categories, amino acid consumption and pH values) were done with Microsoft Excel (Microsoft, Redmond, USA).

## 3. Results and discussion

### 3.1. Growth characteristics of L. hordei in the presence of S. cerevisiae

To investigate interaction of *L. hordei* and *S. cerevisiae* in the water kefir system, the viable cell counts were determined in co-cultivation between *L. hordei* TMW 1.1822 and *S. cerevisiae* TMW 3.221 compared with those of their respective single cultures. The cell yield of *L. hordei* TMW 1.1822 increased from $8.08 \pm 0.013$ $\log_{10}$ CFU/ml in single culture to $8.30 \pm 0.046$ $\log_{10}$ CFU/ml in co-cultivation with *S. cerevisiae* after 8 h fermentation, and increased from $8.29 \pm 0.105$ $\log_{10}$ CFU/ml in single culture to $8.54 \pm 0.019$ $\log_{10}$ CFU/ml in co-cultivation after 12 h fermentation (Fig. 1). Cells grew exponentially until 12 h of fermentation to subsequently enter the stationary phase as indicated by no further OD increase. For comparative proteomic analysis a sampling time point was chosen at the late exponential phase, i.e. at 10 h fermentation time. This ensured a high amount of proteins from a metabolically active status. The viable count of *L. hordei* was insignificantly affected in the presence of *S. cerevisiae* after 24 h, but declined upon co-cultivation as compared to that one in single culture after 48 h. The cell number of *S. cerevisiae* was merely unaffected in the presence of *L. hordei* from 0 h to 24 h, and slightly decreased upon co-cultivation after 48 h (Fig. S2).

### 3.2. General features of differential proteomics

Only three proteins of *S. cerevisiae* were quantified to be significantly differentially expressed in the presence of *L. hordei*, namely inhibitory regulator protein BUD2/CLA2 (3.4 $\log_2$ fold change), probable electron transfer flavoprotein-ubiquinone oxidoreductase (3.8 $\log_2$ fold change) and glutamate decarboxylase (4.2 $\log_2$ fold change). This is consistent with the finding that growth of *S. cerevisiae* was hardly affected upon co-cultivation with *L. hordei* (Fig. S2). In contrast, the growth of *L. hordei* was significantly enhanced in the presence of *S. cerevisiae*, and the impact on the *L. hordei* proteome was significant upon co-cultivation. Consequently the proteomic analysis was focused on any differential proteomic pattern of *L. hordei* resulting from such co-cultivation, to possibly provide predictions for the causes of this growth stimulation.

The proteomic analysis enabled quantification of 1474 proteins out of 2461 proteins predicted from the genome of *L. hordei*. Among these 131 proteins were significantly up-regulated, 102 proteins were significantly down-regulated and 1241 proteins were unregulated in the presence of *S. cerevisiae* (Fig. 2). Most DE proteins were localized in cytoplasmic and cytoplasmic membrane except those categorized for unknown subcellular localization. Furthermore, as shown in Fig. 3, up-regulated proteins of *L. hordei* in the presence of *S. cerevisiae* were abundant in the SEED categories of amino acids and derivatives (21 out of 83), carbohydrates (9 out of 90) and nucleosides and nucleotides (24 out of 80), cell wall and capsule (5 out of 72). A summary of the DE proteins were provided in Fig. S1.

Taken together these up/down-regulated proteins of *L. hordei* involved in carbohydrate and energy metabolism, amino acid metabolism and biosynthesis enabled predictions on the change of the *L. hordei* lifestyle in the nutritionally poor water kefir medium in response to the presence of *S. cerevisiae*.

### 3.3. Sugar transport, central carbohydrate metabolism

The previous overview on sucrose metabolism of *L. hordei* revealed that sucrose can either be transformed into a glucan and fructose by a glycosyltransferase (glucansucrase), or transported into the cell by PTS sugar transport systems and subsequently metabolized through glycolysis (Xu et al., 2019). Whereas the glucansucrase of *L. hordei*, which was characterized as dextransucrase (Xu et al., 2018), was not significantly regulated in the presence of *S. cerevisiae*, the PTS mannose/fructose/sorbose family was significantly up-regulated (shown in Fig. S3). This suggested that in addition to fructose, resulting from the dextransucrase reaction, resulted from yeast invertase splitting of sucrose into glucose and fructose. While the yeast preferentially consumed glucose, (some) additional fructose was available for *L. hordei*, which readily took it up by various PTS, which were annotated as fructose transporters. Depending on the type of PTS, *L. hordei* gained more fructose-6-phosphate or fructose-1-phosphate, which was subsequently metabolized via glycolysis (see Fig. S4) enabling faster growth.

