Comparative genetic and physiological characterisation of *Pectinatus* species reveals shared tolerance to beer-associated stressors but halotolerance specific to pickle-associated strains

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Declaration of interests: None

Abstract

Obligate anaerobic bacteria from the genus *Pectinatus* have been known to cause beer spoilage for over 40 years. Whole genome sequencing was performed on eleven beer spoilage strains (nine *Pectinatus frisingensis,* one *Pectinatus cerevisiiphilus* and one *Pectinatus haikarae* isolate), as well as two pickle spoilage species (*Pectinatus brassicae* MB591 and *Pectinatus sottacetonis* MB620) and the tolerance of all species to a range of environmental conditions was tested. Exploration of metabolic pathways for carbohydrates, amino acids and vitamins showed little difference between beer spoilage- and pickle spoilage-associated strains. However, genes for certain carbohydrate and sulphur-containing amino acid-associated enzymes were only present in the beer spoilage group and genes for specific transporters and regulatory genes were uniquely found in the pickle spoilage group. Transporters for compatible solutes, only present in pickle-associated strains, likely explain their experimentally observed higher halotolerance compared to the beer spoilers. Genes involved in biofilm formation and ATP Binding Cassette (ABC) transporters potentially capable of exporting hop-derived antimicrobial compounds were found in all strains. All species grew in the presence of alcohol up to 5% alcohol by volume (ABV) and hops extract up to 80 ppm of iso-α-acids. Therefore, the species isolated from pickle processes may pose novel hazards in brewing.

Keywords: *Pectinatus*; comparative genomics; environmental adaptation; beer; pickle; spoilage

1 Introduction

Beer spoilage by microorganisms is a problem for breweries. Although not a health risk, spoilt beer is no-longer fit for consumption due to it becoming unpalatable and brand damage may occur when a spoilt product reaches consumers. There is scope for breweries to better understand beer spoilage-associated microorganisms in order to better predict risks, test for the presence of contaminants and establish novel coping mechanisms. Over the last 30 years, bacteria belonging to the genus *Pectinatus* have become a more prevalent cause of beer spoilage. It is thought that this is caused by improvements in filling processes, resulting in less residual oxygen in the product and allowing for an anaerobic environment where these organisms can thrive (Chelack and Ingledew, 1987; Suzuki, 2011). Beer contaminated with *Pectinatus* species is easily recognised by strong off-odours such as hydrogen sulphide and acetate and turbidity of the product. It is therefore important to research *Pectinatus* species to better understand their growth requirements and stress adaptation, with the aim to develop novel ways to prevent contamination of beer with *Pectinatus* species.

The genus *Pectinatus* was first described by Lee et al. (1978) and currently consists of five recognised species. *P. cerevisiiphilus*, *P. frisingensis*, and *P. haikarae* have been isolated from spoilt beer (Juvonen and Suihko, 2006; Lee et al., 1978; Schleifer et al., 1990), whereas *P. brassicae* and *P. sottacetonis* have been isolated from saline waste water and fermentation tanks from commercial pickle processing plants, respectively (Caldwell et al., 2013; Zhang et al., 2012). To date, no natural reservoir for these organisms has been found. The beer spoilage species can be found throughout the brewery environment. How these anaerobic bacteria survive in this varied environment is not yet understood. They seem to be permanently present in the brewery, rather than an occasional contaminant (Hakalehto, 2000; Helander et al., 2004), and they have been described to inhabit mixed species biofilms together with *Saccharomyces cerevisiae* and *Lactobacillus* species (Matoulkova et al., 2012). In this mixed community, the yeast can scavenge oxygen and the lactobacilli can produce exopolysaccharides. *Pectinatus* itself has not been described to produce exopolysaccharides, a critical requirement for biofilm production.

Limited physiological characterisation has been carried out to date, which largely focussed on differences between *Pectinatus* species in nutrient utilisation. Beer spoilage-associated *Pectinatus* species are known to use a wide variety of carbon sources and each species grows on different substrates. *P. frisingensis* for instance is unique in that it can grow on cellobiose, N-acetyl glucosamine and xylitol. *P. cerevisiiphilus* is the only beer spoilage *Pectinatus* species that can grow on ribose and salicin, and *P. haikarae* can grow on lactose whereas the other two species cannot (Juvonen, 2009; Schleifer et al., 1990). *P. frisingensis* and *P. cerevisiiphilus* also cannot grow on amino acids that are prevalent in beer alone (Tholozan et al., 1996). Pickle fermentation-associated species also show differences in carbon source utilisation. Only *P. sottacetonis* is able to ferment lactose and mannose, whereas only *P. brassicae* can ferment aesculin (Caldwell et al., 2013; Zhang et al., 2012). Some of these results contradict previously reported carbon source requirement data (Caldwell et al., 2013; Haikara et al., 1981; Juvonen and Suihko, 2006; Lee et al., 1978; Schleifer et al., 1990; Zhang et al., 2012), therefore there is a need to further study the physiology of these species.

Glucose metabolism in *P. cerevisiiphilus* and *P. frisingensis* results in the formation of propionate and CO_2, with smaller amounts of acetate, acetoin and succinate also being produced (Tholozan et al., 1994). *P. haikarae* has been described to produce acetate, propionate, hydrogen sulphide and acetoin as major metabolites when grown on fructose (Juvonen and Suihko, 2006). Both *P. brassicae* and *P. sottacetonis* have been described to produce mainly propionate and acetate, with some production of hydrogen sulphide, when fermenting glucose or fructose. *P. sottacetonis* produces acetoin as well, while this is not the case for *P. brassicae* (Caldwell et al., 2013; Zhang et al., 2012). In addition to the off-flavour caused by the production of these fermentation products, utilization of lactate by pickle spoilage-associated *Pectinatus* strains causes the pH of brine in the pickle fermentation to rise, which in turn makes the product more susceptible to contamination with other unwanted organisms (Breidt et al., 2013; Franco et al., 2012).

The different species in the genus *Pectinatus* have been distinguished based on differences in the 16S rRNA gene sequence, carbon source utilisation, fermentation products and motility, but no genome-wide comparison study has been performed. Here we present comparative genomic analysis of the genus *Pectinatus* by using a representative of all five species and highlight genetic diversity, as well as key differences between brewery and pickling-associated species. In addition, we carried out within-species comparative genomics with nine *P. frisingensis* strains isolated from different breweries to assess intraspecies diversity. The in-silico analyses are complemented by physiological characterisation of the growth response of all species to a range of environmental conditions to gain a better understanding for the requirements for these organisms to grow in beer.

2 Materials and methods

2.1 Strains

Nine strains of *P. frisingensis* and one strain each of *P. cerevisiiphilus*, *P. haikarae*, *P. brassicae* and *P. sottacetonis* were supplied by Heineken Supply Chain B.V, see Table 1. *Lactobacillus brevis* MB521 was provided by Marine Feyereisen from University College Cork.

2.2 Characterisation of strain growth

Strains were grown in mYPG (modified Yeast Peptone Glucose) medium (Table S1) unless mentioned otherwise. The medium was prepared anaerobically under a CO_2 atmosphere as described in Table S1. Inoculations in Hungate tubes (1% volume) were performed using a constant flow of CO_2 to retain an anaerobic headspace from a culture grown to an optical density (OD, 650 nm) of 1.2 - 1.6 at 30°C. OD measurements were performed in a Novaspec II spectrophotometer (Pharmacia, Stockholm, Sweden) at 650 nm wavelength after vigorous mixing to obtain a homogeneous suspension. Hungate tubes were incubated in a waterbath at 30°C unless mentioned otherwise. To determine the effect of temperature on the growth rate of *Pectinatus* strains, cultures were incubated in water baths at 4°C, 8°C, 12°C, 16°C, 20°C, 26°C, 30°C, 34°C, 37°C, 42°C, 45°C and 52°C. To obtain exponential growth at the same time in all culture conditions, the inoculum for the 30°C - 42°C cultures was diluted between 5 and 20 times prior to inoculation.

Growth experiments were also performed in 96-well format with 200 µl medium per well in a Biotek Epoch 2 microplate reader located in a Don Whitley Scientific MACS-VA500 anaerobic workstation (80% N_2, 10% H_2, 10% CO_2; for details of medium preparation see Table S1). Plates were inoculated (1% volume) from a pre-culture grown in a Hungate tube as specified above and incubated at 30°C. OD (650 nm) measurements were performed every 10 minutes after 10 seconds of double orbital shaking. To test pH tolerance, mYPG media of pH 4 – 7.6 were prepared as detailed in Table S1. Tolerance to salt stress was tested in mYPG media (pH 6.3) with 1%, 3%, 5% and 7% sodium chloride. Tolerance to hops was tested in mYPG media (pH

5.3) supplemented with isomerised hop extract (Hopsteiner, Mainburg, Germany) at 0 to 80 ppm iso-α-acids. Growth of all strains in alcohol-free beer (Heineken 0.0, Heineken, Edinburgh, UK) was compared to growth in regular beer (5% ABV, Heineken, Heineken, Edinburgh, UK). Tolerance to alcohol was tested in alcohol-free beer supplemented with water-alcohol mixtures of different alcohol contents at 5% maximum volume.

Maximum growth rates were determined by calculating the slope of a linear regression fit of the natural logarithm of the absorbance during exponential growth up to an OD of 0.5. To test if the difference in growth rate between conditions is significant (p=0.05), a one-way ANOVA with Tukey post-hoc testing was performed in IBM SPSS Statistics 25. Data are presented as the average of a minimum of triplicate measurements, error bars represent the standard deviation.

Strain identity and purity was confirmed using Gram-staining, phase contrast microscopy and multiplex PCR.

