Identification of beer spoilage microorganisms using the MALDI Biotyper platform

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ABSTRACT

Beer spoilage microorganisms present a major risk for the brewing industry and can lead to cost intensive recall of contaminated products and damage to brand reputation. The applicability of molecular profiling using matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in combination with Biotyper software was investigated for the identification of beer spoilage microorganisms from routine brewery quality control samples. Reference mass spectrum profiles for three of the most common bacterial beer spoilage microorganisms (*Lactobacillus lindneri*, *Lactobacillus brevis* and *Pediococcus damnosus*), four commercially-available brewing yeast strains (top- and bottom-fermenting) and *Dekkera/Brettanomyces bruxellensis* wild yeast were established, incorporated into the Biotyper reference library and validated by successful identification after inoculation into beer. Each bacterial species could be accurately identified and distinguished from one another, and from over 5,600 other microorganisms present in the Biotyper database. In addition, wild yeast contaminations were rapidly detected and distinguished from top- and bottom-fermenting brewing strains. The applicability and integration of

mass spectrometry profiling using the Biotyper platform into existing brewery quality assurance practices within industry was assessed by analysing routine microbiology control samples from a local brewery, where contaminating microorganisms could be reliably identified. Brewery-isolated microorganisms not present in the Biotyper database were further analysed for identification using LC-MS/MS methods. This renders the Biotyper platform a promising candidate for biological quality control testing within the brewing industry as a more rapid, high-throughput and cost effective technology that can be tailored for the detection of brewery-specific spoilage organisms from the local environment.

Keywords: beer spoilage microorganisms, Biotyper, quality control, mass spectrometry, MALDI

INTRODUCTION

Accurate and reliable quality control methods for the early detection and rapid identification of beer spoilage microorganisms are vital for breweries to monitor batch quality. Without effective measures, the recall of contaminated products is not only a monetary burden but also damaging to brand reputation. Current microorganism detection procedures for bacterial and wild yeast contamination involve classical cultivation-based enrichment and optical examination in addition to more recent molecular methods such as polymerase chain reaction (PCR) (Fujii et al. 2005; Hayashi et al. 2001; Iijima et al. 2008; Juvonen et al. 2008; Pfannebecker and Fröhlich 2008; Yasui et al. 1997), riboprinting (Barney et al. 2001; Koivula et al. 2006), rRNA hybridisation (Huhtamella et al. 2007; Weber et al. 2008) and antibody-based techniques (March et al. 2005; Whiting et al. 1999). However, classical methods require specialist technicians for visual examination and are prone to misidentifications (Back 2006), while molecular methods like PCR are cost intensive. An alternative approach to identify microorganisms is proteomic fingerprinting or ‘bio-typing’, which is based on the acquisition of a mass spectrum from the microorganism (Holland et al. 1996). This spectrum is

obtained predominantly from cytosolic ribosomal proteins (Arnold and Reilly 1999; Sato et al. 2011; Teramoto et al. 2007), though further signals can be assigned to proteins involved in metabolism and cell division such as RNA chaperones, DNA-binding proteins and cold shock proteins (Dieckmann et al. 2010; Ryzhov and Fenselau 2001). Despite strong evolutionary conservation within a genus, the spectra generated from ribosomal protein extracts display slight variations as a result of amino acid sequence divergence at the species level (Fagerquist et al. 2006). Moreover, due to the high abundance of ribosomal proteins and RNA chaperones within cells, the mass spectrum profile of a microorganism is relatively stable and largely independent of growth conditions (Valentine et al. 2005; Wunschel et al. 2005a) and technical acquisition factors such as instrumentation, amount of biomass per sample and type of matrix employed (Wunschel et al. 2005b). The Biotyper platform, applying this principle, has recently received 510(k) clearance by the US Food and Drug Administration for the clinical use of specimen processing methods (Sepsityper), MALDI Biotyper library and analysis software. This clearance is based on a multi-site hospital clinical trial where the performance of the Biotyper platform was assessed and compared with molecular sequencing (http://www.accessdata.fda.gov/cdrh_docs/pdf14/k142677.pdf). It was found that Biotyper analyses correctly identified 98.9% of isolates to the genus or species level where only 0.9% of isolates were unable to be identified, results that were consistent with molecular sequencing of ribosomal components and represented the highest identification accuracy for any mass spectrometry-based bacterial and yeast ID system to date (Mellmann et al. 2008). Furthermore, high inter-laboratory reproducibility was achieved (Mellmann et al. 2009). Biotyping is currently utilised in clinical settings (Carbonnelle et al. 2011; Saffert et al. 2011; Schmitt et al. 2013) and the food industry for the identification of microorganism-related infections (Andres-Barrao et al. 2013; Duskova et al. 2012). At time of writing, the Biotyper library covered 5,643 microorganisms. Additionally, own database entries from regional isolates can be established.