As it was reported previously, both analysis of putative functional genome and quantified proteome of *L. hordei* characterized it as a facultative homofermentative LAB (Xu et al., 2019). There were no significantly DE enzymes of *L. hordei* involved in the Embden-Meyerhof pathway (EMP) based on comparative proteomic analysis, when *L. hordei* was co-cultivated with *S. cerevisiae* after 10 h fermentation. Nevertheless, glucose-6-phosphate dehydrogenase (G6PDD, No. 12 in

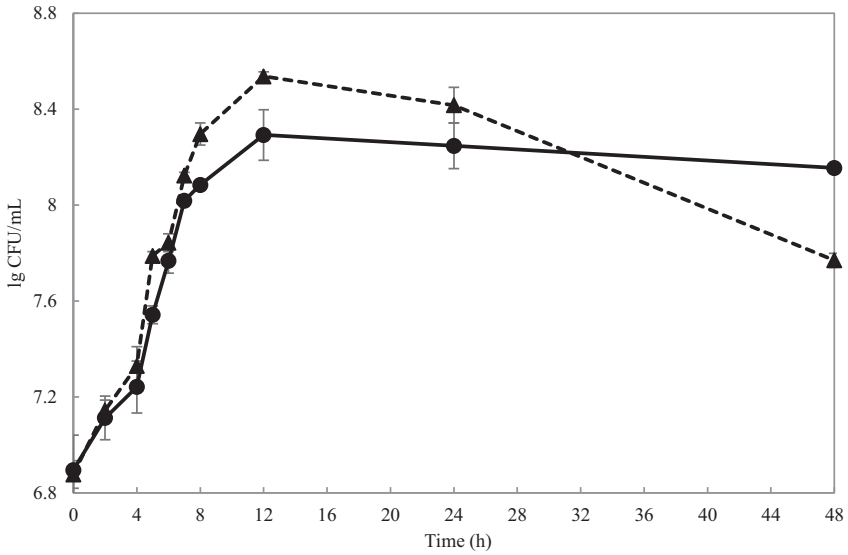
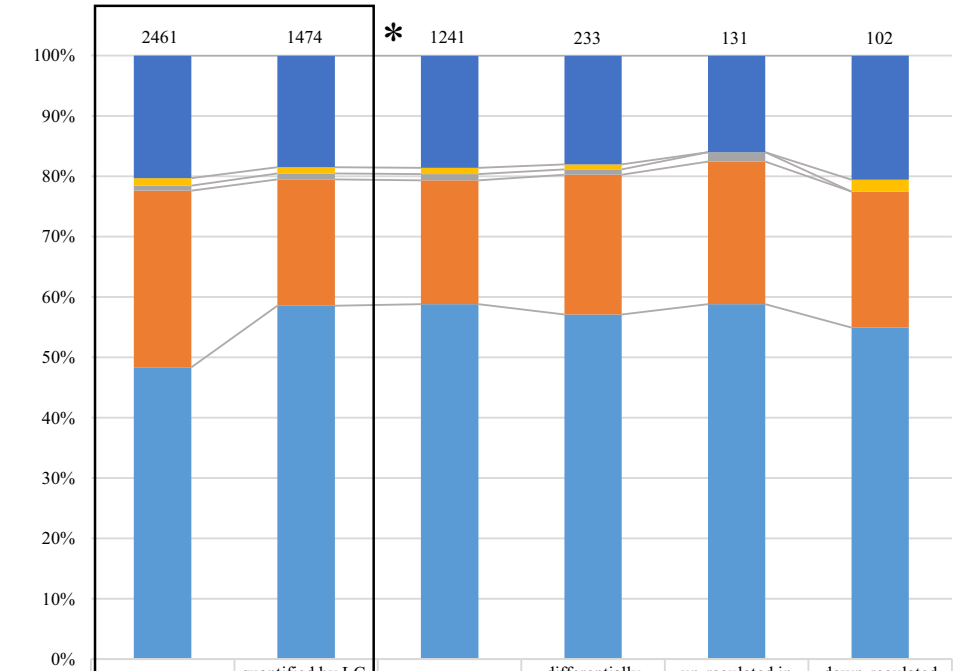



**Fig. 1.** Cell counts of *L. hordei* TMW 1.1822 in single culture (●) and co-cultivation with *S. cerevisiae* TMW 3.221 (▲).