2.3 Multiplex PCR for verification of strain identity

PCR primers were designed for twelve strains with a different amplicon size for each strain using Primer3Plus (Untergasser et al., 2007) in regions of a genome that did not align with any of the other genomes. Although unique genomic regions were present in *P. frisingensis* MB360, very few potential strain-specific primers were found in-silico, none of which resulted in reliable amplification in the multiplex method. Specific primer sequences could therefore not be established for strain *P. frisingensis* MB360. The twelve primer sets were tested for specific amplification of the expected product size and absence of cross-reactivity with the 13 *Pectinatus* strains of this study at a range of annealing temperatures and combined into sets of three multiplex PCRs with four primer sets each (Table S2). PCR reactions were carried out in a total volume of 50 µl with 0.2 nM of each dNTP (Promega, Southampton, UK), 3.5 mM $MgCl_2$ (Bioline, London, UK), 25 mU/µl Taq enzyme (Bioline, London, UK) and 1 µl of stationary phase culture as template. Amplification was performed with a Bio-Rad S1000 thermocycler (Bio-Rad, Watford, UK) as follows: two minutes denaturation (96°C) followed by 30 cycles of 30 seconds denaturation, 30 seconds annealing (60°C) and two minutes extension (72°C) after which a 10-minute extension step and a 10-minute cooling step (4°C) were performed.

5 µl of amplicon was loaded on a gel containing 2.5% agarose (Melford, Chelsworth, UK), Tris Acetate EDTA (TAE) (NBS biologicals, Huntingdon, UK) and 1:20 000 SYBR-Safe (Invitrogen, Carlsbad, USA). A 100 bp ladder was used as a reference (Biotium, Fremont, USA). Electrophoresis was performed for 45 minutes at 120 V in TAE running buffer. Pictures (Fig. S1) were taken using a UVIdoc HD5 (Uvitec, Cambridge, UK).

2.4 Sequencing and genome assembly

Whole genome sequencing was performed by Baseclear B.V (Leiden, the Netherlands). All strains were sequenced using Illumina HiSeq 2000 paired-end mate pair sequencing using 50 bp reads and sequencing data was assembled by BaseClear. Six strains were sequenced using long read sequencing with Pacific Biosciences (PacBio) technology (Table S3). Initially, just *P. frisingensis* MB139 was sequenced using RS-II chemistry and assembled using the PacBio SMRTPortal (version 2.3.1), utilizing the RS_HGAP_Assembly.2 protocol. Later, the other five strains were sequenced using newer Sequel technology. PacBio Sequel based assemblies were assembled using Canu 1.4 (Koren et al., 2017) with settings optimised per strain to obtain an assembly that was both contiguous and was similar in size and number of predicted genes to the BaseClear Illumina assemblies (Table S3). Where both PacBio and Illumina sequencing data was available, the Illumina data was used to improve the base call confidence of PacBio data by mapping Illumina reads onto PacBio assemblies using bwa mem (Li and Durbin, 2009). Base call quality score recalibration and variant calling were both performed using GATK 4.beta.5 (Broad Institute, 2017a). Variants were filtered out if fewer than 50 Illumina reads showed an alternative genotype and if less than 90% of base calls suggested a specific single nucleotide polymorphism. Variants that passed filtering were incorporated in the genome using GATK 3.8 FastaAlternateReferenceMaker (Broad Institute, 2017b).

2.5 Protein prediction, annotation and building protein families

Protein prediction was performed using PROKKA (Seemann, 2014). Protein annotation was performed by running NCBI BLASTp v2.2.31 (Altschul et al., 1990) against the SwissProt KB 2018_09 database (Boeckmann et al., 2003) filtering out annotations at an e-value of 10^{-6}. If several annotations were possible based on these criteria, the annotation with the highest bitscore was used. Proteins with no or unknown annotation were subsequently run against the TrEMBL 2018_09 database (Boeckmann et al., 2003). Families of orthologous proteins were determined using vice versa best blast hits using a Genedata proprietary algorithm based on the NCBI Cluster of Orthologous Groups approach (Tatusov et al., 1997). If no annotation for a protein was available, but an annotation was present for a protein in the same orthologous protein family in a well-annotated genome, this annotation was inherited.

2.6 Phylogenetic relationship between strains

Confirmation of species classification was performed by comparing 16S rRNA gene sequences extracted from the assemblies to published references (Caldwell et al., 2013; Juvonen and Suihko, 2006; Schleifer et al., 1990; Yarza et al., 2013; Zhang et al., 2012) using BLASTn v2.2.31 (Altschul et al., 1990). Genomic diversity among all isolates was determined through Average Amino acid Identity (AAI) using CompareM v0.0.23 (Parks, 2014). Maximum distance measures were determined and a phylogenetic tree was built using complete agglomerative hierarchical clustering with the R package pvclust (Suzuki and Shimodaira, 2006) using multiscale bootstrap resampling of one million iterations. Average Nucelotide Identity (ANI)

was calculated using mumMER/nucMER v3.23 (Kurtz et al., 2004). Euclidean distances were calculated and a phylogenetic tree was built as described above.

2.7 Identification of common genomic features

To determine genes shared among all isolates and strain-specific genes, a core and pan genome was built using Roary v3.7.0 (Page et al., 2015). All proteins with a shared nucleotide sequence identity over 50% were considered homologs and the core genome was defined as genes present in all 13 strains. Proteins present in all beer spoilage strains but absent in the pickle spoilage strains and vice versa were extracted and annotated against the SwissProt database (Bairoch and Apweiler, 2000) using BLASTp (Altschul et al., 1990). Genes involved in antimicrobial resistance were tested for using the Comprehensive Antibiotic Resistance Database online analysis tool Resistance Gene Identifier, CARD-RGI (Jia et al., 2017). Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and their associated enzymes were determined using the development version of CRISPRdisco (Crawley et al., 2018). CRISPR systems were classified according to clustering of CRISPR-associated (Cas) proteins as described by Koonin at al. (2017). Prophages were detected using Phaster (Arndt et al., 2016). An all-vs-all BLASTn search was performed to identify prophages in different strains with a high resemblance in nucleotide sequence and alignments of similar prophages were visualised using Mauve v2.3.1 (Darling et al., 2010). To test for the potential presence of plasmids, contigs were compared with a database of 8.5 million plasmid sequences downloaded from RefSeq (O'Leary et al., 2016) with NCBI BLASTn and hits where more than 80% of both the plasmid and the contig were aligned were considered candidates for plasmids. These candidate plasmids were then investigated for the presence of an origin of replication, conjugation, relaxation and mobilisation genes by blasting these contigs against the SwissProt database using BLASTx v2.2.31.

2.8 Analysis of metabolic pathways and stress response genes

Presence or absence of genes coding for enzymes involved in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa and Goto, 2000) for metabolism of amino acids, vitamins, sugars, lipids, sulphur and fermentation acids was identified using *Pathway Analyser* (Genedata Selector® 5.3 (Genedata AG, Basel, Switzerland)).

Genes commonly associated with biofilm formation, sporulation, hop resistance, alcohol detoxification and osmotic stress were identified in literature (Table S4) and for each of these genes, proteins sharing this annotation were downloaded from UniprotKB (Bateman et al., 2017). Proteins were aligned using Muscle 3.8.31 (Edgar, 2004) and a Hidden Markov Model (HMM) was prepared using HMMer v3.2.1 (Eddy, 1995). This HMM was tested against the protein sequences of all strains and the top hit was extracted and blasted against a well-characterised query protein and the SwissProt database (Boeckmann et al., 2003) to see if the extracted protein matches the initial query. Regardless of the annotation of the closest blast hit, hits with less than 50% query coverage and less than 30% sequence identify were regarded as negative.

3 Results

3.1 General genome characteristics

Genomes of 13 *Pectinatus* strains were assembled into between 1 and 233 contigs (see Table S3 for assembly statistics per strain). The median assembly size was 3.01 Mbp, with a median number of protein coding genes of 2866 and a median GC-content of 38.0%. Seven potential plasmid sequences (between 335 and 3207 base pairs in size) were identified using the RefSeq plasmid database, none of which contained an origin of replication, conjugation, relaxation or mobilisation genes. The core genome of the genus *Pectinatus* (based on 13 isolates) at >50% conserved nucleotide sequence (Fig. S2), consisted of 1261 genes. Within the nine *P. frisingensis* isolates, this number came to 1984 genes. The pan genome (the number of unique genes across all isolates) of the genus *Pectinatus* contained 7582 genes and the *P. frisingensis* pan genome was 5000 genes, 1419 of which were accessory genes unique to a single isolate (ranging between 75 in *P. frisingensis* MB255 and 444 in *P. frisingensis* MB240). Fig. 1 shows the number of genes shared between different species when using *P. frisingensis* MB139 as representative for *P. frisingensis*. No large differences in numbers of shared genes were seen when comparing beer-related to pickle-related species. Type I CRISPR systems were found in ten strains encompassing all *Pectinatus* species, Type III systems were present in seven strains belonging to *P. frisingensis, P. brassicae* and *P. sottacetonis* and Type V systems in six strains, being most predominant in the two pickle spoilage-associated strains (Table S5). Beer spoilage strains *P. frisingensis* MB175 and *P. frisingensis* MB191 contained no Cas proteins. CRISPR repeat sequences were detected in all strains apart from *P. frisingensis* MB175, some of which were not closely located to the Cas proteins. Prophage regions were found in all strains, with 8 strains containing complete prophages (Table S6). A complete prophage region found in *P. frisingensis* MB169 was also present in *P. frisingensis* MB240 (100% identity). Some other prophages were also found in multiple *P. frisingensis* strains at more than 95% identity over more than 95% of the sequence length (Fig. S3). CARD-RGI analysis showed presence of the *adeF* gene in all 13 strains, which codes for an efflux system that may impart antimicrobial resistance to tetracycline and fluoroquinolone (Leus et al., 2018). However, *P. sottacetonis* MB620 has been experimentally described to be tetracycline susceptible (Caldwell et al., 2013).

3.2 Phylogenetic relationship between strains

For all species, the average identity between the 16S rRNA genes extracted from the assembled genomes and the published reference genes for that species was 99.5% or higher (Table S7). AAI analysis showed a difference between the beer spoilage and pickle spoilage strains (Fig. 2A). The *P. frisingensis* isolates were between 98% - 99% identical and the strains from different species were at least 72% identical. ANI showed 95% - 99% identity between *P. frisingensis* strains (Fig. 2B). Two clusters (six and three strains, respectively) were observed within the *P. frisingensis* genomes, which was also observed in the AAI analysis.