The detection and identification of beer spoilage microorganisms using the MALDI Biotyper platform therefore has potential to be developed into a robust, high-throughput, cost and time

effective method for quality control testing within the brewing industry (Kern et al. 2014; Schurr et al. 2015; Wieme et al. 2014). With the inclusion of mass spectrum profiles for common beer spoilage bacteria and yeast species into the Biotyper library, these contaminants can be identified from brewery batch processing samples using MALDI-TOF MS. In this study, mass spectrum profile (MSP) reference spectra were created for three of the most common facultative anaerobic beer spoilage bacterial species (*Lactobacillus lindneri*, *Lactobacillus brevis* and *Pediococcus damnosus* (Hutzler 2013), two strains of wild yeast (*Dekkera/Brettanomyces bruxellensis* and a *Dekkera/Brettanomyces* isolate from brewing production), in addition to four commercially-available brewing yeasts (top- and bottom-fermenting). Method validation was achieved by inoculating microorganisms into beer samples, then employing the MALDI Biotyper software and analysis platform to successfully identify the microorganisms by matching generated sample spectra against the combined library database and the in-house established reference spectra. This was further extended to assess the Biotyper platform for industrial application through the analysis of samples from a brewery environment where wild yeast, bacteria and fungi could be successfully detected and identified.

MATERIALS AND METHODS

Yeast and bacterial strains

Liquid yeasts Munich Lager (Wyeast 2308), Czech Pils (Wyeast 2278), Kölsch (Wyeast 2565), Weihenstephan Weizen (Wyeast 3068), wild yeast *Brettanomyces bruxellensis* (Wyeast 5112) (Wyeast, Odell, Oregon, USA) were purchased from Beerbelly Brewing Equipment (Adelaide, Australia) and cultured in NBB®-B Bouillon growth medium (Doehler GmbH, Darmstadt, Germany) at 27 ºC. Facultative anaerobic beer spoilage microorganisms *Lactobacillus lindneri* (DSM20690), *Lactobacillus brevis* (DSM20054) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany), while *Pediococcus damnosus* (Wyeast 5733)

was purchased from Beerbelly Brewing Equipment, and cultured in NBB®-B Bouillon growth medium at 27 ºC. Streak plates were made utilising NBB®-A Agar (Doehler GmbH) and were incubated at 27 ºC.

Brewery provided samples

Brewery quality control samples were collected and provided by Coopers Brewery Ltd., Adelaide, Australia. Samples consisted of streak / spread agar plates and filtration membranes on agar and were sourced from beer production processes and equipment.

Protein extraction

Proteins for MALDI Biotyper analyses were extracted from yeast or bacterial colonies grown on NBB®-A Agar, cultured in NBB®-B broths, from inoculated beer samples or from brewery provided samples. Large single agar colonies (approximately 10^6 cells)(or at least 5 x 10^4 cells in the case of small colonies from brewery provided agar plates) were harvested into 1 ml water and centrifuged for 5 min at 3,300 × g. Liquid cultures were established by inoculation of a single colony into 1 ml NBB®-B broth and incubation overnight at 27 ºC. 1 ml liquid cultures (approximately 10^6 cells/mL) were centrifuged for 5 min at 3,300 × g. Samples were washed three times in 400 µl 75% (v/v) ethanol (Merck, Darmstadt, Germany) by resuspension and centrifugation (5 min, 3,300 × g) and allowed to partially dry at room temperature for 5 min to remove residual ethanol. Pellets were resuspended in 30 µl 70% (v/v) formic acid (Sigma-Aldrich, St. Louis, USA), then 30 µl 100% acetonitrile (Merck, Darmstadt, Germany) was added and samples were mixed well. HPLC grade reagents were used. Samples were centrifuged at 20,000 × g for 5 min and cleared protein lysates (supernatant) were transferred to fresh tubes for spotting onto a MALDI target plate and storage of remaining sample at 4 °C.