Table 1 and Fig. S4) involved in the phosphoketolase pathway (PKP) and pentose phosphate pathway (PPP) was significantly up-regulated, leading to high production of 6-phosphogluconolactone and reductive power (NADPH). This supports the previous interpretation that *L. hordei* not only possess PKP but also indeed employs the PKP to adapt to changing environmental conditions. Furthermore, this could be interpreted to that the yeast induced a different redox potential in the water kefir system and delivered additional electron acceptors (fructose). As a result, *L. hordei* switched from EMP to PKP producing gluconate. Fructose could act as electron acceptor and be reduced to mannitol. Also, in the full consortium harboring also *Gluconobacter* spp. *L. hordei* was likely offered additional exogenous gluconate resulting




| | in silico | quantified by LC-MS/MS | unregulated | differentially expressed | up-regulated in co-cultivation | down-regulated in co-cultivation |
|---|---|---|---|---|---|---|
| unknown | 500 | 273 | 231 | 42 | 21 | 21 |
| extracellular | 31 | 15 | 13 | 2 | 0 | 2 |
| cell wall | 20 | 15 | 13 | 2 | 2 | 0 |
| cytoplasmic membrane | 721 | 308 | 254 | 54 | 31 | 23 |
| cytoplasmic | 1189 | 863 | 730 | 133 | 77 | 56 |

**Fig. 2.** Subcellular localization of proteins (in silico, quantified by LC-MS/MS, unregulated, differentially expressed, up-regulated in co-cultivation, down-regulated in co-cultivation) which were predicted by PSORTb. 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. A bias can be obviously seen for the proportion of cytoplasmic and cytoplasmic membrane proteins. Asterisk content was cited from Xu et al. (2019).

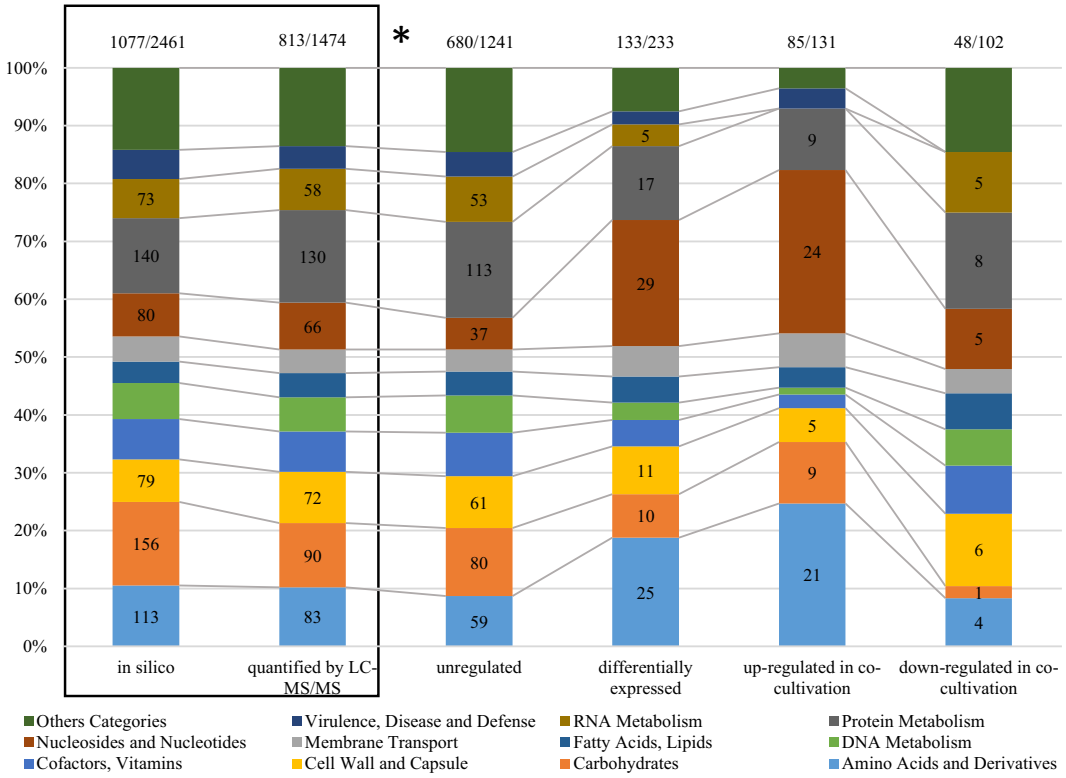



**Fig. 3.** SEED categories of proteins (in silico, quantified by LC-MS/MS, unregulated, differentially expressed, up-regulated in co-cultivation, down-regulated in co-cultivation) which were predicted by SEED. The proportion of proteins assigned to each respective categories of metabolism and the group "other categories", which is the sum of several small categories with respect to the total number of proteins is shown by the bar chart. The ratio on the top of each column is the number of predicted SEED categories accounts for the number of all coding DNA sequence (CDS). Asterisk content was cited from Xu et al. (2019).