3.3 Metabolic pathway analysis

Investigation of the presence of genes involved in core metabolism revealed that all 13 *Pectinatus* strains had complete pathways for the synthesis of the 20 proteinogenic amino acids and for vitamin B1 (thiamine), B2 (riboflavin), B3 (niacin), B7 (biotin) and B12 (cobalamin). The *P. cerevisiiphilus* and *P. haikarae* isolates are missing one gene for vitamin B5 (pantothenate) production (Fig. S4A), dehydropantoate hydroxymethyltransferase. No genes for one step in vitamin B6 (pyridoxine) production were detected in any strain apart from the *P. cerevisiiphilus* isolate, which had a pyridoxine 5'-phosphate oxidase (*pdxH*) gene and therefore a complete B6 synthesis pathway (Fig. S4B). All *Pectinatus* isolates had complete vitamin B9 (folate) production pathways but lacked the dihydrofolate reductase (Fig. S4C).

The capacity to ferment 32 different carbon sources was predicted based on gene presence. *P. frisingensis* was considered positive for utilisation of a carbon source when the majority of the nine isolates contained all genes required for fermentation of this carbon source. When comparing the genetic capability of all isolates to utilise specific carbon sources, differences between strains from different species were found (Table 2). All strains had the required genes to use dulcitol, fructose, galactitol, galactose, glucosamine, glucose, glycerol, mannitol, mannose, melibiose, ribose, salicin, sorbitol, sorbose, sucrose, xylitol and xylulose based on KEGG-map analysis. *P. sottacetonis* MB620 was unique in lacking genes for fermenting rhamnose. Only the two pickle-associated isolates showed the presence of genes required for utilisation of raffinose and stachyose. All isolates of the three beer spoilage species contained genes for the use of galacturonate while the pickle-associated strains lacked these genes. While some discrepancies to literature-based data were found, most of the genome-based data in agreement with the literature (Table 2).

Regarding sulphur metabolism, none of the *Pectinatus* strains investigated had genes that catalyse conversion of molecular sulphur or sulphate into sulphite, but genes for conversion of sulphite to hydrogen sulphide were found in all 13 isolates (Fig. S5A). All 13 strains contained the genes required for the production of methyl mercaptan. A likely source of hydrogen sulphide formation is the breakdown of sulphur-containing amino acids, specifically cysteine, as all strains contained genes that code for enzymes capable of breaking down L-cysteine into pyruvate, hydrogen sulphide and ammonia (EC 4.4.1.8), and *cysK* and *cysE* (E.C. 2.5.1.47 and E.C. 2.3.1.30), which catalyse conversion of L-cysteine to L-serine via O-acetyl-L-serine. A gene for D-cysteine desulfhydrase (EC 5.1.1.10) for the conversion of D-cysteine into hydrogen sulphide and pyruvate was only found in the *P. haikarae* isolate (Fig. S5B). Both pickle spoilage strains lacked genes encoding cystathionine gamma lyase (EC 4.4.1.1) while all beer spoilage strains contained these genes (Fig. S5B).

Pathways for the production of propanoyl-CoA were found in all strains via succinate (Fig. S5C). No enzymes were found that catalyse the conversion of propanoyl-CoA to propionate via either a CoA-transferase or kinase, the two routes commonly found in fermentative organisms (Louis and Flint, 2017). Succinate-CoA transferase was found to be present in all strains and might be able to catalyse the

conversion from propanoyl-CoA to propionate as well, as low substrate specificity in CoA-transferases has been described before (Louis and Flint, 2017; Mullins and Kappock, 2012). All strains contained complete pathways for the production of lactate, acetate and acetoin, but lacked pathways for the production of diacetyl, butyrate, longer chain or branched chain fatty acids. *P. brassicae* was the only organism containing a pathway for the production of acetoacetate.

3.4 Identification of genes involved in response to brewery and pickling-associated stress

Growth in beer poses several challenges due to the low availability of nutrients, low pH, anaerobic conditions, presence of alcohol and hop-derived antimicrobial substances. The presence of a range of genes that may aid survival in the diverse environment of a brewery or commercial pickle production site was investigated (details for all genes searched for are given in Table S4). As *Pectinatus* species have routinely been found in mixed-species biofilms in breweries where they are able to persist and can cause contamination of products, all *Pectinatus* genomes were mined for the presence of biofilm-associated genes. It was found that all *Pectinatus* isolates have genes for exopolysaccharide production as well as associated regulatory genes. Alcohol dehydrogenases were found in all isolates. ABC-type transporters with resemblance to HorA and a divalent cation transporter with resemblance to HitA, well-known to be involved in resistance to hop stress in *Lactobacillus* species (Kullen and Klaenhammer, 2002; Sakamoto and Konings, 2003), were found to be present in all strains. Few oxidative stress tolerance-related genes were found. Catalase was only found in *P. haikarae* MB233, whereas an alkyl hydroperoxide reductase was found in all strains apart from *P. brassicae* MB591. Superoxid dismutase or alternative (NADH-dependent) alkyl hydroperoxide reductase were not found in any of the strains. Acid tolerance systems were found in all strains, most of which are predicted to be involved in alkali production or a F1-F0-ATPase proton pump. Heat tolerance-associated chaperones were found in all strains, apart from the small heat shock protein IlpB, which was absent in all *P. frisingensis* strains. Although *P. brassicae* MB591 has been described to be non-motile, the same genes for flagellar proteins and motor proteins were found in all strains, apart from FliO which was found in strains of all species but only one *P. frisingensis* strain, MB255.

3.5 Genes unique to beer spoilage or pickle spoilage organisms

To investigate differences between beer spoilage-associated and pickle spoilage-associated *Pectinatus* strains, genes unique to each of these groups were examined. Only genes present in all isolates in one group but absent in all isolates in the other group were investigated, as these may shed light on the genes required to survive in these two different environments. In total, 71 genes were present in all beer spoilage strains but absent in pickle spoilage strains and 60 of these had bacterial matches in the SwissProt database at an e-value below 10^{-6} (Fig. 3, Table S8). The beer spoilage strains contained a number of amino acid active enzymes that were missing in the pickle spoilage strains. In addition, a range of unique carbohydrate active enzymes, and transporters were also found in the beer spoilage strains. In total, 100 genes were

present in pickle spoilage strains but absent in beer spoilage strains, 65 of which had hits to bacterial SwissProt entries with an e-value of 10^{-6} or less (Fig. 3, Table S9). Genes for the transport of glycine betaine, a universal osmoprotectant (Empadinhas and da Costa, 2008), were uniquely found in the pickle-associated strains. Nine unique regulatory genes were also found, however analysis of surrounding genes did not reveal clear indications of which genes they may regulate (data not shown).

3.6 Physiological response to brewery and pickling-relevant stress

The growth characteristics of seven *Pectinatus* strains were determined under a range of different conditions. For *P. frisingensis* three strains were chosen to include the strain with a complete genome sequence (MB139), the type strain (MB174) and the strain with the largest number of genes (MB240, Table S3). Final optical densities largely depended on the growth medium (mYPG or beer) rather than the effect of alcohol or hops, with growth in mYPG in Hungate tubes resulting in ODs of over 1.6 for all strains examined at optimal growth conditions (Table S10). Final optical densities obtained in 96-well plates were generally lower (OD ≈1.0 – 1.5, Table S10). Clear differences in OD were seen between growth in alcohol-free and alcoholic beer, with the former ranging between OD 0.43 and OD 0.95 and the latter between OD 0.11 and OD 0.33. Cold temperatures, high concentrations of salt and high pH all negatively influenced the final OD (Table S10).

Growth at different temperatures revealed that most strains grew best between 30 and 37°C, with the exact optimum temperature differing somewhat between strains (Fig. 4A and Table S10). *P. cerevisiiphilus* MB134 showed the highest temperature tolerance of all strains, with the fastest growth between 37 and 42°C. All strains displayed good growth at 26°C with *P. sottacetonis* MB620 growing fastest, but the growth rate of all strains markedly decreased at 22°C. Very slow growth was still observed for *P. cerevisiiphilus* MB134, *P. frisingensis* MB139, *P. haikarae* MB233 and *P. brassicae* MB591 at 12 and 16°C. At 8°C growth was only observed for *P. haikarae* MB233 during the nine-week test period (Fig. 4A). The influence of pH on the growth rate of the seven *Pectinatus* strains was determined between pH 4.02 and 7.63 (Fig. 4B). A broad growth optimum was found for most strains, with the growth rate not being significantly different between 6.3 and 7.27 for *P. frisingensis* MB139, MB174, MB240, *P. brassicae* MB591 and *P. sottacetonis* MB620. Optimal growth was observed between pH 5.58 and 6.30 for *P. cerevisiiphilus* MB134, and between 4.96 and 6.93 for *P. haikarae* MB233 (Fig. 4B).

The influence of brewery-specific stress factors on the growth of all *Pectinatus* species was investigated. No significant effect on maximum growth rate was observed in any strain when supplementing media (pH 5.3) with up to 80 ppm iso-α-acids from hop extract (shown for *P. frisingensis* MB139 in Fig. 5) while in the positive control (*Lactobacillus brevis* MB521) inhibition of growth was obtained at 20 ppm (Fig. 5). All strains grew in alcohol-free beer and regular beer (5% ABV), although a large and significant ($p < 0.05$) difference in growth rate (Fig. 6A) was observed between the two conditions in all strains apart from *P.*

frisingensis MB139, where the difference was not significant. As the alcohol-free beer and regular beer differ in composition (Table S1), the effect of adding up to 5% ethanol to alcohol-free beer on the growth of *Pectinatus* was investigated. No significant decrease in maximum growth rate was observed up to 5% ABV in any strain apart from *Pectinatus frisingensis* MB174, which showed a small but significant ($p = 0.032$) decrease in maximum growth rate at 2-5% ABV (Fig. 6B).

