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# Comparative gene analysis of beer tolerant and sensitive *Lactobacillus brevis*

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**Abstract**

*Lactobacillus brevis* is the major spoiler in beer, affecting product quality and causing economic losses in breweries. Although several genes involved in beer-spoilage have been identified, there are still unresolved questions about which genes that are associated with growth in beer and the potential roles of these genes. In this study, 21 *Lactobacillus brevis* strains were tested for beer spoilage potential; three beer-tolerant and three beer-sensitive strains were selected for comparative genomic analysis. One of the tolerant strains was exposed to growth conditions containing novobiocin that favor loss of plasmids, and subsequently became more sensitive to beer as determined by growth experiments. The genetic difference between the wildtype and the sensitive mutant confirmed that the previously identified beer (hop)-tolerance genes *horA* and *hitA*, which are usually localized on plasmids, play important roles in beer spoilage. Interestingly, *horA* and *hitA* were present in another sensitive strain whereas these genes were absent in another tolerant strain. This indicates that a beer-spoilage phenotype cannot be easily identified from the presence of a few reported beer-spoilage genes. The potential roles of additional genes involved in beer tolerance were discussed, including a ClpX protease and a manganese transporter different from *hitA*.

**Keywords:** whole genome sequence; comparative; genomics; beer spoilage; *L. brevis*.

**Practical Application:** Rapid detection of beer spoilage bacteria.

## 1 Introduction

Beer is an inhospitable environment for most microorganisms due to the presence of ethanol, hop compounds, low pH, and the lack of nutrients as well as oxygen. Usually, ethanol and hops will prevent microorganisms from interfering the essential cell membrane functions, the low pH will prevent most Gram-negative bacteria from destroying the enzyme activity, and the lack of nutrients and oxygen will starve many potential pathogens (Rodríguez-Saavedra et al., 2021). However, a few bacterial species are able to tolerate all of these selective stresses, such as *Lactobacillus brevis*, *Pediococcus damnosus*, *Lactobacillus lindneri*, *Lactobacillus paracasei* and so on (Suzuki et al., 2020a). Among these species, *L.brevis* is the predominant beer spoilage bacteria, and it has been reported that this species account for more than half of the spoilage incidents in breweries in Germany (Munford et al., 2020; Rodríguez-Saavedra et al., 2020). Spoilage bacteria typically cause turbidity and off-flavors in beer, which eventually leads to customer dissatisfaction and economic losses for the brewers (Rodríguez-Saavedra et al., 2021). Thus, there is an increasing demand to rapidly identify and thoroughly understand organisms involved in beer spoilage.

Hop compounds are the main inhibitory compounds in beer, which cause intracellular pH decrease and elevated cellular oxidative stress. In the past two decades, several studies have investigated different physiological aspects of the bacterial response to hop compounds (Feyereisen et al., 2020a; Gomes et al., 2022; Piraine et al., 2023). But a strong emphasis has also been directed towards identifying the genes that are responsible for beer spoilage. Genes such as *horA*, *horC* and *hitA* from *L. brevis* and *fabZ* from *Ped. damnosus* have been suggested to be associated with hop tolerance, and all of these genes were located on plasmids (Feyereisen et al., 2020b; Suzuki et al., 2020b). The gene products HorA and HorC have been proposed to work as efflux pumps for hop compounds (Asano et al., 2019; Zheng et al., 2019), HitA is involved in manganese transport, and FabZ contributes to fatty acid biosynthesis. Since these plasmid borne genes can be easily acquired or lost due to the environmental conditions (Schneiderbanger et al., 2020), ecotype-specific chromosomal genes such as signal transduction histidine kinase and *arsR/ cinA* were proposed to be the most stable predictors of spoilage potential (Suzuki et al., 2020b). Nevertheless, disagreements still exist as to which genes or gene combinations play the most significant role in beer spoilage (Deng et al., 2019; Schneiderbanger et al., 2020)

Next generation sequencing (NGS) technology is becoming faster and less expensive. At present, more than 20 *L. brevis* strains, isolated from e.g. beer, cheese and fermented vegetables have been sequenced and studied. Comparative genomic analysis of beer spoilage and non-spoilage bacteria (at strain level) can provide novel insights into the specific genetic characteristics that confer spoilage ability to bacteria (Wang et al., 2019). This will also provide a broader understanding of spoilage mechanisms and, more importantly, facilitate in the early detection and identification of potential beer spoilage bacteria in breweries.

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In this study, we selected three beer-tolerant and three beer-sensitive strains among 21 *L. brevis* strains. Furthermore, we sequenced the genomes of all six strains and, through comparative analysis, demonstrated novel genomic markers that may contribute to beer spoilage.

## 2 Materials and methods

### *2.1 Bacterial strains*

A total of 21 *L. brevis* strains isolated from beer were included in the present study. *L. brevis* MI2158 (DSM 20054$^T$) isolated from faeces was purchased from the German Collection of Micro-organisms and Cell Cultures GmbH (Braunschweig, Germany). *L. brevis* A, B, D, E, F, G, H, I, J, L, M, N, O, P, Q, R, JK09 and HF01 were isolated from Danish craft beer in our laboratory and were identified as *L. brevis* by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Wieme et al., 2014). *L. brevis* G430 was isolated from Czech beer, and was kindly provided by Dr. J.J. Leisner (Department of Veterinary Disease Biology, University of Copenhagen). A partial plasmid-cured derivative of *L. brevis* JK09 (called *L. brevis* JK09--) was produced by subculturing the former in de Man Rogosa Sharpe (MRS) broth containing novobiocin, a plasmid-curing agent (Ruiz-Barba et al., 1991). The success of plasmid curing was assessed by testing the absence of previously suggested plasmid borne genes *horA* and *hitA* by PCR (Geissler et al., 2016; Asano et al., 2019)