MALDI-TOF MS

Protein samples extracted from yeast and bacterial samples were spotted onto an MTP 384 steel BC target plate (Bruker Daltonik, Bremen, Germany) for acquisition and analysis using an ultrafleXtreme MALDI-TOF/TOF MS instrument (Bruker Daltonik). 2 µl protein sample was spotted onto a target spot, allowed to dry, then 2 µl alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix (10 mg/ml HCCA (Bruker Daltonik) in 70% (v/v) acetonitrile (Merck), 0.1% (v/v) trifluoroacetic acid (Merck)) was overlaid and allowed to crystalise. Bacterial Test Standard (Bruker Daltonik) was used as an external calibrant and prepared according to manufacturer's protocol. Acquisition was conducted according to the manufacturer provided Biotyper standard procedure in the m/z range from 2,000 to 20,000 with variable laser power in linear positive mode. 200 single laser shots were accumulated and this spectrum was checked if the masses between m/z 4,000 to 10,000 had a resolution higher than 400. When the resolution was above 400, this spectrum was accumulated into a sum spectrum until a total of six spectra (6 × 200 single laser shots) were accumulated.

Biotyper MSP creation

Twenty biological replicates of each microorganism were grown and their proteins extracted as described above. Each extract was spotted on a MALDI target plate, resulting in twenty acquisition points representing the twenty biological replicates. Two sum spectra per biological replicate were acquired as described above, resulting in 40 distinct sum spectra of the respective yeast and bacterial strain. MSPs for each microorganism were created from their respective 40 sum spectra, using the MALDI Biotyper software (version 3.1.66; Bruker Daltonik) and incorporated into the local Biotyper MSP organism database library. A separate MSP for each growth method (agar plate and broth culture) was created. A workflow for Biotyper MSP creation is presented in Fig. 1.

Biotyper identification from spiked beer samples

Microorganisms were spiked into an American pale lager style beer at 10^5 cfu / 100 ml and incubated at 27 ºC for 48 hours. Cultured yeast or bacteria were isolated using 2 methods; either harvested directly from 100 ml spiked beer by centrifugation at 3,300 × g for 10 min; or harvested by membrane

filtration of 100 ml using a 0.45 µm pore membrane (PALL Corporation, Ann Arbor, MI, USA), which was subsequently placed onto an NBB®-A Agar plate and incubated for 24-48 hours at 27 °C. Proteins were extracted from isolated microorganism samples according to the ethanol/formic acid extraction method, then samples were spotted as four technical replicates onto a MALDI target plate and analysed by MALDI-TOF MS, as described above. Spectra were loaded into the Biotyper software and identified against the MSP database library (5,643 MSP entries including 16 additional entries of in-house established MSPs representing brewing yeast and beer spoilage microorganisms, refer to Biotyper MSP creation above). Explanation of the Biotyper derived scores as provided by the manufacturer's manual are shown in Table 1. A workflow for Biotyper identification from spiked beer samples is presented in Fig. 1.

Liquid chromatography coupled tandem mass spectrometry (LC-MS/MS)