When investigating salt tolerance, which is relevant for pickling, differences were observed between strains. At 1% salt, a significant decrease in maximum growth rate (Fig. 7A) was observed in the beer-spoilage-associated strains *P. cerevisiiphilus* MB134 ($p < 0.001$), *P. frisingensis* MB139 ($p < 0.001$) and *P. frisingensis* MB240 ($p < 0.001$) compared to medium without added salt. For some of the beer spoilers and the pickle spoilage-associated strains (*P. frisingensis* MB174, *P. haikarae* MB233, *P. brassicae* MB591 and *P. sottacetonis* MB620), no significant difference in growth rate was observed between 0% and 3% salt. Only a single beer spoilage-associated strain (*P. frisingensis* MB174) and the pickle spoilage strains (*P. brassicae* MB591 and *P. sottactonis* MB620) were showing growth at 5% salt but exhibited a significant reduction of the maximum growth rate compared to medium without added salt ($p \leq 0.004$). Differences in final biomass were observed between these strains. Only the pickle-associated strains grew to an OD of over 1.0 at 3% salt, with the most halotolerant beer spoilage isolate only reaching 0.55 (Table S10).

The beer spoilage strain with the largest salt tolerance, *P. frisingensis* MB174, was further investigated in Hungate tubes in comparison to the pickle-associated strain *P. brassicae* MB591 (Fig. 7B). Growth of *P. frisingensis* MB174 was not observed at 5% salt under these conditions and only one out of three replicates grew at 3% salt, whereas for *P. brassicae* MB591 the results were similar to those obtained in 96-well format. Strain identity and purity were confirmed using microscopy and multiplex PCR for both sets of experiments.

4 Discussion

Here we have performed the first comparative genomic analysis of the genus *Pectinatus*. AAI and ANI analysis show more than 95% of genetic material is shared between the nine isolates of *P. frisingensis*. Apart from individual strains lacking the metabolic pathways for utilization of dulcitol, xylitol or xylulose, very little difference was found between *P. frisingensis* strains. A separate indicator for the degree of conserved identity between the *P. frisingensis* strains is that several strains share prophage sequences. Although no phage is shared between all *P. frisingensis* strains, the presence of an exact copy in *P. frisingensis* MB169 and *P. frisingensis* MB240 may indicate that the infection event occurred prior to strain differentiation. It is known that prophages acquired in the past may be lost when a new infection causes the old phage to be replaced (Bobay et al., 2014). Finding the same prophage sequences in several strains further suggests that the strains of *P. frisingensis* had a recent mutual ancestor, even though they were isolated from Singapore, the Netherlands, Finland, Indonesia and Croatia. Two *P. frisingensis* clusters were

observed in the dendrograms of the AAI and ANI analysis. As these strains share more than 95% ANI, this separation between the two clusters is not strong enough to consider them different species based on recently proposed classification (Chun et al., 2018). This is in line with findings based on 16S rRNA gene sequence homology.

Pectinatus species are distinguishable based on their ability to produce acid from different carbon sources, however publications do not agree on which carbon sources are used by the different species. Strain-specific differences in the presence of carbon source utilisation genes were found here for the different *P. frisingensis* isolates. This, together with differences in methodology, may explain why different groups working with different isolates obtained variable results. Several metabolic pathways are present according to KEGG analysis that indicate consumption of carbon sources that these organisms were reported to not be able to ferment. These discrepancies could be due to these genes not being expressed or functional, annotation errors or to specific growth conditions employed during physiological tests. Additionally, the absence of specific transport proteins for these carbon sources may also explain discrepancies between the genomic and experimental findings, which requires further investigation. Previous research indicated that certain carbon sources can be used by *Pectinatus* species to grow, even though the relevant genes were not found in the genome (Table 2), which too could be due to an error in gene prediction or misannotation. A second possibility is the presence of promiscuous enzymes that catalyse reactions with a range of different substrates. Interestingly, *P. cerevisiiphilus* MB134 lacks the required enzymes to break down maltose, one of the main sugars found in beer, which is in agreement with some published growth data, although others have reported *P. cerevisiiphilus* MB134 fermenting maltose (Caldwell et al., 2013; Lee et al., 1978). *P. brassicae* has been described not to produce acetoin, unlike the other *Pectinatus* species (Zhang et al., 2012). Genetically, no difference was found in acetoin production pathways between *P. brassicae* MB591 and the other species.

Several genes that are associated with detoxification of alcohol were found in all strains. This is in line with our findings that all strains tolerated up to 5% ABV. Growth rate differences seen between alcohol-free and alcoholic beer are likely mainly due to the differences in the available substrates, as little or no growth rate differences were found in alcohol-free beer supplemented with alcohol. Watier et al. reported lower growth rates for *P. frisingensis* DSM20465 during growth in a modified MRS medium in the presence of 2.5 and 5% ethanol compared to no addition of ethanol (Watier et al., 1996). It is possible that the limited total growth seen in beer due to the low substrate availability masks potential effects on the growth rate. No effect of hops on the growth of any *Pectinatus* strains was found. This could be due to the Gram-negative like cell wall structure of the bacteria, or the presence of genes (*horA* & *hitA*) linked to hop tolerance in lactic acid bacteria (Bergsveinson et al., 2017). When mining the genome for beer spoilage specific genes, differences in transporters were found between the beer spoilage and pickle spoilage strains. The presence of these transporters may reflect the environments these organisms evolved in. The carbohydrate-specific

transporters found in beer spoilage-associated species could indicate that these organisms evolved in a more carbon-source-poor environment than the pickle spoilage strains and therefore require higher numbers and variety of transporters to scavenge nutrients from the environment. One of these transporters is a D-xylose transporter, which is unexpected as none of the species are expected to consume xylose according to Table 2. Several genes involved in carbohydrate and amino acid utilisation were exclusively found in the beer spoilage organisms, however none of the substrates associated with these enzymes is in high abundance in beer (Meilgaard and Denmark, 1981). This higher diversity of carbohydrate utilization genes and transporters was also found in comparative genomic analyses of Gram-positive beer spoilage organisms when compared to strains obtained from other food and clinical settings (Behr et al., 2016; Bergsveinson and Ziola, 2017). These publications also list beer spoilage-associated diagnostic marker genes, genes that are indicative of a strain's ability to spoil beer. However, these diagnostic marker genes were not found to be different between beer spoilage- and pickle spoilage-associated *Pectinatus* strains in our study. Beer spoilage strains have additional pathways to produce hydrogen sulphide compared to the pickle spoilage strains through the breakdown of cystathionine, which may contribute to the characteristic rotten egg smell of beer that is contaminated with *Pectinatus*.

The ability of the *P. brassicae* and *P. sottacetonis* strains to grow to a high biomass (OD > 1.0) in medium with 3% salt is likely linked to their ability to take up osmoprotectants, as these two isolates contained genes related to compatible solute transporters. Interestingly, two beer-associated strains, *P. frisingensis* MB174 and *P. haikarae* MB233 also showed growth at higher salt concentrations. However, the final OD was much lower compared to that of pickle-associated organisms. *P. haikarae* MB233 exhibited strongly reduced final OD already at 1% and *P. frisingensis* MB174 at 3% salt (Table S10), which suggests that they cannot counteract the salt stress effectively in the absence of the compatible solute transporters. Testing salt tolerance of the latter strain in Hungate tubes did not result in the same osmotolerance as in multiwell plates. Growth at 3% salt was only observed in one out of three replicates, with a lower final OD compared to the low-salt conditions and no growth was observed at 5% salt. This is likely due to differences in methodology such as agitation and gas phase as in both the Hungate and the 96-well experiment cultures were confirmed to be pure *P. frisingensis* MB174 through microscopy and strain-specific PCR.

While *Pectinatus* strains are regularly found in biofilms in breweries, the production of extracellular polysaccharides is commonly attributed to other inhabitants of the biofilm, such as lactic acid bacteria (Matoulková et al., 2012). From our genomic analysis it appears that all 13 strains of *Pectinatus* investigated contain genes involved in the production of these extracellular polymeric substances. This may help *Pectinatus* to persist in the brewery for longer and explain the high detection rate by surface swabbing in breweries (Matoulkova et al., 2012; Paradh et al., 2011). All *Pectinatus* strains grew at below-room temperature conditions, but the growth rate dropped substantially and it could take weeks or even months for a low-level contamination to become noticeable under these conditions. Refrigeration of the product

should stop growth of the bacteria and could be considered for situations where longer term storage is required.

5 Conclusions

Here, we found low intra-species diversity of *P. frisingensis*, with the main differences observed in carbohydrate utilisation between strains of this species. Isolates from different *Pectinatus* species investigated here also displayed few differences, despite the fact that they were isolated from two different environments. One key difference between strains from different environments was the presence of transporters for the osmoprotectant compound glycine betaine. They were found exclusively in the pickle spoilage organisms, which likely causes the increased halotolerance of these strains. Genes required for formation of biofilms, detoxification of alcohol and proteins that may facilitate export of hop antimicrobials were found in all strains. Biofilm formation in *Pectinatus* has not been described before but could play a large role in the survival and persistence of *Pectinatus* contaminants in brewery environments. Although not associated with beer spoilage, both genetic analysis and physiological tests show that the *P. brassicae* and *P. sottacetonis* isolates are able to tolerate brewing-related stresses, produce off-flavours commonly found in spoiled beer and are potential new hazards for the brewing industry.

6 Acknowledgements

This work was supported by Heineken Supply Chain B.V. PL, SD & HJF receive support from the Scottish Government Rural and Environment Sciences and Analytical Services (RESAS). We would like to express thanks to Nicola Smith for her help formatting Fig. 1, Dr Paul Sheridan for bioinformatics-based advice and help with troubleshooting scripts, Dr Kenneth Forbes for his advice on core and pan genome analysis, Sandro Cocuzza for providing the hop extract and Marine Freyereisen for supplying *Lactobacillus brevis* MB521.

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Table headers:

Table 1. List of strains used and the culture collection reference for all strains that have been deposited.

Table 2. Carbon source utilisation according to KEGG analysis and published information.