### *2.2 Assessment of beer spoilage potential*

Advanced beer-spoiler detection (ABD) medium (Suzuki et al., 2020a) was used to assess the beer spoilage potential of the 21 *L. brevis* strains. Bacteria taken from -80 °C frozen stock cultures were grown overnight in MRS medium at 30°C and 100µl of this culture was subsequently inoculated into 10 mL of ABD medium. Growth was assessed by measuring $OD_{600}$ in 96-wells microplates using a Varioskan™ Flash (Thermo Fisher Scientific Oy, Finland). Each well contained 200µl suspension and the microplates were sealed with parafilm (Sigma-Aldrich) and incubated at 30˚C. The growth was monitored daily for five days. For each strain, the mean $OD_{600}$ value was calculated from 3 wells. One-way analysis of variance (ANOVA) with a post-hoc Tukey Honestly Significant Difference (HSD) test was employed to assess differences in $OD_{600}$ values within more than two tested strains ($\alpha$=0.05). Independent sample t-test ($\alpha$=0.05) was used to assess the difference between the $OD_{600}$ value of tolerant and sensitive strains (IBM SPSS Statistic v24).

### *2.3 DNA extraction, sequencing and genome assembly*

Strains A, HF01, JK09, JK09--, Q and G430 were selected for sequencing. Cells were grown overnight in MRS broth at 30°C and then lysed with bead-beating using a FastPrep-24TM 5G (MP Biomedicals, CA, USA). DNA was extracted with the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, CA, USA) according to the manufacturer's standard instructions. The concentration and quality of the extracted DNA was assessed by Qubit® dsDNA Broad range assay kit (Thermo Fisher Scientific) and agarose gel electrophoresis, respectively. The extracted DNA was stored at -20°C until further analysis.

Libraries for DNA sequencing were prepared using the Nextera XT library preparation kit according to the manufacturer's protocol. The libraries were sequenced using Illumina paired-end sequencing on the MiSeq platform as a part of a flowcell (2x250 cycles; Illumina). Reads were subjected to quality trimming and possible contaminations were removed using Cutadapt v1.6 (Kechin et al., 2017) and AdapterRemoval v1.5.2 (Kennedy et al., 2018). Trimmed reads were then assembled using SPAdes v3.5.0. For plasmid component prediction, plasmidSPAdes included in SPAdes v3.5.0 release was used (Kazachenka & Kassiotis 2021), and the corresponding contigs were confirmed by using BLAST (using default parameters against NCBI database).

### *2.4 Genome Annotation, comparative genomics and BLAST*

tRNA genes were predicted by tRNAscan-SE v1.21 (Chan & Lowe 2019) and rRNA genes were identified by RNAmmer v1.2 (Lagesen et al., 2007). CRISPRFinderwas used for identification of clustered regularly interspaced short palindromic repeats (CRISPR). Open reading frames (ORFs) were predicted using Prodigal program v2.6.3 (Hyatt et al., 2010). Functional annotation and metabolic reconstruction were carried out with the Rapid Annotations using Subsystems Technology (RAST) server using default settings (Overbeek et al., 2014) and Blast2go (Conesa et al., 2005) . Comparative genomic analysis was performed by the CMG-Biotools package (Vesth et al., 2013). An all-against-all protein comparison was conducted using BLAST to describe homology. A homolog was considered significant if there was a minimum of 50% identity over a coverage of at least 50% of the longer sequence. Annotated contigs were analyzed by BLASTP against custom-built database of 21 annotated proteins using default parameters. These 21 proteins were suggested to be the beer spoilage related proteins (Behr et al., 2016; Rodríguez-Saavedra et al., 2020) and downloaded from RefSeq non-redundant proteins (Date 23.09.2016). Analysis of gene synteny of regions harboring *horA* and *hitA* genes was done using progressiveMauve algorithm (Ryoo et al., 2018). For each comparison, a relevant plasmid sequence of *L. brevis* BSO 464 (Bergsveinson et al., 2015) was compared with corresponding contigs of strains HF01, JK09 and Q. Additionally gene synteny of the closest, chromosomal homologs of horA and hitA were checked in a similar manner between BSO 464 strain and all six strains used in the study.

### *2.5 Accession numbers*

Assemblies of the six *L. brevis* strains are deposited at the European Nucleotide Archive under the Project PRJEB17528. The accession numbers for strains A, HF01, JK09, JK09--, Q and G430 are ERS1427100–105, respectively.

## 3 Results

### *3.1 Beer spoilage potential of different L. brevis strains*

In order to assess beer spoilage potential, a total of 21 *L. brevis* strains were grown for 5 days in ABD broth.

Strains that could grow above the average $OD_{600}$ value were considered strong beer spoilers. The $OD_{600}$ value ranged from 0.07 to 0.29 (mean=0.19) for all the strains after 5 days, with 13 strains showing $OD_{600}$ value above the mean (Figure 1). Analysis by one-way ANOVA indicated that the difference in $OD_{600}$ among the 21 strains was statistically significant ($p$ value < 0.05). Importantly, strains A, HF01, JK09 grew significantly faster than JK09--, Q and G430 ($p$ value < 0.05), and therefore strains A, HF01 and JK09 were selected as relative beer tolerant strains and JK09--, Q and G430 as relative beer sensitive strains. Further analysis of the growth characteristics in MRS (pH 4.3) containing 55.2μM hop compounds ($MRS_{4.3+H}$) suggested that strains A, HF01 and JK09 were hop tolerant and strains JK09--, Q and G430 were hop sensitive. Strain A grew the fastest among the three tolerant strains in ABD medium but, interestingly, it displayed the slowest growth of the tolerant strains in $MRS_{4.3+H}$.

These six strains(strains A, HF01, JK09, JK09--, Q and G430) were used for sequencing and for further comparative genomic analysis.