Microorganisms were harvested from agar plates (one large single colony harvested; approximately 10^6 cells) and proteins were extracted using 200 µl 20% (v/v) trichloroacetic acid (Sigma-Aldrich), while cell disruption and DNA shearing was assisted using a Bioruptor ultrasonic bath (Diagenode, Seraing, Belgium). Following settings were used: Power: high, 30 s continuous treatment followed by 1 min pause for a 10 min cycle. Afterwards the volume was increased to 1 ml with 100% ice-cold acetone (Merck) and stored at -20 °C overnight. Proteins were pelleted by centrifugation (Eppendorf, Hamburg, Germany) at 18,000 × g for 30 min at -9 °C. The pellet was washed twice with 1 ml 80% (v/v) ice-cold acetone. The resulting protein pellet was resuspended in 1% (w/v) sodium dodecyl sulphate (Sigma-Aldrich), 50 mM Tris (Biochemicals, Gymea, Australia), pH 8 and 100 mM dithiothreitol (Sigma-Aldrich), sonicated for 5 min then heated to 56 °C for 20 min followed by 98 °C for 5 min. Tryptic digest was done according to previously published protocols (Wisniewski et al. 2009). Tryptic peptides were resuspended in 2% (v/v) acetonitrile (Merck), 0.1% (v/v) formic acid (Sigma-Aldrich) to a final concentration of 1 µg/µl. LC−MS/MS was performed on an Ultimate 3000 RSLC system (Thermo-Fisher Scientific, Waltham, Massachusetts, USA) coupled to an Impact HD™

Q-TOF mass spectrometer (Bruker Daltonics). One µg of injected peptides were desalted for 10 min using a C18 trapping column (Acclaim PepMap100 C18 75 µm × 20 mm, Thermo-Fisher Scientific), in 2% acetonitrile, 0.1% trifluoroacetic acid at a flow rate of 5 µl/min. Peptides were separated by a 75 µm inner diameter C18 column (Acclaim PepMap100 C18 75 µm × 50 cm, Thermo-Fisher Scientific) applying a linear gradient from 5 to 45% B (A: 5% (v/v) acetonitrile 0.1% (v/v) formic acid, B: 98% (v/v) acetonitrile 0.1% (v/v) formic acid) over 80 min, with a flow rate of 300 nl/min, this was followed by a 20 min column wash step with 90% B, and 20 min equilibration step with 5% A. MS scans were acquired in the mass range of 150 to 2200 m/z, MS/MS was carried out on m/z features picked by the manufacturer's supplied Shotgun Instant Expertise™ algorithm.

LC-MS/MS data analysis

Acquired spectra were processed using Compass DataAnalysis for OTOF (Version 1.7, Bruker Daltonics). Detected compounds were exported as Mascot generic format and submitted to Mascot (Version 2.3.02) for protein identification. Following search parameters were used: NCBInr database (Version 01/04/2015), bacteria and fungi taxonomy (48,735,875 sequences searched), trypsin with up to 2 missed cleavages was specified as protease, fixed modification: carbamidomethylation of cysteine. Oxidation of methionine was set as variable modification; MS mass tolerance was set to 30 ppm, and MS/MS mass tolerance to 0.2 Da. The Mascot standard scoring algorithm in combination with the homology threshold was used to calculate cut-offs for statistical significance of peptide identification. Results were exported as comma separated values; data was analysed using Excel 2010 (Microsoft, Redmond, USA) and R (Version 3.2.2, The R Foundation for Statistical Computing). Identification of microorganisms was based on the number of top-scoring proteins (as by Mascot derived "total ions score", from individual protein families) associated with a unique microorganism.

RESULTS

In order to develop the MALDI Biotyper platform for the detection and identification of spoilage microorganisms from brewery process samples, MSPs for common beer spoilage bacteria, wild yeast and brewing yeast strains were created and incorporated into the local Biotyper MSP library database. Commercially-available brewing strains Munich Lager, Czech Pils, Kölsch and Weihenstephan Weizen were chosen to represent two bottom-fermenting and two top-fermenting yeast strains, respectively, in addition to a commercially-available strain of wild yeast, *D./B. bruxellensis*. Bacterial strains *L. lindneri*, *L. brevis* and *P. damnosus* were chosen as they represent three of the most common beer spoilage bacteria (Hutzler 2013), accounting for more than 75% of consumer complaints relating to the brewing industry (Back 1994). Twenty biological replicates were selected for analysis from each culture method (growth on agar; growth in broth) (refer to workflow in Fig. 1 (a)). Proteins were extracted, spotted onto a MALDI target plate and two sum spectra were acquired from each biological replicate giving a total of 40 individual sum spectra consisting of 1,200 single spectra each. These sum spectra were processed using the Biotyper software to generate a single MSP for each microorganism (for each growth method). MSPs for brewing yeasts, wild yeast and bacterial spoilage microorganisms were incorporated into the local MSP library that, after inclusion, consisted of 5,643 database entries across bacterial, fungal and mould species. Representative spectra from yeast and bacterial strains analysed are presented in Fig. 2.