from their fructosyltransferases and subsequent oxidative metabolism of remaining glucose.

On the other hand, *L. hordei* UCC125, UCC126, UCC127, UCC128 isolated from barley have been reported as homofermentative since they are capable of producing acid from glucose, fructose, maltose, sucrose, mannose, mannitol, cellobiose but not from pentose, arabinose, galactose, sorbitol et al. (Rouse et al., 2008). In the lack of respective genomic data of *L. hordei* UCC125, UCC126, UCC127, UCC128, one cannot define whether similar metabolic switches could occur in isolates from that barley. But *L. hordei* DSM 19519 also isolated from barley (Sun et al., 2015), had no sucrose specific PTS and extracellular glycosyltransferases and less transporters involved in fructose import found in the genome compared to *L. hordei* TMW 1.1822 as mentioned previously (Xu et al., 2019). So *L. hordei* likely expresses specific lifestyles in the different food systems and responds to the presence of other consortium members.

### 3.4. Pyruvate and citrate metabolism

Beyond the indicated switch from EMP to PKP more redox related metabolic reactions were affected upon co-cultivation of *L. hordei* with *S. cerevisiae*. Such reactions are generally regulated by the redox-sensing repressor Rex. Initially described in *S. coelicolor*, Rex regulated metabolism of Gram-positive bacteria in response to the cellular NADH/$NAD^+$ levels. The core conserved part of the Rex regulons as a set of 22 genes and operons were preceded by candidate Rex-binding sites in 11 taxonomic groups of bacteria (Ravcheev et al., 2012). Although the Rex proteins of *L. hordei* were not significantly DE in the presence of *S. cerevisiae*, Rex apparently sensed a change in the cellular NAD/NADH ratio and induced DE of enzymes in reconstructed Rex regulons (Bitoun et al., 2012; Ravcheev et al., 2012; Zhang et al., 2014), which were involved in pyruvate metabolism. As shown in Fig. 5, these included bifunctional acetaldehyde/alcohol dehydrogenase (ADHE, No. 19/20), pyruvate formate lyase (PFL, No. 25), and acetoin reductase (AR, No. 28). ADHE involved in PKP and pyruvate metabolism was significantly down-regulated in the presence of *S. cerevisiae*, reducing

**Table 1**
Significantly differentially expressed proteins in *L. hordei* TMW 1.1822 in response to co-cultivation with *S. cerevisiae* TMW 3.221 involved in carbohydrate metabolism (PKP, pentose phosphate pathway, pyruvate metabolism).

| Number | Enzyme | EC number | $Log_2$ fold change (co-cultivation vs single culture) | -Log(*P*-value) |
|---|---|---|---|---|
| Up-regulated | | | | |
| 12 | Glucose-6-phosphate dehydrogenase | EC 1.1.1.49 | 4.2 | 5.7 |
| 21 | Citrate lyase | EC 4.1.3.6 | 4.8 | 6.7 |
| 25 | Pyruvate formate lyase | EC 2.3.1.54 | 2.7 | 3.1 |
| 28 | Acetoin reductase | EC 1.1.1.4 | 4.7 | 3.3 |
| Down-regulated | | | | |
| 19/20 | Bifunctional acetaldehyde/alcohol dehydrogenase | EC 1.2.1.10/EC 1.1.1.1 | −1.9 | 2.9 |