Figure legends:

Fig. 1. Venn diagram showing the number of genes shared between the different species of *Pectinatus.* The number underneath the strain names indicate the total number of genes in this strain. [a]For *P. frisingensis* isolate MB139 was used as complete genome information was available for this strain. Beer spoilage strains are indicated in blue, pickle spoilage strains are in red.

Fig. 2. Cluster dendrogram for the Average Amino acid Identity based on a minimum of 1652 proteins in the pairwise comparison of 13 *Pectinatus* strains (A) and Average Nucleotide Identity (B) values of 9 *P. frisingensis* strains based on a minimum alignment of 2.3 Mbp. Approximately Unbiased (AU) values (in red) denote the p-value based on multiscale bootstrap resampling expressed as a percentage. Bootstrap Probability (BP) values (in green) denote the probability based on normal bootstrap resampling, also expressed as a percentage. Beer spoilage strains are indicated in blue, pickle spoilage strains are in red.

Fig. 3. Number and category of genes unique to beer spoilage and pickle spoilage strains.

Fig. 4. Optimum growth temperature and medium pH of seven *Pectinatus* isolates. A: Maximum growth rate in mYPG medium (pH 6.3) at temperatures ranging from 4°C to 52°C. B: Maximum growth rate in mYPG medium at pH values ranging from 4.02 to 6.78 at 30°C. Beer spoilage strains are indicated in blue, pickle spoilage strains are in red. Error bars indicate the standard deviation from the mean. Triangles indicate values do not significantly differ from maximum observed growth rate, circles indicate values are significantly lower than the maximum observed growth rate.

Fig. 5. Growth of *P. frisingensis* MB139 (circles) and *L. brevis* MB521 (triangles) when grown on mYPG medium (pH 5.3) supplemented with increasing concentrations of iso-α-acids from hop concentrate. Error bars indicate the standard deviation from the mean.

Fig. 6. Growth of *Pectinatus* isolates in beer. A: Maximum growth rate of 13 *Pectinatus* isolates on beer and alcohol-free beer. B: Maximum growth rate of seven *Pectinatus* isolates in alcohol-free beer supplemented with different concentrations of alcohol. Beer spoilage strains are indicated in blue, pickle spoilage strains are in red. Error bars indicate the standard deviation from the mean. Significant differences in comparison to alcohol-free beer are indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Fig. 7. Growth of *Pectinatus* isolates in the presence of salt. A: Maximum growth rate of seven *Pectinatus* isolates in mYPG medium (pH 6.3) supplemented with salt. Error bars indicate the standard deviation from the mean for triplicates. Significant differences in comparison to medium without salt are indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. B: Growth of *P. frisingensis* MB174 (circles) and *P. brassicae* MB591 (triangles) when grown in Hungate tubes in mYPG with different concentrations of salt. Data points represent the average of triplicate measures with error bars indicating the standard deviation. Beer spoilage strains are indicated in blue, pickle spoilage strains are in red. The dashed line for *P. frisingensis* MB174 at 3% salt indicates the growth curve of the only replicate of the three cultures to grow under these conditions. No further change in growth was observed over the next 3 days (data not shown).

Supplementary table headers:

Table S1. Media composition and preparation

Table S2. Primers used in strain-specific multiplex PCR

Table S3. Assembly statistics for all strains, assembly parameters for PacBio Sequel assemblies and parameters for incorporating Illumina data into the PacBio assembly.

Table S4. List of stress response genes, presence of these genes in *Pectinatus* isolates and the BLAST alignment percentages and identity percentages for hits with a reference gene as well as the best match in Swissprot

Table S5. Presence of CRISPR-associated (Cas) Proteins, Classification of CRISPR systems into types and location of CRISPRs. Loci represented in bold indicate repeats that are co-localised with Cas proteins

Table S6. Overview of complete and incomplete prophage regions in the genome of 13 *Pectinatus* isolates.

Table S7. Average percentage identity between 16S rRNA genes extracted from the genome assemblies for all 13 strains compared to the published *Pectinatus* rRNA gene sequences. The eight-character alphanumeric code below the species name is the GenBank accession number for the 16S rRNA gene sequence used. Values in bold highlight the percentage identity match between the 16S rRNA gene extracted from the genome with the reference gene of the same species as the isolate.

Table S8. Beer spoilage strain specific genes with accession number, gene name and annotation according to SwissProt

Table S9. Pickle spoilage strain specific genes with accession number, gene name and annotation according to SwissProt

Table S10. Maximum OD obtained during growth experiments

Supplementary figure legends:

Fig. S1. Gel electrophoresis of strain specific amplicons obtained from pure cultures. MB numbers are used to indicate individual strains (Table 1). Letters A-C indicate different mixtures of primers (Table S2). A Biotium 100 bp ladder was used (catalog #31032), the size of main bands is indicated in number of basepair.

Fig. S2. Plots of total genes and conserved genes for the *Pectinatus* genus (all 13 strains; A) and *P. frisingensis* (nine strains, B) when using a 50% identity cut-off during pan genome generation.

Fig. S3. Prophage sequences shared between different strains of *P. frisingensis*. The amount of red indicates the degree of conservation of nucleotides at this position.

Fig. S4A. KEGG MAP00770 Pantothenate and CoA biosynthesis: Blue represents metabolic pathways present in all strains, purple denotes a step absent in the *P. cerevisiiphilus* and *P. haikarae* isolates but present in all other strains, orange denotes a pathway absent in all strains.

Fig. S4B. KEGG MAP00750 Vitamin B6 metabolism: Blue represents metabolic pathways present in all strains, orange denotes a conversion present in the *P. cerevisiiphilus* isolate but absent in all other strains.

Fig. S4C. KEGG MAP00790 Folate biosynthesis: Blue represents metabolic pathways present in all strains, orange denotes a conversion absent in all strains.

Fig. S5A. KEGG MAP00920 Sulfur metabolism: Orange arrow indicates a pathway absent in all strains, blue arrow indicates a pathway present in all strains.

Fig. S5B. KEGG MAP00270 Cysteine and methionine metabolism: Blue represents metabolic pathways present in all strains, orange denotes pathways present in beer spoilage strains but absent in pickle spoilage strains, purple denotes a reaction unique to the *Pectinatus haikarae* strain.

Fig. S5C. KEGG MAP00640 Propanoate metabolism: Pathway in blue indicates pathway from succinate to propanoyl-CoA present in all strains, pathway in orange indicates missing pathways from propanoyl-CoA to propanoate.

Table 1

List of strains used and the culture collection reference for all strains that have been deposited.

Species	Internal strain reference	Country of isolation	Culture collection reference
Beer spoilage associated strains			
Pectinatus frisingensis	MB139†‡	Singapore	
Pectinatus frisingensis	MB169	The Netherlands[1]	
Pectinatus frisingensis	MB174^{T}‡	Finland	DSM 6306 / ATCC 33332
Pectinatus frisingensis	MB175	The United Kingdom	
Pectinatus frisingensis	MB191	The Netherlands[2]	
Pectinatus frisingensis	MB240†‡	Indonesia	
Pectinatus frisingensis	MB255	The Netherlands[2]	
Pectinatus frisingensis	MB273	Croatia	
Pectinatus frisingensis	MB360	The Netherlands[3]	
Pectinatus cerevisiiphilus	MB134^{T}†‡	The United States of America	DSM 20467 / ATCC 29359
Pectinatus haikarae	MB233†‡	Germany	DSM 20764 / VTT E-97914
Pickle spoilage associated strains			
Pectinatus brassicae	MB591^{T}†‡	China	DSM 24661
Pectinatus sottacetonis	MB620^{T}†‡	The United States of America	ATCC BAA-2501 / VTT E-113163

^{T} Type strain

† Strain has been PacBio sequenced

‡ Strain was used in physiological experiments

[1, 2, 3] Different breweries in the Netherlands

Table 2

Carbon source utilisation according to KEGG analysis and published information.

Carbon source	*P. cerevisiiphilus*[T]			*P. frisingensis*			*P. haikarae*			*P. brassicae*[T]			*P. sottacetonis*[T]		
	Gen.[a]	Lit +[b]	Lit -[b]	Gen.[a]	Lit +[b]	Lit -[b]	Gen.[a]	Lit +[b]	Lit -[b]	Gen.[a]	Lit +[b]	Lit -[b]	Gen.[a]	Lit +[b]	Lit -[b]
Arabinose	-	abf		-	bf		-	f		-			-		**f**
Cellobiose	-	abdf	**c**	9	**bcdf**		-		**df**	-			+	**f**	
Dulcitol	+	**acdf**		8/9	**bcdf**		+	**df**		+			+		f
Fructose	+	**abd**		9	**bd**		+	**d**		+			+		
Fructuronate	-			-			-			-			-		
Galactose	+	**abd**		9	**bd**		+	**d**		+			+	**f**	
Galacturonate	+			9			+			-			-		
Glucosamine	+			9			+			+			+		
Glucose	+	**abdf**		9	**bdf**		+	**df**		+			+	**f**	
Glucuronate	-			-			-			-			-		
Glycerol	+	**abdef**		9	**bdf**	e	+	**df**	e	+	**e**		+	**f**	
Inositol	-	c	**df**	-	c	**bdf**	-	df		-			-	f	
Lactose	+	**bdf**	e	-		**bdef**	+	**def**		-		**e**	-	f	
Maltose	-	af	**bd**	9	**bf**	d	-		**df**	+			+	**f**	
Mannitol	+	**abc**	def	9	**bcdef**		+	**def**		+	**e**		+	**f**	
Mannose	+	**abdef**		9	**bdef**		+	**def**		+		e	+	**f**	
Melibiose	+	**cdf**		9		bcf	+	**df**		+			+		f
N-acetyl glucosamine	-	f	**cd**	9	**cdf**		+		f	+			-		**f**
Raffinose	-		**abd**	-		**bd**	-		**df**	+			+		
Rhamnose	+	**abdf**		9	**cdf**		+	**df**		+			-		**f**
Ribitol	-	f		-	f		-	f		-			-	f	
Ribose	+	**abdf**		9	**bdf**		+	**df**		+			+	**f**	
Salicin	+	**f**	ad	9	**f**	bd	+		df	+			+	**f**	

Sorbitol	+	**bf**	a	9	**bf**		+	**f**		+			+	**f**		
Sorbose	+	**b**	a	9	**b**		+			+			+			
Stachyose	-			-			-			+			+			
Sucrose	+		abef	9	**f**	be	+		ef	+	**e**		+	**f**		
Trehalose	-		**abf**	9	**f**	b	-		**f**	+			+	**f**		
Xylitol	+	**df**	c	8/9	**df**		+	**df**		+			+	**f**		
Xylose	-	cdef	**ab**	-	df	**bcef**	-	def		-		**e**	-		**f**	
Xylulose	+			7/9			+			+			+			

[a]Genome based carbon source utilization. + indicates this organism is able to ferment this carbon source, – indicates the organism is unable to ferment this carbon source based on KEGG map analysis. For *P. frisingensis*, the number of strains containing all required genes for the utilization of this compound is given.