### *3.2 Draft genome sequences of L. brevis strains*

The genomic features of the six sequenced *L. brevis* strains are summarized in Table 1.

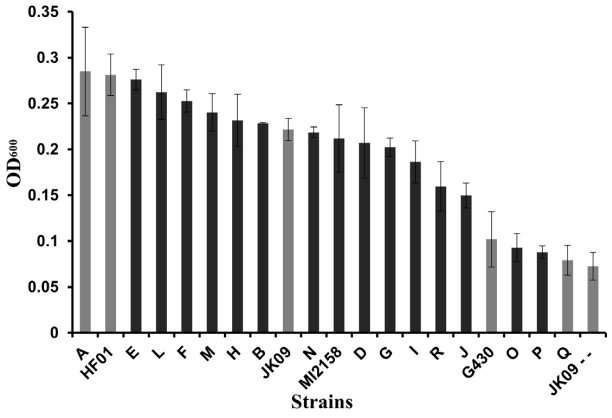


**Figure 1.** $OD_{600}$ values of 21 *L. brevis* strains in ABD medium after 5 Days incubation. The values are the means of $OD_{600}$ of 3 wells in 96-wells microplate, and the error bars indicate the standard deviations. Strains that have gray bars were selected for further sequencing and comparative genomic analysis.

The predicted genome sizes were in the range of 2.45-2.75 Mb, with G+C contents of approx. 45.5%, which is in accordance with previously published *L. brevis* genomes. For strain JK09--, which is a partial plasmid-cured derivative of the wild-type strain JK09, the number of contigs and consequently the expected genome size as well as the predicted coding sequences (CDSs) appeared to be reduced. According to RAST, the closest relative to all the strains from this study was the fully sequenced reference strain *L. brevis* ATCC 367. Further analysis using RAST revealed that carbohydrate and protein metabolism were the most dominant subsystem categories (Fig. S1). Compared to JK09--, the products of the unique CDSs present in JK09 appeared to be involved in metabolism of amino acids and derivatives thereof, virulence, disease and defense and code for phage and prophage genes. All the strains except G430, which contained only one unique CRISPR array, appeared to contain two CRISPR arrays that have the same repeats and spacers (Fig. S2). Apart from these two CRISPR arrays, strain A, HF01 and Q carried a third II-A CRISPR locus as reported previously in other strains. Besides, there is a difference in the number of three spacer-repeat units between strain HF01 and the other two (strain A and Q) at the leader end.

### *3.3 Comparison of predicted proteomes*

A pan- and core-genome plot analysis was performed as described in Figure 2A. Strain A was plotted first, followed by the other tolerant strains, JK09-- was added after the wildtype strain JK09 and then the other two sensitive strains. The accumulative number of pan genome slightly increased from strain A to JK09. As expected, addition of the plasmid-cured strain JK09-- caused an obvious decrease (241 protein families) in the core-genome. Besides, both the pan- and core-genome curves were not influenced by the addition of strain Q. However, the addition of strain G430 increased the pan-genome with 248 new genes, such as malate dehydrogenase, gluconokinase, phosphoenolpyruvate-phosphotransferase and several prophages (Table S1). The final core genome of six strains was estimated to include 2077 gene families and the pan genomes contains 2866 gene families. Furthermore, BLAST matrix analysis was conducted as described in Figure 2B. It illustrates that there is considerably higher level of conservation among the genomes of strains A, HF01, JK09 and Q (>92% similarity). Strains JK09-- and G430 showed lower level of similarity to the other strains (≤88% similarity) and between each other (~79% similarity). In conclusion, the pan- and core-genome analysis revealed that strain JK09-- lost a certain amount

**Table 1**. Sequencing statistics for six *L. brevis* strains.

| Strain | A | HF01 | JK09 | JK09-- | Q | G430 |
|---|---|---|---|---|---|---|
| Accession No. | ERS1427100 | ERS1427101 | ERS1427102 | ERS1427103 | ERS1427104 | ERS1427105 |
| Origin | Danish craft beer | Danish craft beer | Danish craft beer | Plasmid-cured from JK09 | Danish craft beer | Czech beer |
| Size (Mbp) | 2.69 | 2.72 | 2.67 | 2.45 | 2.75 | 2.59 |
| GC% | 45.4 | 45.4 | 45.6 | 45.9 | 45.4 | 45.6 |
| Number of Contigs | 173 | 192 | 181 | 144 | 198 | 250 |
| Number of CDS | 2723 | 2755 | 2920 | 2454 | 2772 | 2645 |
| Number of tRNA | 64 | 62 | 62 | 60 | 64 | 62 |
| Number of rRNA | 7 | 7 | 6 | 6 | 7 | 7 |
| Number of CRISPR loci | 3 | 3 | 2 | 2 | 3 | 1 |

of genes in comparison with the parental strain JK09 and strain G430 was most different from the others.

An all-against-all protein comparison was conducted using BLAST to describe homology. A homolog was considered significant if there was a minimum of 50% identity over a coverage of at least 50% of the longer sequence. For the plots calculation (A), if two proteins within a genome matching the 50-50% cutoff, they were considered as one protein family. If a protein family contains proteins from all compared genomes, this family is a core protein family. For the matrix (B), the top part (green) consists of pairwise genome comparisons and the number represents the proportion of shared protein families (more green, more shared proteins); the bottom part (red) reflects self-comparison where a hit within a genome to protein other than the query is identified as the internal homologs (more red, more similar proteins within one genome).