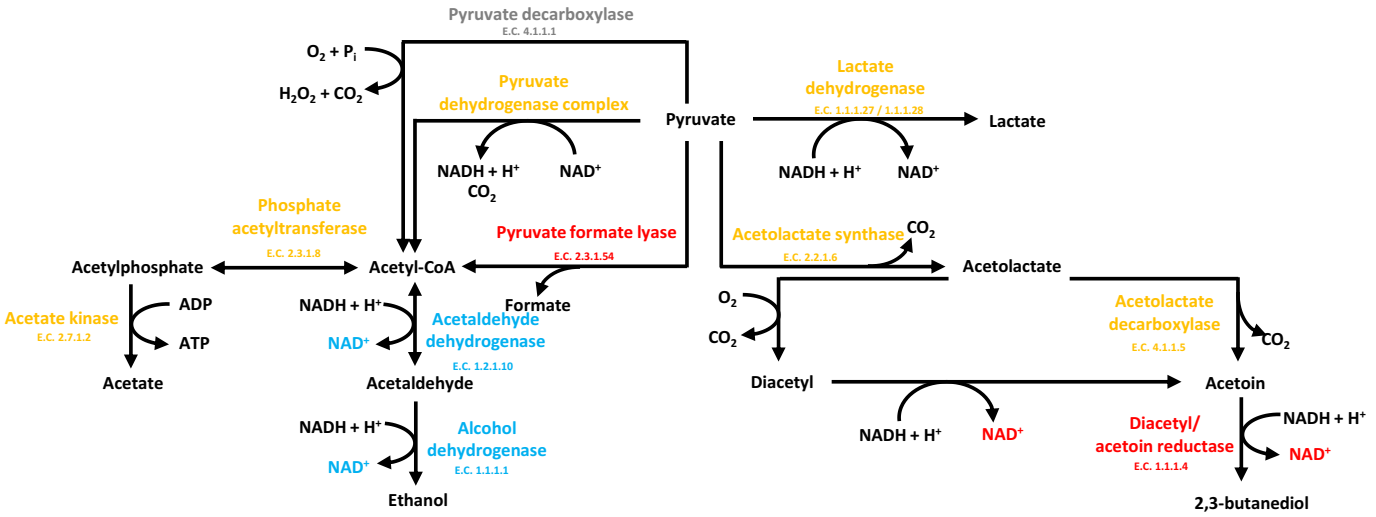



**Fig. 4.** Predicted outline of pyruvate metabolism of *L. hordei* TMW 1.1822 in the presence of *S. cerevisiae* TMW 3.221. The enzymes colored in red represent up-regulated, in blue represent down-regulated enzymes or proteins according to proteomic data, while enzymes colored in yellow are both annotated by genomic and quantified by proteomic, enzymes colored in grey are neither present in genome nor proteome. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the utilization of NADH, $H^+$ and production of ethanol. In the presence of ethanol, which is mainly produced by the yeast in alcoholic water kefir (Beshkova et al., 2003), *S. cerevisiae* limits production of ethanol by *L. hordei* in the water kefir consortium. Furthermore, three enzymes (citrate lyase, PFL, AR) involved in pyruvate metabolism were highly abundant in *L. hordei* when co-cultivated with *S. cerevisiae* (shown in Table 1 and Fig. 4). The up-regulated citrate lyase (*citEF*) promoted the production of acetate and oxaloacetate converted from citrate. In addition, triphosphoribosyl-dephospho-CoA synthase (*citG*), which could activate citrate lyase from the apo to the holo enzyme, was significantly up-regulated, while citrate lyase transcriptional regulator (*citI*) was significantly down-regulated. Taken together, this suggested that citrate present in the water kefir was readily consumed by *L. hordei* preferentially in the presence of *S. cerevisiae* and can be used as carbon source and electron acceptor. Over-expressed PFL enabled an electron acceptor reaction and generates a high amount of NADH, $H^+$, which was available for the also over-expressed AR to produce 2,3-butanediol instead of producing ethanol. This re-direction of pyruvate metabolism in the presence of citrate was consistent with the results that high diacetyl production from *L. helveticus* MP12 and *Lactococcus lactis* subsp. *lactis* C15 isolated from (milk) kefir grains were recorded (Beshkova et al., 2003). Accordingly, the acidification of co-cultivated *L. hordei* and *S. cerevisiae* became limited through the switch from lactic and acetic acid to butanediol formation. Taken together, this group of regulated enzymes of *L. hordei* in the presence of *S. cerevisiae* was associated with maintenance of redox homeostasis targeted at optimization of energy generation and reduction of ethanol and acid stress.