[b]Based on experimental findings from literature. Letters in column Lit + indicate the publication reports fermentation of this carbon source, letters in the column Lit – indicate the publication reports this carbon source is not fermented. Publications used are a (Lee et al., 1978), b (Haikara et al., 1981), c (Schleifer et al., 1990), d (Juvonen and Suihko, 2006), e (Zhang et al., 2012) and f (Caldwell et al., 2013). Letters in bold with a dark background indicate these publications and the genome are in agreement, a light background and non-bold letters indicate a discrepancy between the publication and the genome.

Fig. 1

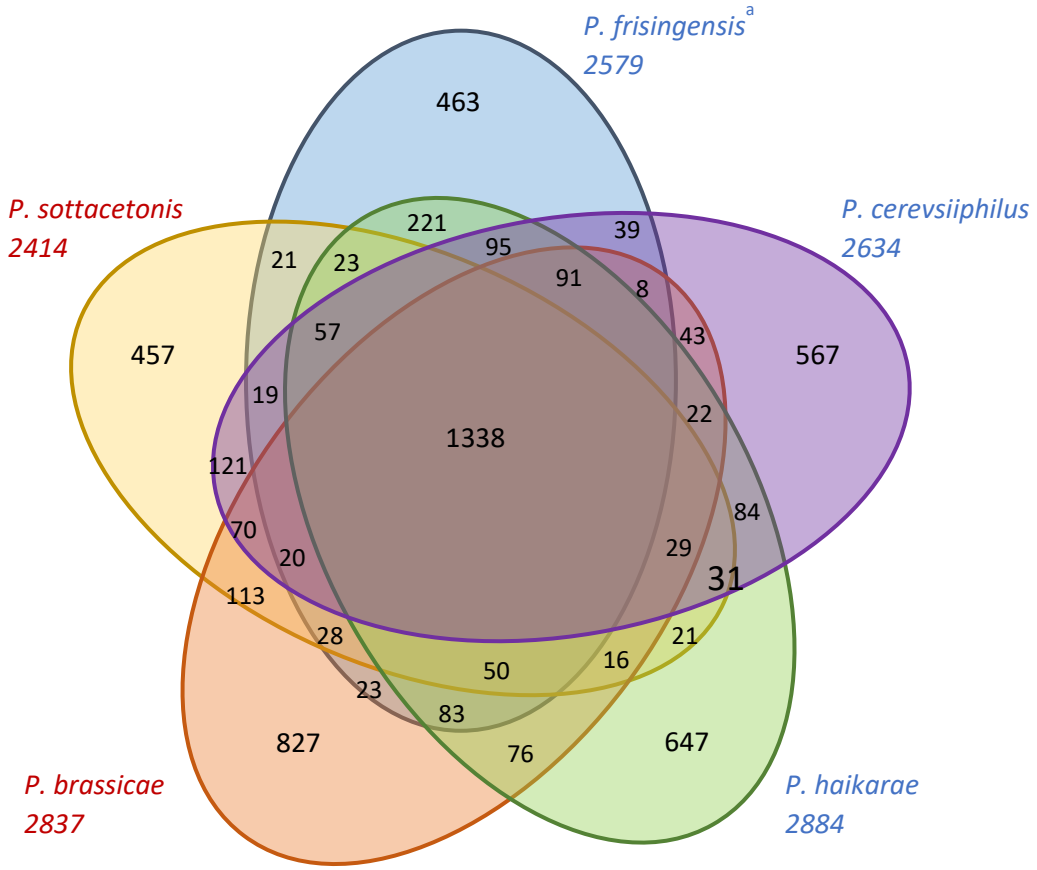

P. frisingensis[a]
2579
463
P. sottacetonis
2414
P. cerevsiiphilus
2634
221
95
39
21
23
91
8
57
43
457
567
19
22
1338
121
84
70
20
29
31
113
28
21
50
16
23
83
827
76
647
P. brassicae
2837
P. haikarae
2884

Fig. 2

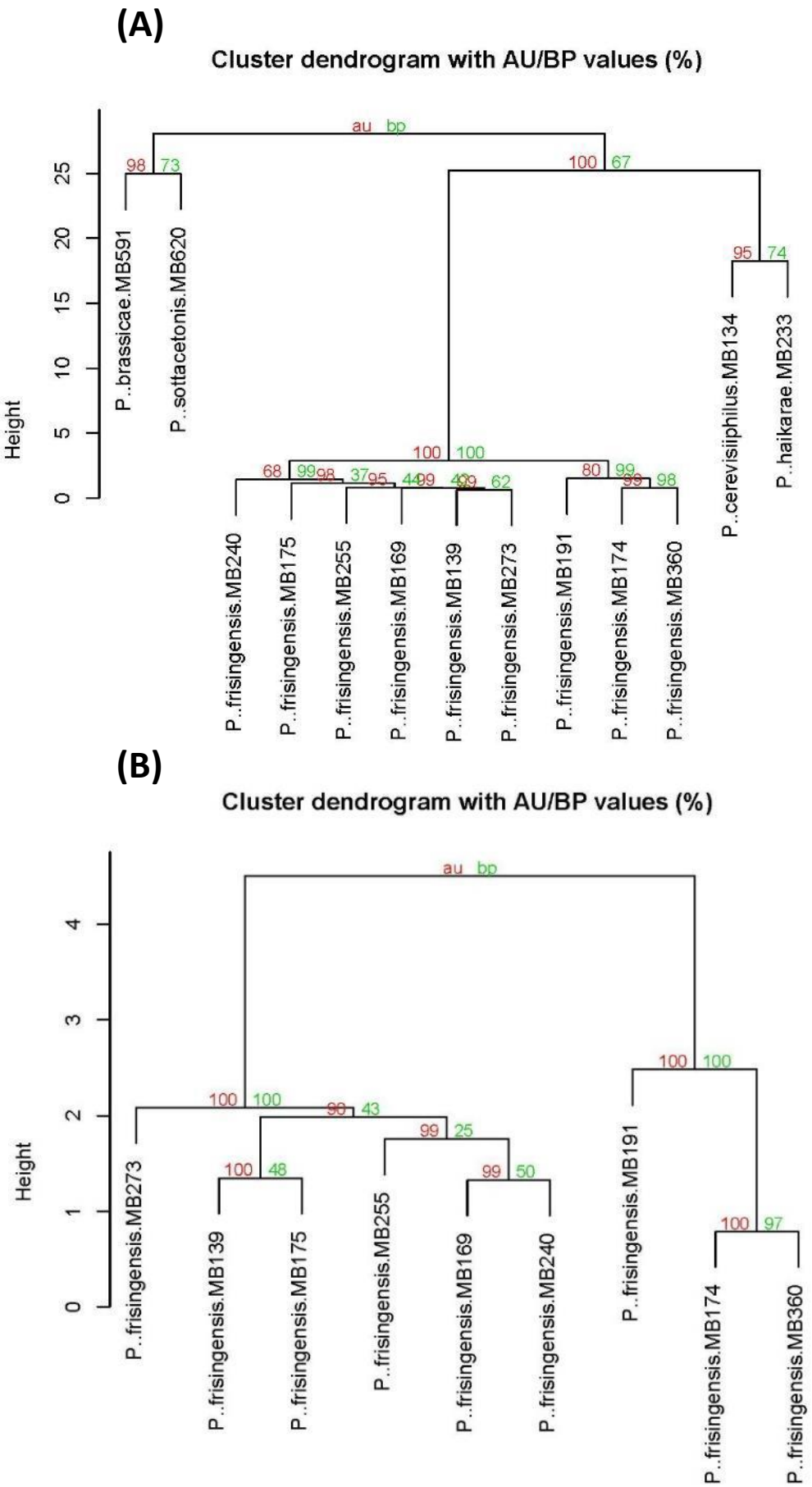

(A)
Cluster dendrogram with AU/BP values (%)
au bp
Height
0
5
10
15
20
25
98 73
100 67
95 74
100 100
68 99 98 37 95 44 99 62
80 99 99 98
P..brassicae.MB591
P..sottacetonis.MB620
P..cerevisiiphilus.MB134
P..haikarae.MB233
P..frisingensis.MB240
P..frisingensis.MB175
P..frisingensis.MB255
P..frisingensis.MB169
P..frisingensis.MB139
P..frisingensis.MB273
P..frisingensis.MB191
P..frisingensis.MB174
P..frisingensis.MB360
(B)
Cluster dendrogram with AU/BP values (%)
au bp
Height
0
1
2
3
4
100 100
100 100
99 43
99 25
100 48
99 50
100 100
100 97
P..frisingensis.MB273
P..frisingensis.MB139
P..frisingensis.MB175
P..frisingensis.MB255
P..frisingensis.MB169
P..frisingensis.MB240
P..frisingensis.MB191
P..frisingensis.MB174
P..frisingensis.MB360