### *3.4 Genes potentially involved in beer spoilage*

Annotated contigs were analyzed by BLASTP to examine potential beer (hop)-tolerance genes that were described previously (Table S2). The results for the six most important beer tolerance genes are presented in Table 2. In all six strains, CDSs

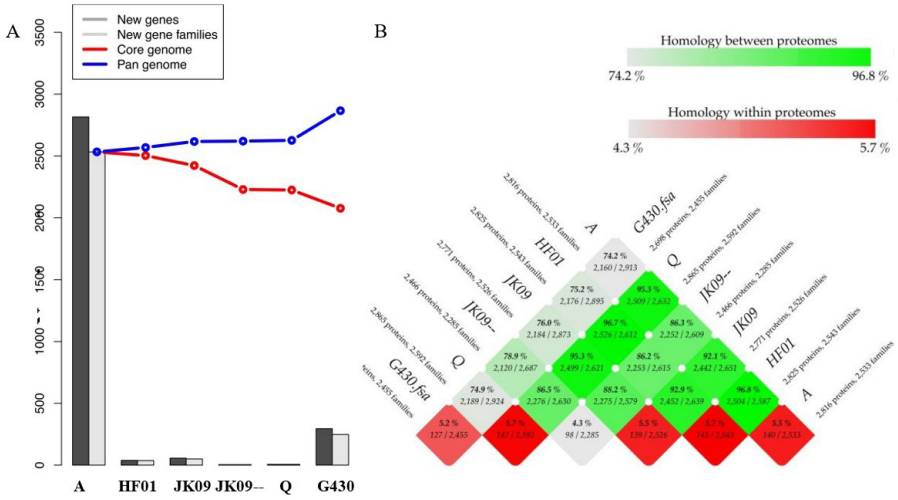


**Figure 2.** Pan- and core- genome plots (A) and BLAST matrix (B) of six *L. brevis* strains.

**Table 2**. Six potential beer (hop)- tolerance genes analyzed by BLASTP.

| Strain | Accession No | AFR11464 | AFR11467 | AFR11466 | KIO95014 | KIO97109 | KIP01033 |
|---|---|---|---|---|---|---|---|
| | Predicted gene function | ABC-type multidrug transporter (horA) | PMF-dependent multidrug transporter (HorC) | putative divalent cation transporter (HitA) | Signal transduction histidine kinase | ArsR family Transcriptional regulator | 3-hydroxyacyl-[acyl-carrier-protein] dehydratase (FabZ) |
| **A** | **Identity** | 59% | 97% | 76% | 100% | 100% | 100% |
| | **Coverage** | 97% | 100% | 98% | 100% | 100% | 100% |
| | **Contig** | 10 | 90 | 66 | 107 | 17 | 44 |
| **HF01** | **Identity** | 99% | 97% | 100% | 100% | 100% | 100% |
| | **Coverage** | 100% | 100% | 100% | 100% | 100% | 100% |
| | **Contig** | 87 | 92 | 100 | 108 | 18 | 42 |
| **JK09** | **Identity** | 99% | 97% | 100% | 100% | 100% | 100% |
| | **Coverage** | 100 | 100 | 100 | 100 | 100 | 100 |
| | **Contig** | 86 | 93 | 76 | 36 | 17 | 43 |
| **JK09--** | **Identity** | 59% | 97% | 76% | 100% | 100% | 100% |
| | **Coverage** | 97% | 100% | 98% | 100% | 100% | 100% |
| | **Contig** | 13 | 84 | 59 | 92 | 16 | 40 |
| **Q** | **Identity** | 99% | 97% | 100% | 100% | 100% | 100% |
| | **Coverage** | 100% | 100% | 100% | 100% | 100% | 100% |
| | **Contig** | 90 | 97 | 92 | 35 | 17 | 44 |
| **G430** | **Identity** | 62% | 91% | 76% | 100% | 100% | 100% |
| | **Coverage** | 49% | 99% | 98% | 100% | 100% | 100% |
| | **Contig** | 58 | 147 | 83 | 54 | 42 | 3 |

were found that showed 100% identity to signal transduction histidine kinase, ArsR family transcriptional regulator and 3-hydroxyacyl-acyl-carrier-protein dehydratase (FabZ form). Based on the BLAST analysis, all these CDSs were predicted to be on the chromosome. Moreover, all the strains also contained CDSs that showed significant homology (≥97%) to HorC, but these CDSs were predicted by BLAST to be plasmid-derived, and the CDSs were flanked by transposons.

Strains HF01, JK09 and Q, contained CDSs with high similarity (>99%) to HorA and HitA. These CDSs were also flanked by mobile genetic elements and found on contigs that were predicted to be plasmid-derived. Interestingly, none of the CDSs in the predicted plasmids of strains A, JK09-- and G430 show identity to HorA and HitA. Some of the CDSs contained in the predicted chromosome of all six strains also showed lower identity to HorA and HitA.

In comparison with the *horA* sequence from *L. brevis* BSO 464, 3 strains (HF01, JK09, Q) have it almost identical (5 amino acids changes). Furthermore, the genetic context is identical both upstream and downstream of *horA*. Both side flanking genes have an opposite orientation to *horA* indicate that *horA* is under its own promoter and there is no changes between the BSO 464 and the analyzed strains (Fig. S3A). Downstream to the *horA* there is only one gene that is in common between all four strains. *L. brevis* BSO 464 has afterwards a small hypothetical protein followed by *repB* gene. The three analyzed strains have a cytosine deaminase gene instead. Moreover, analysis of synteny of the closest, chromosomally located homolog of *horA* revealed that all six strains and BSO 464 have it in highly identical sequence and genetic synteny (Fig. S3B).

Similar to *horA*, the *hitA* gene also has a conserved synteny and it is identical when comparing three plasmid carrying strains (HF01, JK09 and Q) to *L. brevis* BSO 464 (Fig. S3C). Furthermore, the analysis of synteny of the chromosomally located homolog of *hitA* showed that all tested strains and BSO 464 have it in highly identical sequence (Fig. S3D). However, in the strain BSO464, two genes upstream from *hitA* homolog, there is an integrase gene inserted.