### 3.5. Peptide transport systems, peptidases, amino acids biosynthesis and metabolism

Periplasmic oligopeptide - binding protein A (OppA, TC 3.A.1.5.1) and oligopeptide transport system permease protein B (OppB) of the peptide transport system (OppABCDF) were 2.5 $\log_2$fold and 2.2 $\log_2$fold up-regulated, respectively. As demonstrated for *Lactococcus lactis* the Opp system is over expressed upon peptide limitation, and repressed in their abundance (Guédon et al., 2001). This suggests that the competition for the limited nitrogen source in this environment increases in the presence of *S. cerevisiae*. Once uptaken, peptides were degraded by a variety of peptidases (Christensen et al., 1999; Kunji et al., 1996). *L. hordei* was predicted to have 13 genes encoding peptidases (Xu et al., 2019). Among them, alpha-aspartyl dipeptidase E (*pepE*) was up-regulated (2.7 $\log_2$ fold change) catalyzing the hydrolysis of dipeptides Asp-|-Xaa. It did not act on peptides with N-terminal Glu, Asn or Gln, nor did it cleave isoaspartyl peptides. While aminopeptidase S (*pepS*) belonging to the peptidase M29 family was down-regulated ($-1.9$ $\log_2$ fold change), which decreased the ability of displaying broad substrate specificity towards peptides containing leucine, valine, phenylalanine, tyrosine.

The predictive analysis of the complete genome of *L. hordei* combined with physiological results revealed that *L. hordei* is prototrophic for 11 amino acids and auxotrophic for 6 amino acids (Xu et al., 2019). In total, 16 identified and quantified enzymes of *L. hordei* involved in the biosynthesis of histidine, tryptophan, methionine, arginine, glutamate, glutamine, proline were up-regulated in the presence of *S. cerevisiae*, while only 2 enzymes involved in the biosynthesis of aromatic amino acids (tryptophan, tyrosine, phenylalanine) and serine were down-regulated (listed in Table 2, visualized in Fig. S5). However, from the KEGG in silico metabolic pathways, the direction of a respective metabolic reaction often remains ambiguous. Still, together with metabolic data on the amino acids consumption/formation of *L. hordei* and *S. cerevisiae* this can be solved for some of the predicted cases. For example, as shown in Fig. 5, about 68.6% of glutamine (4.82 mM) in CDM was consumed by *L. hordei* after 24 h. In contrast, 6.15 mM glutamine was produced by *S. cerevisiae* after 24 h compared to initial concentration in CDM. This suggested that *L. hordei*, even though it is prototrophic for glutamine, takes advantage of glutamine provided by *S. cerevisiae* via the up-regulated glutamate synthase (6.4 $\log_2$ fold change) and possibly used this reaction for anaplerotic sequences in transamination reactions in biosynthetic pathways of other amino acids. Ponomarova et al. (2017) also predicted that glutamate/glutamine are secreted by *S. cerevisiae* and consumed by *Lactococcus lactis* and *L. plantarum*. In a targeted LC-MS/MS approach they quantified the amounts of secreted amino acids in yeast-conditioned medium and further suggested cross-feeding of phenylalanine and proline (in concentration below the limit of exact quantification) from the yeast to *L. plantarum*. However, in our case, except glutamine, other amino acids were not produced but partly consumed by *S. cerevisiae* after 24 h fermentation in CDM. Therefore, we were unable to prove the real-time metabolic exchange (release/uptake) between LAB and yeast based on physiological results. Still, the label-free quantitative comparative proteomic analysis enabled investigation of real-time, dynamic, and integrated metabolic exchange and molecular response between microbial communities in fermented foods along DE. The up-regulated

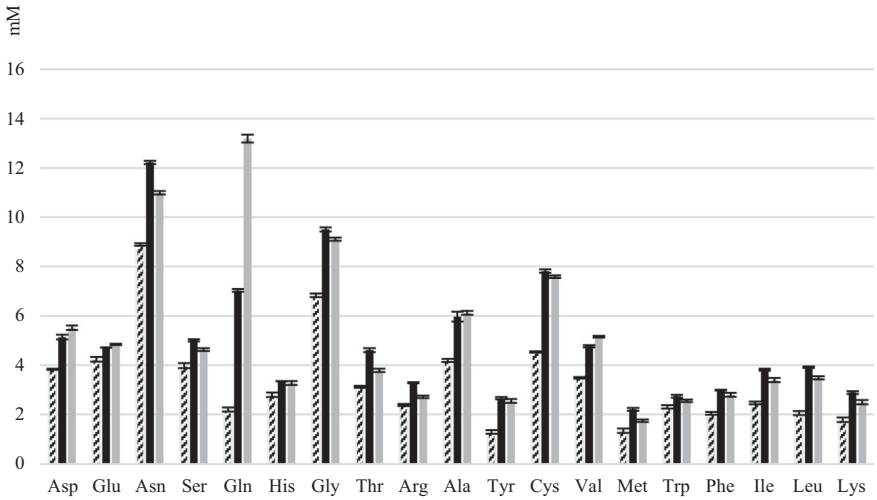



**Fig. 5.** Consumption of amino acids of *L. hordei* TMW 1.1822 and *S. cerevisiae* TMW 3.221 isolated from water kefir grown in CDM after 24 h. Black histogram represented CDM, slash histogram represented *L. hordei*, grey histogram represented *S. cerevisiae*.