The following experimental series was designed to provide proof-of-concept via the identification of brewing-related microorganisms from spiked beer samples using the Biotyper analysis and software platform. Bacterial and yeast strains used to establish newly-generated MSPs were inoculated into an American pale lager style beer and incubated in the bottle, to emulate typical secondary contaminations at the bottle filling stage of brewery production. Microorganisms were then harvested from the beer by two parallel methods: 1) by direct centrifugation and 2) by membrane filtration and cultivation on nutrient agar (refer to workflow in Fig. 1 (b)). Protein extracts from harvested cells

were spotted as four technical replicates onto the target plate, analysed by MALDI-TOF mass spectrometry and matched against the Biotyper MSP library database. The performance of Biotyper identification for yeast and bacterial species is presented in Table 2. Contaminating beer spoilage microorganisms could be readily identified; for example, *D./B. bruxellensis* wild yeast contamination could be identified with 100% accuracy. Additionally, spoilage bacteria from multiple species were identified with 100% accuracy, exhibiting Biotyper scores indicating secure genus and probable species identification. Moreover, top-fermenting yeasts such as Kölsch and Weihenstephan Weizen could be distinguished from bottom-fermenting Lager and Pils strains (100% accuracy). However, within the bottom-fermenting group of yeasts, distinction between Munich Lager and Czech Pils strains was less accurate (68% accuracy), as shown in Table 2.

To demonstrate relevance to industry application, wild yeast, bacterial contaminations and/or other unknown contaminations would need to be detected and identified from brewery process samples. In order to assess the feasibility and accuracy of the Biotyper platform for this application, biological quality control samples exhibiting microorganism growth were sourced from a local brewery for analysis. Samples with uncharacterised microbial and fungal growth were provided in the form of streak and spread agar plates, agar plates with membrane filters from brewing process or equipment samples. Plates were visually assessed and each distinct growth type was sampled for Biotyper analysis according to pre-established methods (refer to Fig. 1 (c)). Sample descriptions, Biotyper identification results and consistency of identification as the top-ranking score from 5 technical replicates (performance) are shown in Table 3. In addition to brewing yeast, which was identified with scores representing secure genus identification and highly probable species identification, 9 bacterial species and 8 yeast species were identified, including an isolate of *D./B. bruxellensis* wild yeast.

Representative spectra for bacterial and yeast species identified from brewery process samples are depicted in Fig. 3 (a). Several samples isolated from membrane filtration of production process samples were shown to produce distinct spectra that could not be identified by the Biotyper software

(refer to Table 3; samples from plates 4, 5, 11, 12 and 13). Further analysis of these samples by LC-MS/MS revealed the putative identity of these microorganisms to be *Acidomonas methanolica* (plate 4; small green colonies), an acidophilic facultative methylotrophic bacterium, and predominantly *Enterobacter* sp. Bisph2 (plate 5, 11, 12 and 13; green viscous growth), a species first isolated from soil from Algeria (http://www.ncbi.nlm.nih.gov/bioproject/270819). Representative MALDI-TOF MS spectra for putatively identified *A. methanolica* and *E.* sp. Bisph2 are depicted in Fig. 3 (b). Putative organism identification by LC-MS/MS and Mascot was determined based on the consistent taxonomy assignment of 10 out of 10 identified protein families in the case of *A. methanolica* (data not shown), while *Enterobacter* sp. Bisph2 (from plate 5) was the dominantly assigned organism with 280 unique protein matches, however further matches to other bacteria and yeast indicate a mixture of various microorganisms and a possible explanation for the failure of Biotyper to identify these samples. However, the degree of influence of the non-dominant microorganisms onto the derived spectra was not assessed. In total, four phenotypically similar samples (green viscous growth; plates 5, 11, 12 and 13) sourced from independent, brewery-derived membrane filter agar plates were analysed by Biotyper. All four independent samples were found to possess similar mass spectrum patterns, depicted in Fig. S1 in the Supplementary Material, and could not be identified using the current Biotyper database. Consistent with the high similarity of their MALDI-TOF MS spectra, each of these samples was subsequently identified by LC-MS/MS as dominantly containing *Enterobacter* sp. Bisph2, as well as a set of additional microorganisms highly similar to those identified from plate 5, as shown in Fig. S2 in the Supplementary Material**.**