Comparative analysis of the plasmid component of JK09 and JK09-- revealed the absence of a number of proteins in JK09-- (Table S3). In addition to HorA and HitA, a NAD (FAD)-dependent dehydrogenase, a FAD-dependent pyridine nucleotide-disulphide oxidoreductase, a DNA-binding ferritin DPS family protein, a ferric reductase, a manganese transporter, a cadmium-manganese-transporting P-type ATPase, a glycosyltransferase, and many phage related proteins (e.g. prophage P1 protein 30), plasmid replication proteins, transcriptional regulators and transposases are absent in JK09--. Most of these proteins can be found in the plasmids of the beer-spoilage strain *L. brevis* BSO 464 (pLb464-1, pLb464-2, pLb464-3, pLb464-4 and pLb464-8).

The beer tolerant strains A, HF01 and JK09 and the more sensitive strain Q have a very high level of similarity (>95%) (Figure 2), and it is therefore interesting to further elucidate the genetic determinants that may affect or modify the tolerance and/or sensitivity of these strains to beer. A BLASTP analysis of unique protein/peptide sequences revealed that the three beer-tolerant strains have a set of nine proteins/peptides that were absent in strain Q (Table 3). Three of these proteins/peptides (ClpX protease, manganese transporter and replication protein) were also absent in the other two beer-sensitive strains JK09-- and G430. These three proteins are also located on contigs that are predicted as plasmid-derived.

## 4 Discussion

In this study, a beer-based medium supplemented with a small amount of MRS medium called ABD broth was used to test the spoilage potential of *L. brevis* strains (Suzuki et al., 2020a). As expected, strains that grow faster in ABD broth (beer-tolerant strains) also exhibited faster growth in hop stress medium ($MRS_{4.3+H}$), albeit with some inconsistencies (Zhao et al., 2017). This supports the previous notion that bacteria should tolerate additional pressures such as ethanol and nutrient limitation in order to be able to grow in beer, although hop is the key stress factor in beer (Schneiderbanger et al., 2020). The strain MI2158 was originally isolated from feces, and was included in this study because it was expected to demonstrate poor beer tolerance. However, MI2158 demonstrated an intermediate tolerance towards beer, which further underlines that many strains of *L .brevis* has an innate tolerance to beer, that can not only be attributed to adaptation in breweries.

The results from CRISPR analysis (Fig. S2) and protein comparisons (Figure 2) indicated that among the original strains (A, HF01, JK09, Q and G430), strain G430 appeared to be more different from the others. This result is in agreement with previous result where only G430 was significantly inhibited by low pH (Zhao et al., 2017). G430 has unique proteins like

**Table 3**. Predicted proteins that are present in beer tolerant strains but absent in strain Q.

| Accession No | Predicted protein | Identity | E-value | Coverage |
|---|---|---|---|---|
| WP_042253659 | Clp protease ClpX [*Lactobacillus brevis*] | 99% | 0 | 100% |
| WP_003554836 | Manganese transporter [*Lactobacillus*] | 98% | 4E-49 | 100% |
| AJA81579 | Replication protein [Lactobacillus brevis BSO 464] | 98% | 5E-68 | 99% |
| WP_052256260 | Cell wall hydrolase [*Lactobacillus brevis*] | 99% | 0 | 100% |
| KIO96091 | Putative glycosyltransferase [*Lactobacillus brevis*] | 100% | 7E-71 | 100% |
| WP_057879142 | Hypothetical protein [ *Lactobacillus paucivorans*] | 100% | 2E-118 | 100% |
| AJA81671 | FAD-dependent pyridine nucleotide-disulfide oxidoreductase protein [*Lactobacillus brevis* BSO 464] | 100% | 0 | 100% |
| BAN06996 | Conserved hypothetical protein [*Lactobacillus brevis* KB290] | 100% | 8E-42 | 100% |
| WP_02452676 | DeoR family transcriptional regulator [*Lactobacillus brevis*] | 99% | 0 | 100% |

malate dehydrogenase, gluconokinase, phosphoenol pyruvate-phosphotransferase that are involved in sugar metabolism and several prophage genes that improve the surviving under adverse environmental conditions (Kelleher et al., 2018). It could also reflect the different origins and biogeographic distribution of the strains, where G430 was isolated from beer from the Czech Republic, while the rest were all isolated from Danish craft beer. Therefore, the four strains from Danish craft beer might have evolved from a common ancestor.

Analysis of synteny of the chromosomally located homologs of *horA* and *hitA* revealed that all analyzed strains and *L. brevis* BSO 464 have them in highly identical sequence and genetic synteny (Fig S3B-S3D). Therefore, these *horA* and *hitA* homologs likely do not contribute to beer tolerance and possess another biological function since they are present in both beer tolerant and sensitive strains.

Plasmid curing of strain JK09 strongly supports that key components of hop tolerance are plasmid borne. These genes include (i) *horA* and *hitA*, which have previously been suggested to play important roles in hop tolerance and beer spoilage (Bergsveinson et al., 2017; Suzuki et al., 2020b); (ii) genes coding for NAD (FAD)-dependent dehydrogenase, FAD-dependent pyridine nucleotide-disulphide oxidoreductase, DNA-binding ferritin DPS family protein and ferric reductase. These genes were previously reported to contribute to the bacterial defense system against oxidative stress due to hop compounds (Bergsveinson et al., 2016, Ilari et al., 2020); (iii) the manganese transporter and cadmium-manganese-transporting P-type ATPase, which play important roles in manganese homeostasis and oxidative stress response (Tong et al., 2017); (iv) glycosyltransferase that is involved in glycosylation and modification of teichoic acids was suggested to serve as a genetic marker for discriminating the wine spoilage ability within a species (Snauwaert et al., 2015; Suzuki et al., 2020b); (v) several phage related proteins showing significant similarity to plasmid pLb464-4 that was suggested to provide important functions for the growth of *L. brevis* BSO 464 in beer (Bergsveinson et al., 2015); and (vii) transcriptional regulators and transposases, which might provide genetic flexibility in response to different environmental stresses (Behr et al., 2015).