enzymes involved in amino acid metabolism or catabolism suggested that *S. cerevisiae* secreted glutamine, glutamate, arginine, histidine, tryptophan, methionine and proline, which were subsequently assimilated by *L. hordei* for its enhanced growth in this symbiotic water kefir environment.

### 3.6. Acid tolerance by ADI pathway

Arginine catabolism via arginine deiminase (ADI) system, which is widely distributed in LAB is considered an important characteristic conferring acid tolerance, was identified and quantified in *L. hordei* (Xu et al., 2019). In the lack of carbamate kinase, this partial operon cannot deliver additional ATP. As shown in Fig. 6A, ADI (5.5 $\log_2$ fold change), ornithine transcarbamoylase (OTC, 6.9 $\log_2$ fold change) of *L. hordei* were significantly up-regulated leading to high production of ammonia in the presence of *S. cerevisiae*. This was also demonstrated by pH determination of single-cultivated and co-cultivated *L. hordei* and *S. cerevisiae* after 72 h fermentation (Fig. 6B). The pH value of co-cultivated *L. hordei* and *S. cerevisiae* increased to around 3.49 from 3.28 determined with single-cultivated *L. hordei*. Partial arginine conversion contributed to maintenance of intracellular pH in *L. hordei*, and alleviated acid stress of *S. cerevisiae*. In conclusion, *S. cerevisiae* provides arginine for *L. hordei*, whose conversion is beneficial for both partners.

**Table 2**
Significantly differentially expressed proteins in *L. hordei* TMW 1.1822 in response to co-cultivation with *S. cerevisiae* TMW 3.221 involved in amino acids biosynthesis.

| Number | Enzyme | EC number | $\log_2$ fold change (co-cultivation vs single culture) | -Log (*P*-value) | SEED subcategory |
|---|---|---|---|---|---|
| Up-regulated | | | | | |
| 1 | ATP phosphoribosyltransferase | EC 2.4.2.17 | 3.0 | 3.8 | Histidine biosynthesis |
| 2 | Imidazole glycerol phosphate synthase cyclase | EC 4.1.3.- | 3.6 | 4.8 | |
| 3 | Histidinol-phosphate aminotransferase | EC 2.6.1.9 | 3.5 | 3.3 | |
| 4 | Histidinol dehydrogenase | EC 1.1.1.23 | 1.8 | 5.0 | |
| 5 | Tryptophan synthase | EC 4.2.1.20 | 3.6 | 5.4 | Tryptophan synthesis |
| 6 | S-ribosylhomocysteine lyase | EC 4.4.1.21 | 2.1 | 3.1 | Methionine biosynthesis |
| 7 | Cystathionine beta-lyase | EC 4.4.1.8 | 3.5 | 2.0 | |
| 8 | O-succinylhomoserine sulfhydrylase | EC 2.5.1.48 | 2.1 | 4.2 | |
| 9 | 5-methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase | EC 2.1.1.14 | 3.3 | 3.0 | |
| 10 | Glutamate synthase | EC 1.4.1.13 | 6.4 | 2.8 | Glutamate, arginine biosynthesis |
| 11 | Glutamine synthetase | EC 6.3.1.2 | 2.2 | 4.6 | Glutamine, arginine biosynthesis |
| 12 | Acetylornithine aminotransferase | EC 2.6.1.11 | 7.9 | 5.4 | Arginine biosynthesis |
| 13 | Ornithine carbamoyltransferase | EC 2.1.3.3 | 6.9 | 5.6 | |
| 14 | Argininosuccinate synthase | EC 6.3.4.5 | 4.5 | 7.2 | |
| 15 | Argininosuccinate lyase | EC 4.3.2.1 | 3.3 | 4.9 | |
| 16 | Ornithine cyclodeaminase | EC 4.3.1.12 | 2.6 | 5.8 | Proline biosynthesis |
| Down-regulated | | | | | |
| 17 | Chorismate synthase | EC 4.2.3.5 | −1.5 | 4.0 | Aromatic amino acid biosynthesis |
| 18 | D-3-phosphoglycerate dehydrogenase | EC 1.1.1.95 | −1.7 | 2.8 | Serine biosynthesis |