Interestingly, a brewery isolate of wild yeast (as shown in Table 3; plate 7) was identified where it was noticed that although attributed to *D./B. bruxellensis* with scores representing secure genus identification and highly probable species identification for 4 of 5 technical replicates (average score 2.354), the mass spectrum profile showed small deviations from the commercially-available strain, as shown in Fig. 4 (a). Consistent with this, when analysed with the inclusion of an MSP generated from this brewery-specific isolate, the brewery wild yeast could be identified with an improved

293 average score of 2.416. To investigate the difference in the mean of the two distributions of the scores,
294 8 further biological replicate clones from the agar plate were processed and two spectra from each
295 biological replicate were acquired and scored using Biotyper methods. The arithmetic means of the
296 scores from the two technical replicates per biological replicate were tested using a two-tailed paired
297 student's *t*-test. The probability for the scores of the commercial and brewery-specific isolate being
298 from the same distribution was found to be $p = 6.94*10^{-06}$ (see Fig. 4 (b)), indicating an improved
299 Biotyper score by using the MSPs from in-house derived *D./B. bruxellensis*.
300
301 **DISCUSSION**
302 This study represents, to the best of our knowledge, the first application of the Biotyper platform for
303 the identification of beer spoilage microorganisms in an industry setting. As seen from Table 2 and
304 Table 3, a wide range of microbial contaminations could be easily identified and distinguished from
305 each other and from brewing yeast using the Biotyper database consisting of over 5,643
306 microorganisms. However, within the bottom-fermenting brewing yeast group, distinguishing
307 between different commercial yeast strains, Munich Lager and Czech Pils, proved to be difficult (see
308 Table 2). This could be attributed to the closely-related nature of lager-type *Saccharomyces*
309 *pastorianus* yeast strains, where it has been shown previously that intragroup members of the Saaz
310 or Frohberg sub-types of *S. pastorianus* could not be distinguished by genetic methods (Fernadez-
311 Espinar et al. 2000; Manzano et al. 2004; Pham et al. 2011). MALDI-TOF MS spectra generated from
312 these strains were indistinguishable from one another, resulting in both strains being identified by the
313 Biotyper software with scores in the highest score range (2.3-3.0). Specifically, although Czech Pils
314 isolated from filtered beer was incorrectly identified as Munich Lager as the top scoring
315 microorganism (refer to Table 2), the scores for identification against the Czech Pils MSP were
316 equally within the highest score range (scores 2.527, 2.628, 2.611, 2.597). This leads us to speculate
317 that both Czech Pils and Munich Lager yeast are from the same subgroup of *S. pastorianus*, where
318 the occurrence of different subgroups correlates to geographical location (Dunn and Sherlock 2008).
319 Collectively, these proof-of-concept data from controlled laboratory inoculations provide evidence

that the Biotyper platform is suitable for the detection and identification of beer spoilage microorganisms and brewing yeast strains.