It is not clear which genes have the most significant effect on beer spoilage of JK09, and the underlying mechanisms remain to be investigated. However, all the above mentioned genes seem to have individual protective functions against different beer related stresses and therefore contribute to the beer tolerance phenotype.

Controversy also exists with regard to whether the reported beer tolerance genes can predict the spoilage potential of bacteria (Behr et al., 2016; Feyereisen et al., 2020a). In our study, the genes *arsR*, *fabZ*, *horC* and the encoding signal transduction histidine kinase were present in all strains, beer tolerant as well as sensitive. According to many previous reports, beer tolerant strains are expected to possess *horA* and/or *hitA* (Suzuki et al., 2020b). Surprisingly, this was not the case in our study; the beer tolerant strain A did not contain the two genes, while the beer sensitive strain Q did, as was also confirmed by colony PCR (results not shown). Therefore, our findings disagree somewhat with the reports of Asano et al (Asano et al., 2019) which suggested that e.g. *horA* can be used for highly accurate detection of beer-spoilage bacteria. Yet, our findings are in accordance with the reports of Bergsveinson, Baecker (Bergsveinson et al., 2015), which suggested that growth in beer is a multifactorial process requiring complex genetics and cellular regulation.

In our study, three proteins were unique to the beer-tolerant strains A, HF01 and JK09, ClpX protease, a manganese transporter different from HitA and a replication protein. ClpX is a chaperone and has been shown to play a role in stress resistance in several bacteria (Saunders et al., 2020; Vahidi et al., 2020). Since the majority of hop-regulated enzymes are pH and manganese dependent, the changes in intracellular pH and/or manganese level that is caused by hop compounds could produce conformational changes in proteins. As ClpX proteases may reactivate, remodel or degrade misfolded proteins, its absence could render the cells more sensitive to growth in beer. Feyereisen et al. (2020b) suggested that HitA imports manganese into cells to maintain the cellular manganese homeostasis (Feyereisen et al., 2020b). Thus, it is likely that the unique manganese transporter different from HitA that we have identified could complement or replace HitA. For example, the beer tolerant strain A does not possess HitA, but is still able to grow well in beer. The replication protein is an essential factor for plasmid replication. Indeed, this unique protein increases the copy number of the plasmids and it is likely that it affects the expression level of beer tolerance proteins. However, Bergsveinson et al. (2015) demonstrated that the increase in plasmid copy number does not necessarily correlate with an increase in bacterial growth in degassed beer. Thus, the role of the replication protein present in beer tolerant strains needs further investigation. Nevertheless, the association of these three unique proteins with plasmids highlights the importance of plasmids in beer tolerance.

## 5 Conclusions

The results presented in this study indicate that the ability of *L. brevis* to grow in beer is complex and multifactorial. Our findings suggest that many genes can be linked to beer spoilage, but it is difficult to absolutely predict the beer spoilage potential solely on the presence or absence of previously described genes. In this study we identified two novel genes (ClpX protease and a manganese transporter different from HitA) that appear to be involved in beer spoilage, but it is impossible to predict if these two genes will always be present in beer spoilage strains. It is therefore likely that a future system to predict beer spoilage should consist of two parts. A monitoring part, where a larger number of potential spoilage genes are routinely investigated in strains that are isolated in breweries. This will probably lead to the detection of a significant number of false positives, where the genetic makeup suggest that the strain possess one or more genes associated with beer spoilage. These suspect isolates will then have to be investigated by alternative strategies that focus on either gene expression/protein translation or physiological responses such as change in intracellular pH to predict if the strain actually has the potential to spoil beer.

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## References


Asano, S., Shimokawa, M., & Suzuki, K. (2019). PCR Analysis methods for detection and identification of beer-spoilage lactic acid bacteria. *Methods in Molecular Biology (Clifton, N.J.)*, 1887, 95-107. http://dx.doi.org/10.1007/978-1-4939-8907-2_9. PMid:30506252.

Behr, J., Geissler, A. J., Preissler, P., Ehrenreich, A., Angelov, A., & Vogel, R. F. (2015). Identification of ecotype-specific marker genes for categorization of beer-spoiling Lactobacillus brevis. *Food Microbiology*, 51, 130-138. http://dx.doi.org/10.1016/j.fm.2015.05.015. PMid:26187837.

Behr, J., Geissler, A. J., Schmid, J., Zehe, A., & Vogel, R. F. (2016). The Identification of novel diagnostic marker genes for the detection of beer spoiling pediococcus damnosus strains using the BlAst diagnostic gene findEr. *PLoS One*, 11(3), e0152747. http://dx.doi.org/10.1371/journal.pone.0152747. PMid:27028007.

Bergsveinson, J., Baecker, N., Pittet, V., & Ziola, B. (2015). Role of plasmids in lactobacillus brevis BSO 464 hop tolerance and beer spoilage. *Applied and Environmental Microbiology*, 81(4), 1234-1241. http://dx.doi.org/10.1128/AEM.02870-14. PMid:25501474.

Bergsveinson, J., Friesen, V., & Ziola, B. (2016). Transcriptome analysis of beer-spoiling Lactobacillus brevis BSO 464 during growth in degassed and gassed beer. *International Journal of Food Microbiology*, 235, 28-35. http://dx.doi.org/10.1016/j.ijfoodmicro.2016.06.041. PMid:27394184.