Fig. 3

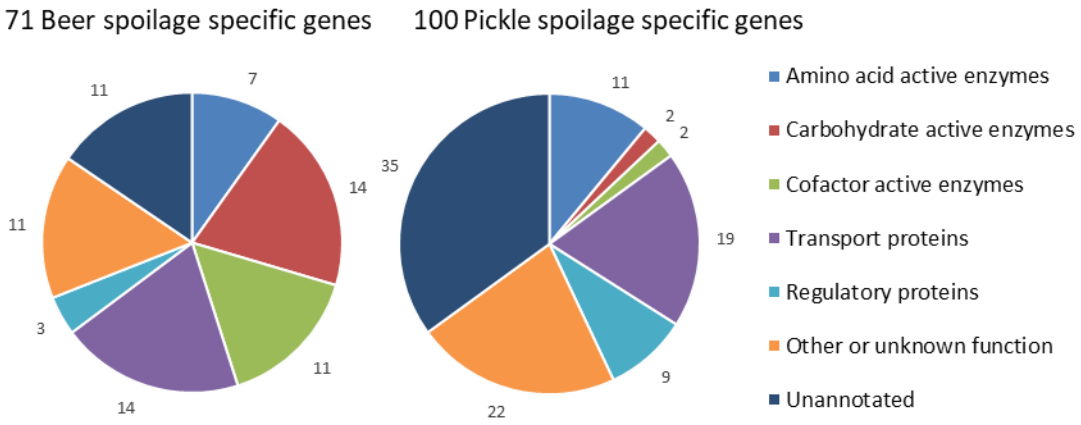
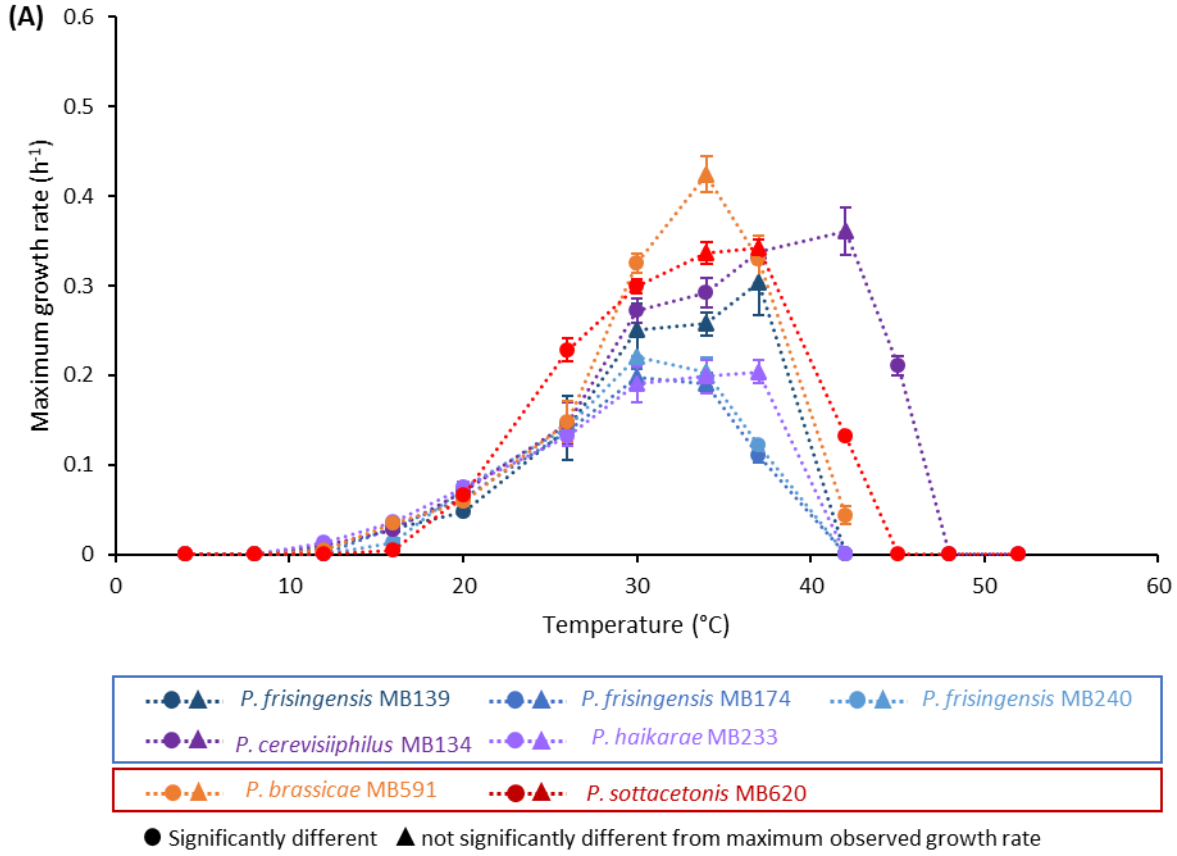

71 Beer spoilage specific genes
100 Pickle spoilage specific genes
7
14
11
14
3
11
11
11
2
2
19
9
22
35
Amino acid active enzymes
Carbohydrate active enzymes
Cofactor active enzymes
Transport proteins
Regulatory proteins
Other or unknown function
Unannotated

Fig. 4


(A)
Maximum growth rate (h-1)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
Temperature (°C)
P. frisingensis MB139
P. frisingensis MB174
P. frisingensis MB240
P. cerevisiiphilus MB134
P. haikarae MB233
P. brassicae MB591
P. sottacetonis MB620
Significantly different
not significantly different from maximum observed growth rate

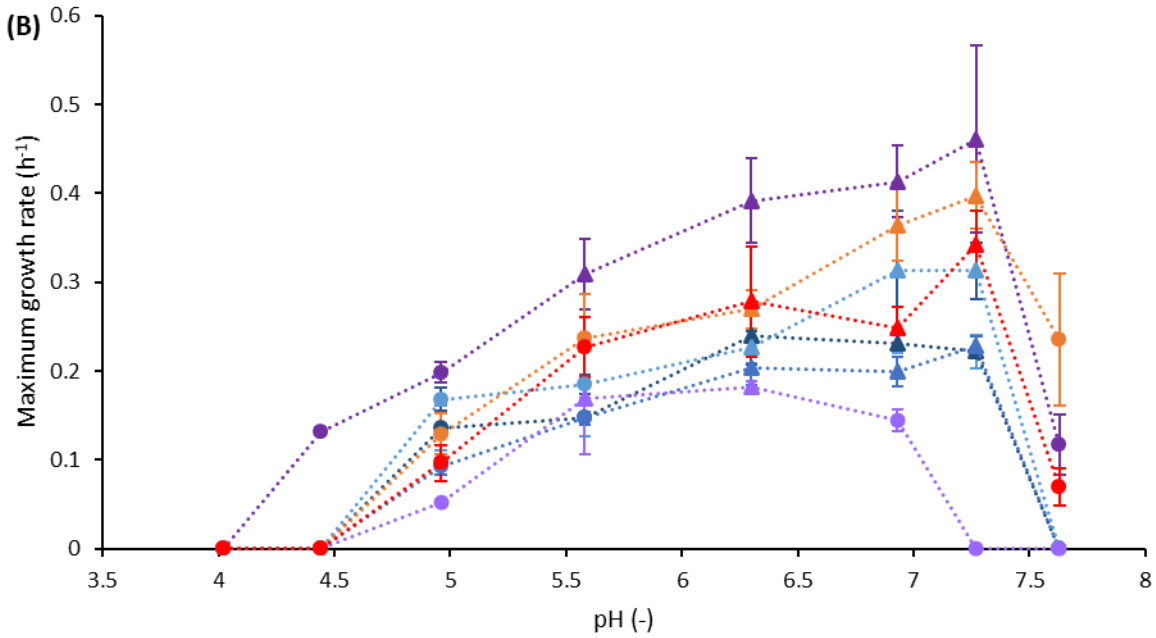

(B)
Maximum growth rate (h-1)
0.6
0.5
0.4
0.3
0.2
0.1
0
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
pH (-)

Fig. 5

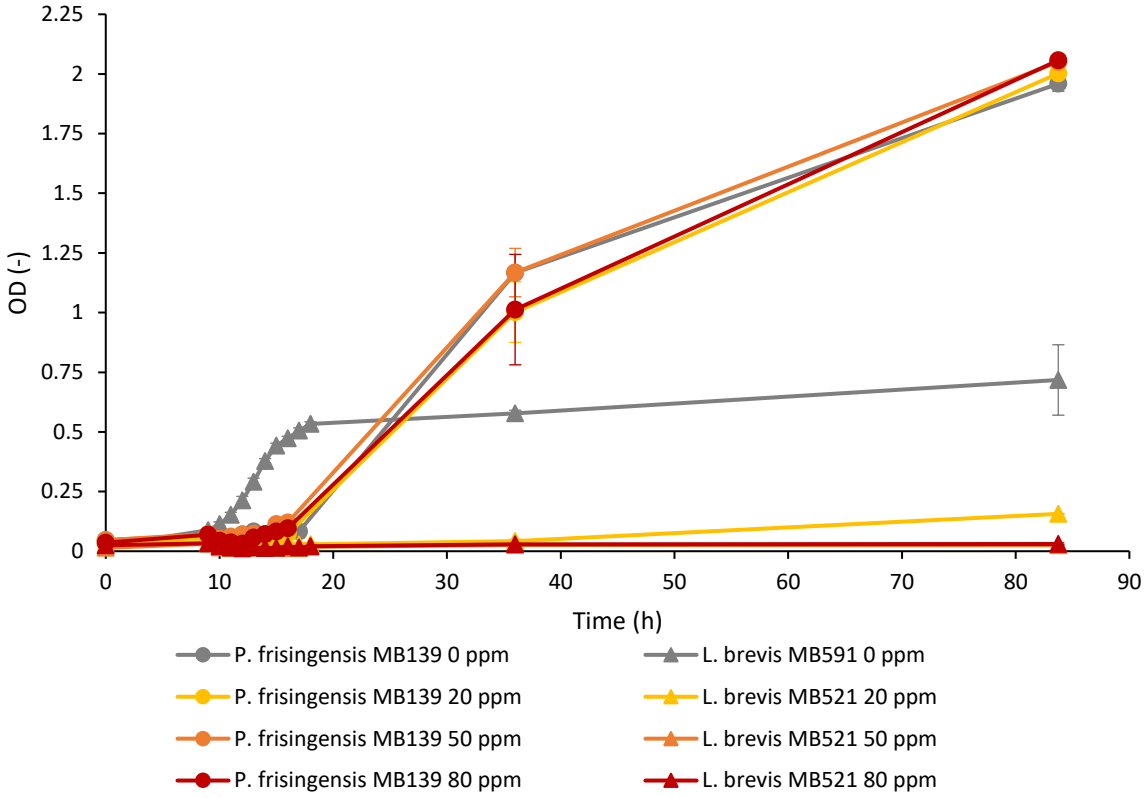

2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
OD (-)
0
10
20
30
40
50
60
70
80
90
Time (h)
P. frisingensis MB139 0 ppm
P. frisingensis MB139 20 ppm
P. frisingensis MB139 50 ppm
P. frisingensis MB139 80 ppm
L. brevis MB591 0 ppm
L. brevis MB521 20 ppm
L. brevis MB521 50 ppm
L. brevis MB521 80 ppm