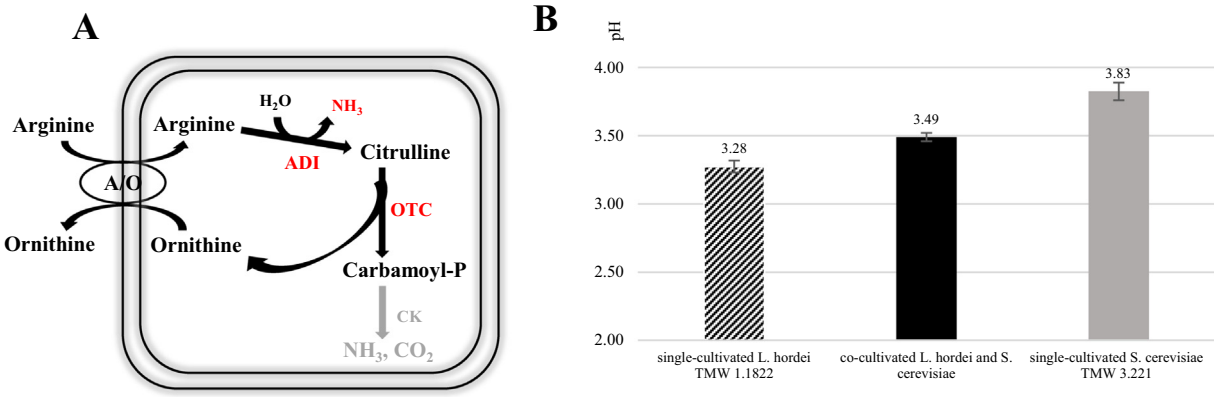



**Fig. 6.** Predicted outline of ADI pathway of *L. hordei* TMW 1.1822 in the presence of *S. cerevisiae* TMW 3.221 (A). ADI, OTC colored in red showed up-regulation. pH value of single-cultivated and co-cultivated *L. hordei* and *S. cerevisiae* after 72 h fermentation in WKM (B). Black histogram represented pH of co-cultivated *L. hordei* and *S. cerevisiae*, slash histogram represented single-cultivated *L. hordei*, grey histogram represented single-cultivated *S. cerevisiae*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### *3.7. Fatty acid biosynthesis*

Although *L. hordei* should not be able to synthesize any unsaturated fatty acids by itself in the absence of FabB, alternative functional enzymes for the biosynthesis of unsaturated fatty acids must exist, which enabled growth under strict limits of fatty acids in water kefir. This should likely be FabF (Xu et al., 2019). In the presence of *S. cerevisiae* the significant up-regulation of acetyl-CoA carboxylase (EC 6.3.4.14, 2.4 $\log_2$ fold change) and biotin carboxylase (2.1 $\log_2$ fold change) of *L. hordei* was observed, which are involved in providing the malonyl-CoA substrate for the biosynthesis fatty acids.

## 4. Conclusions

While this study is limited to the response of *L. hordei* to *S. cerevisiae* in co-culture of a water kefir model, it demonstrates the potential of label-free quantitative proteomics to study microbial interaction within consortia and provides first insight into interactions of water kefir inhabitants. The predicted functional genome and protein regulations of *L. hordei* TMW 1.1822 in the presence of *S. cerevisiae* TMW 3.221 revealed the adaptation and link to *S. cerevisiae* in this water kefir environment. This way, the PKP using gluconate as additional carbon source and simultaneous generation of NADPH for redox maintenance was up-regulated. The neutralization of the cytoplasm protected *S. cerevisiae* in this water kefir consortium against an acidic external pH via over-expressed formation of butanediol instead of lactate or acetate and over-expressed ammonia production upon the partial arginine conversion. Furthermore, many regulated enzymes belong to regulons of Rex related to redox homeostasis. The strict limits of amino acids in water kefir medium were attenuated by the uptake of peptides or amino acids such as glutamine from *S. cerevisiae*, and biosynthesis of some essential amino acids by *L. hordei*, as indicated by up-regulation of related enzymes. This differential proteomic analysis provides a powerful tool to understand modulations in the lifestyle of bacteria in the presence of other microbes sharing the environment or under different environmental stimuli.

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