Bergsveinson, J., Kajala, I., Goerzen, S., & Ziola, B. (2017). Detection of a Hop-tolerance gene hora insertion variant in lactic acid bacteria that results in a truncated HorA lacking the Walker B motif necessary for transport function. *Journal of the American Society of Brewing Chemists*, 75(4), 339-344. http://dx.doi.org/10.1094/ASBCJ-2017-4682-01.

Chan, P. P., & Lowe, T. M. (2019). tRNAscan-SE: searching for tRNA genes in genomic sequences. *Methods in Molecular Biology (Clifton, N.J.)*, 1962, 1-14. http://dx.doi.org/10.1007/978-1-4939-9173-0_1. PMid:31020551.

Conesa, A., Gotz, S., Garcia-Gomez, J. M., Terol, J., Talon, M., & Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics (Oxford, England)*, 21(18), 3674-3676. http://dx.doi.org/10.1093/bioinformatics/bti610. PMid:16081474.

Deng, Y., Lim, J., Nguyen, T. T. H., Mok, I. K., Piao, M., & Kim, D. (2019). Composition and biochemical properties of ale beer enriched with lignans from Schisandra chinensis Baillon (omija) fruits. *Food Science and Biotechnology*, 29(5), 609-617. http://dx.doi.org/10.1007/s10068-019-00714-5. PMid:32419959.

Feyereisen, M., Mahony, J., O'Sullivan, T., Boer, V., & van Sinderen, D. (2020a). Beer spoilage and low pH tolerance is linked to manganese homeostasis in selected Lactobacillus brevis strains. *Journal of Applied Microbiology*, 129(5), 1309-1320. http://dx.doi.org/10.1111/jam.14730. PMid:32478894.

Feyereisen, M., Mahony, J., O'Sullivan, T., Boer, V., & van Sinderen, D. (2020b). A Plasmid-encoded putative glycosyltransferase is involved in hop tolerance and beer spoilage in Lactobacillus brevis. *Applied and Environmental Microbiology*, 86(3), e02268-19. http://dx.doi.org/10.1128/AEM.02268-19. PMid:31757821.

Geissler, A. J., Behr, J., & Vogel, R. F. (2016). Multiple genome sequences of important beer-spoiling lactic acid bacteria. *Genome Announcements*, 4(5), e01077-16. http://dx.doi.org/10.1128/genomeA.01077-16. PMid:27795248.

Gomes, F. D., Guimaraes, B. P., Ceola, D., & Ghesti, G. F. (2022). Advances in dry hopping for industrial brewing: a review. *Food Science and Technology (Campinas)*, 42, 8. http://dx.doi.org/10.1590/fst.60620.

Hyatt, D., Chen, G.-L., LoCascio, P. F., Land, M. L., Larimer, F. W., & Hauser, L. J. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics*, 11, 119. http://dx.doi.org/10.1186/1471-2105-11-119. PMid:20211023.

Ilari, A., Bellapadrona, G., Carbonera, D., & Di Valentin, M. (2020). Disclosing the molecular mechanism of iron incorporation in listeria innocua Dps by EPR spectroscopy. *Applied Magnetic Resonance*, 51(11), 1543-1557. http://dx.doi.org/10.1007/s00723-020-01287-x.

Kazachenka, A., & Kassiotis, G. (2021). SARS-CoV-2-Host chimeric RNA-Sequencing reads do not necessarily arise from virus integration into the host DNA. *Frontiers in Microbiology*, 12, 676693. http://dx.doi.org/10.3389/fmicb.2021.676693. PMid:34149667.

Kechin, A., Boyarskikh, U., Kel, A., & Filipenko, M. (2017). cutPrimers: a new tool for accurate cutting of primers from reads of targeted next generation sequencing. *Journal of Computational Biology*, 24(11), 1138-1143. http://dx.doi.org/10.1089/cmb.2017.0096. PMid:28715235.

Kelleher, P., Mahony, J., Schweinlin, K., Neve, H., Franz, C. M., & van Sinderen, D. (2018). Assessing the functionality and genetic diversity of lactococcal prophages. *International Journal of Food Microbiology*, 272, 29-40. http://dx.doi.org/10.1016/j.ijfoodmicro.2018.02.024. PMid:29524768.

Kennedy, P. G., Cline, L. C., & Song, Z. W. (2018). Probing promise versus performance in longer read fungal metabarcoding. *The New Phytologist*, 217(3), 973-976. http://dx.doi.org/10.1111/nph.14883. PMid:29334600.

Lagesen, K., Hallin, P., Rodland, E. A., Staerfeldt, H.-H., Rognes, T., & Ussery, D. W. (2007). RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Research*, 35(9), 3100-3108. http://dx.doi.org/10.1093/nar/gkm160. PMid:17452365.

Munford, A.R.G., Chaves, R. D., & Sant'Ana, A. S. (2020). Inactivation kinetics of beer spoilage bacteria (Lactobacillus brevis, Lactobacillus casei, and Pediococcus damnosus) during acid washing of brewing yeast. *Food Microbiology*, 91, 103513. http://dx.doi.org/10.1016/j.fm.2020.103513. PMid:32539960.

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Parrello, B., Shukla, M., Vonstein, V., Wattam, A. R., Xia, F. F., & Stevens, R. (2014). The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Research*, 42(D1), D206-D214. http://dx.doi.org/10.1093/nar/gkt1226. PMid:24293654.

Piraine, R. E. A., Retzlaf, G. M., Goncalves, V. S., Cunha, R. C., Conrad, N. L., Bochman, M. L., & Leite, F. P. L. (2023). Brewing and probiotic potential activity of wild yeasts Hanseniaspora uvarum PIT001, Pichia kluyveri LAR001 and Candida intermedia ORQ001. *European Food Research and Technology*, 249, 133-148.

Rodríguez-Saavedra, M., González de Llano, D., & Moreno-Arribas, M. V. (2020). Beer spoilage lactic acid bacteria from craft brewery microbiota: Microbiological quality and food safety. *Food Research International*, 138(Pt A), 109762. http://dx.doi.org/10.1016/j.foodres.2020.109762. PMid:33292943.