Fig. 6

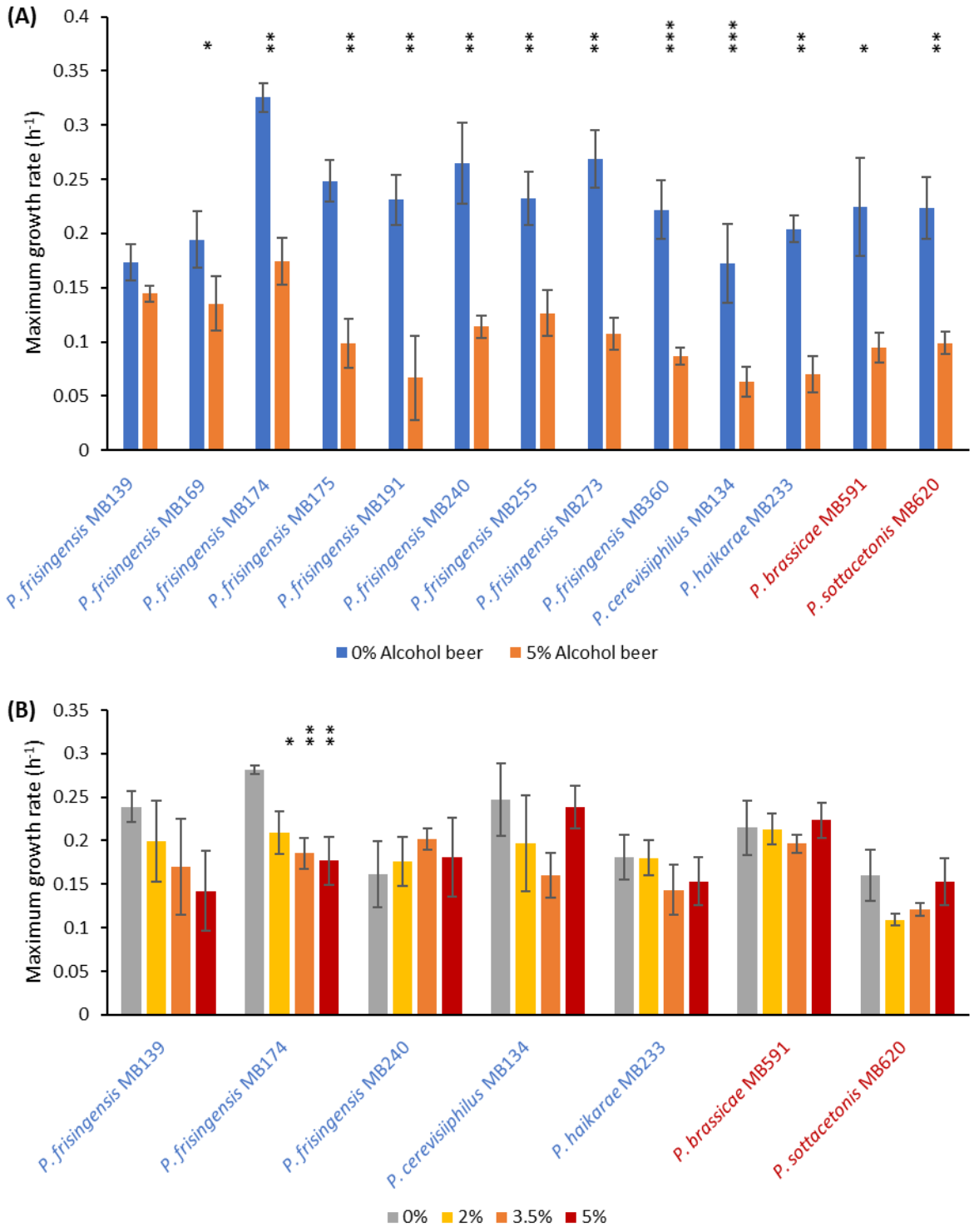
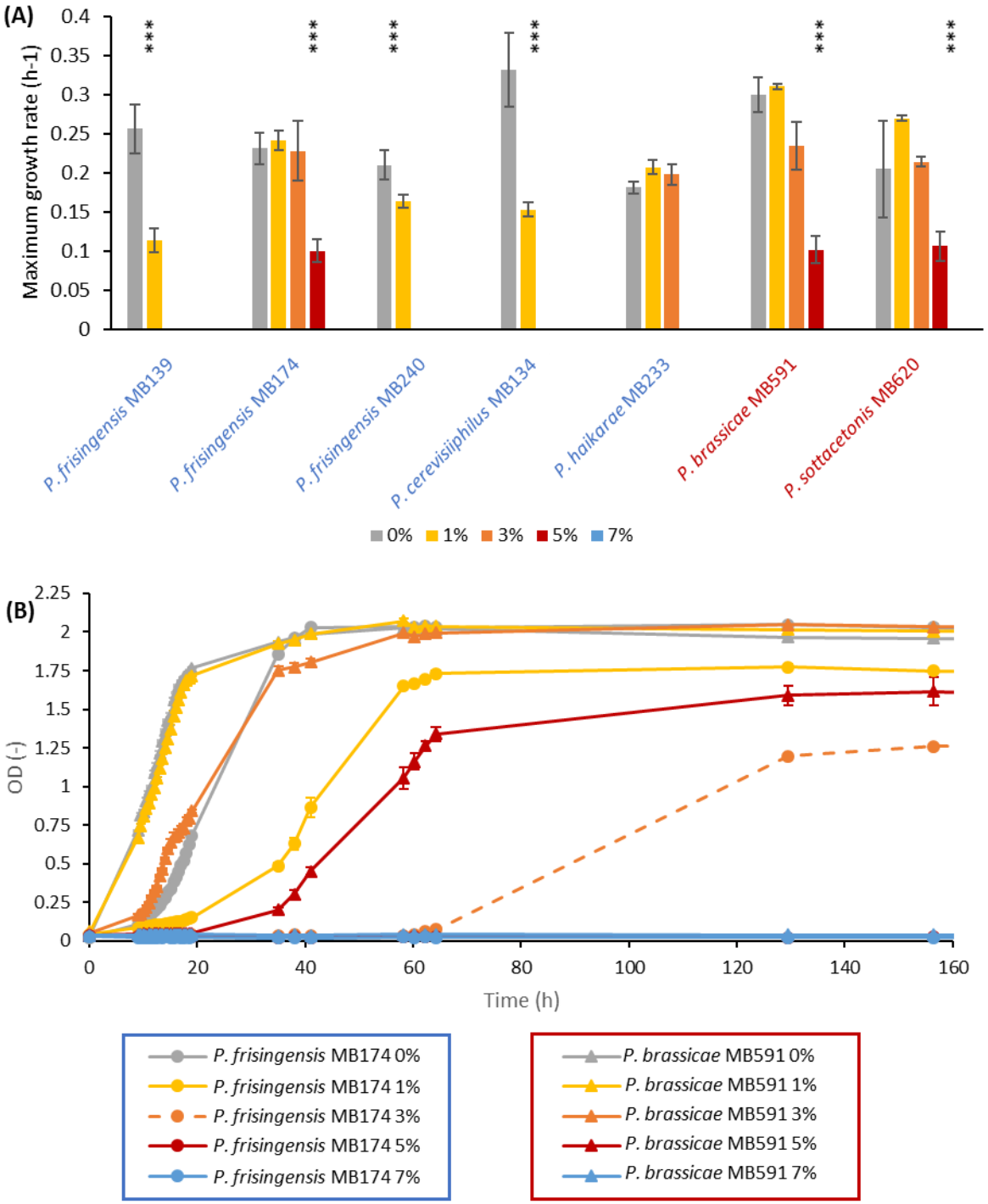

(A)
Maximum growth rate (h-1)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
P. frisingensis MB139
P. frisingensis MB169
P. frisingensis MB174
P. frisingensis MB175
P. frisingensis MB191
P. frisingensis MB240
P. frisingensis MB255
P. frisingensis MB273
P. frisingensis MB360
P. cerevisiiphilus MB134
P. haikarae MB233
P. brassicae MB591
P. sottacetonis MB620
0% Alcohol beer
5% Alcohol beer
(B)
Maximum growth rate (h-1)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
P. frisingensis MB139
P. frisingensis MB174
P. frisingensis MB240
P. cerevisiiphilus MB134
P. haikarae MB233
P. brassicae MB591
P. sottacetonis MB620
0%
2%
3.5%
5%

Fig. 7


(A)
Maximum growth rate (h-1)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
***
P. frisingensis MB139
P. frisingensis MB174
P. frisingensis MB240
P. cerevisiiphilus MB134
P. haikarae MB233
P. brassicae MB591
P. sottacetonis MB620
0%
1%
3%
5%
7%
(B)
OD (-)
2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
0
20
40
60
80
100
120
140
160
Time (h)
P. frisingensis MB174 0%
P. frisingensis MB174 1%
P. frisingensis MB174 3%
P. frisingensis MB174 5%
P. frisingensis MB174 7%
P. brassicae MB591 0%
P. brassicae MB591 1%
P. brassicae MB591 3%
P. brassicae MB591 5%
P. brassicae MB591 7%