Rodríguez-Saavedra, M., Perez-Revelo, K., Valero, A., Moreno-Arribas, M. V., & Gonzalez de Llano, D. (2021). A binary logistic regression model as a tool to predict craft beer susceptibility to microbial spoilage. *Foods*, 10(8), 1926. http://dx.doi.org/10.3390/foods10081926. PMid:34441703.

Ruiz-Barba, J. L., Piard, J. C., & Jimenez-Diaz, R. (1991). Plasmid profiles and curing of plasmids in Lactobacillus plantarum strains isolated from green olive fermentations. *The Journal of Applied Bacteriology*,

71(5), 417-421. http://dx.doi.org/10.1111/j.1365-2672.1991.tb03810.x. PMid:1761434.

Ryoo, S., Lee, J., Oh, J. Y., Kim, B. K., Kim, Y., Kim, J. H., Shin, C., & Lee, S. H. (2018). Comparing two mycobacterium tuberculosis genomes from chinese immigrants with native genomes using mauve alignments. *Tuberculosis and Respiratory Diseases*, 81(3), 216-221. http://dx.doi.org/10.4046/trd.2017.0091. PMid:29926541.

Saunders, R. A., Stinson, B. M., Baker, T. A., & Sauer, R. T. (2020). Multistep substrate binding and engagement by the AAA plus ClpXP protease. *Proceedings of the National Academy of Sciences of the United States of America*, 117(45), 28005-28013. http://dx.doi.org/10.1073/pnas.2010804117. PMid:33106413.

Schneiderbanger, J., Jacob, F., & Hutzler, M. (2020). Mini-Review: the current role of lactic acid bacteria in beer spoilage. *Brewing Science (Nurnberg)*, 73(1-2), 1-5.

Snauwaert, I., Stragier, P., De Vuyst, L., & Vandamme, P. (2015). Comparative genome analysis of Pediococcus damnosus LMG 28219, a strain well-adapted to the beer environment. *BMC Genomics*, 16(1), 267. http://dx.doi.org/10.1186/s12864-015-1438-z. PMid:25880122.

Suzuki, K., Shinohara, Y., & Kurniawan, Y. (2020a). Recent progress of microbiological quality control methods in unpasteurized beer production. *Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi*, 67(11), 411-423. http://dx.doi.org/10.3136/nskkk.67.411.

Suzuki, K., Shinohara, Y., & Kurniawan, Y. N. (2020b). Role of plasmids in beer spoilage lactic acid bacteria: a review. *Journal of the American Society of Brewing Chemists*, 79(1), 1-16. http://dx.doi.org/10.1080/03610470.2020.1843899.

Tong, Y., Zhai, Q., Lu, W., Tian, F., Zhao, J., Zhang, H., & Chen, W. (2017). New insights in integrated response mechanism of Lactobacillus plantarum under excessive manganese stress. *Food Research International*, 102, 323-332. http://dx.doi.org/10.1016/j.foodres.2017.10.014. PMid:29195955.

Vahidi, S., Ripstein, Z. A., Juravsky, J. B., Rennella, E., Goldberg, A. L., Mittermaier, A. K., Rubinstein, J. L., & Kay, L. E. (2020). An allosteric switch regulates Mycobacterium tuberculosis ClpP1P2 protease function as established by cryo-EM and methyl-TROSY NMR. *Proceedings of the National Academy of Sciences of the United States of America*, 117(11), 5895-5906. http://dx.doi.org/10.1073/pnas.1921630117. PMid:32123115.

Vesth, T., Lagesen, K., Acar, Ö., & Ussery, D. (2013). CMG-Biotools, a free workbench for basic comparative microbial genomics. *PLoS One*, 8(4), e60120. http://dx.doi.org/10.1371/journal.pone.0060120. PMid:23577086.

Wang, J. H., Tseng, S. Y., & Tung, K. C. (2019). Genomic investigation of emerging zoonotic pathogen Shewanella xiamenensis. *Tzu-Chi Medical Journal*, 32(2), 162-166. http://dx.doi.org/10.4103/tcmj.tcmj_69_19. PMid:32269948.

Wieme, A. D., Spitaels, F., Aerts, M., De Bruyne, K., Van Landschoot, A., & Vandamme, P. (2014). Identification of beer-spoilage bacteria using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *International Journal of Food Microbiology*, 185, 41-50. http://dx.doi.org/10.1016/j.ijfoodmicro.2014.05.003. PMid:24929682.

Zhao, Y., Knochel, S., & Siegumfeldt, H. (2017). Heterogeneity between and within strains of lactobacillus brevis exposed to beer compounds. *Frontiers in Microbiology*, 8, 239. http://dx.doi.org/10.3389/fmicb.2017.00239. PMid:28261191.

Zheng, F., Niu, C., Zhu, L., Tang, D., Li, Y., Liu, C., Wang, J., & Li, Q. (2019). Evaluation of genotypic diversity in hop resistance of beer spoilage bacteria. *Journal of Food Science and Biotechnology*, 38(12), 1-8.

## Supplementary Material

Supplementary material accompanies this paper.

**Fig. S1.** Subsystem features of six L. brevis strains.

**Fig. S2.** CRISPR arrays of six L. brevis strains.

**Fig. S3.** The synteny comparison of horA (A) and hitA (C) of L. brevis BSO464 and HF01, JK09, Q , the synteny comparison of homolog of horA (B) and hitA (D) of L. brevis BSO464 and all the tested strains.

**Table S1.** Unique proteins in G430.

**Table S2.** 21 potential beer (hop)-tolerance genes analyzed by BLASTP.

**Table S3.** Unique proteins in JK09, compared to JK09—

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