From analysis of brewery production samples sourced from routine industry testing, a number of microorganisms for which MSPs were not established in-house during this study, were identified due to their relevance in human clinical microbiology and were therefore pre-established in the Biotyper MSP database. Of the yeast and bacterial species identified from brewery production and processing samples (Table 3), many are air-borne or environmental contaminants and some have been previously associated with beer spoilage or production contamination. Specifically, *Candida* species (*C. krusei* and *C. inconspicua*) and *Rhodotorula mucilaginosa* are common environmental air-borne contaminants, *Exophiala dermatitidus* is a thermophilic black yeast and *Pichia manshurica* is a member of the *Saccharomycetaceae* family, which is known to interfere with fermentation whilst producing volatile phenols (Saez et al. 2011). *Staphylococcus capitis* and *Staphylococcus hominis* are known human skin-derived bacteria (Kloos and Schleifer 1975). Both species are relevant in the brewing industry, as *S. hominis* was identified earlier by Silvetti *et al.* to occur in bottom-fermented lager beer (Silvetti et al. 2010), while *S. capitis* was identified in traditional indigenous-style beer from South Africa (Lues et al. 2011). *Candida guilliermondii* is the anamorphic form of *Pichia guilliermondii*, a spoilage wild yeast commonly found in beer (Timke et al. 2008; van der Aa Kuhle and Jespersen 1998). *Lactococcus lactis* is a common, potential beer-spoilage bacteria and responsible for approximately 1% of consumer complaints in beer (Back 1994). *Candida pelliculosa* is the teleomorph form of *Pichia anomala*, a routinely encountered wild yeast in the brewing industry (van der Aa Kuhle and Jespersen 1998). *Enterococcus gilvus* (Tyrrell et al. 2002) has previously been identified in meat (Fracalanzza et al. 2007), pasteurised milk (Fracalanzza et al. 2007), fermented sausages (Martin et al. 2009) and cheese (Zago et al. 2009), although it has never been identified in a brewery setting. *Pandoraea apista* was firstly isolated from sputum of cystic fibrosis patients (Coenye et al. 2000) and has never before been described in relationship with beer. However, the identification of both *E. gilvus* and *P. apista* are only putative as the scores derived by Biotyper

analyses are below 2.3 (Table 3), therefore the species level identification would need to be confirmed by additional methods like PCR. Together, these data represent detection and identification of beer spoilage contamination from routine industry samples to a more extensive and greater level of genus and species detail using the Biotyper platform than currently possible for brewery microbiology laboratories using conventional testing methods.

Of note, several samples produced mass spectra that the Biotyper software was not able to assign identity to a respective microorganism (see Plates 4, 5, 11, 12 and 13 in Table 3). We hypothesise that MSPs for these microorganisms were not present within the Biotyper database or consisted of a mixture of microorganisms. This was confirmed by LC-MS/MS analysis of respective samples, where it was shown that these samples consisted dominantly of *A. methanolica* (Plate 4, Table 3) and *Enterobacter* sp. Bisph2 (Plates 5, 11, 12 and 13, Table 3 and Fig. S1), species that were not (at time of writing) included within the pre-established Biotyper database used in this study (version 3.1.66). In order to expand the Biotyper database and allow the rapid identification of isolates such as these additional species, MSP reference spectra of pure isolates should be created for inclusion into the database. This would further allow analysis of the influence of various proportions of microorganisms typically encountered concurrently as biofilm (e.g. *Enterobacteriaceae* (Timke et al. 2005)) onto the resulting mass spectrum and possible identifications of mixtures. Further, as evidenced in Fig. 4 (b), the generation of in-house MSPs for critical spoilage microorganisms could be of advantage**,** leading to higher Biotyper scores and therefore more reliable identifications.

In summary, the major advantages of detection and identification of beer spoilage microorganisms using mass spectrometry within the brewing industry is the high-throughput capacity, simplicity and robustness of the method. However, as biological quality control of brewery production encompasses almost exclusively the detection of trace contaminations, a pre-enrichment of all samples by cultivation on agar plates is necessary to achieve a reasonable sensitivity. This is a pre-requirement for all spoilage detection methods and is established industry practice. However, after

standard cultivation steps, Biotyper sample processing and analysis procedures are both rapid (<30 minutes) and cost effective (low consumables and labor requirements) relative to molecular techniques such as PCR and rRNA-hybridisation. Biotyper analyses can additionally be up-scaled; here, acquisition was performed on 384 sample MALDI target plates and can be automated. Another advantage of the Biotyper platform is the ability to search and identify isolates across an extensive database of microorganisms, providing detailed and informative data. This stands in contrast to assays such as PCR, hybridisation probe- or antibody-based methods, which are target-specific and provide solely binary positive/negative results. On the occasion that an unknown isolate produces a distinct mass spectrum profile, but does not have an entry within the MSP database and can therefore not be identified, the reference database can be readily extended and updated to include newly isolated species. Specifically, the organism can be identified using genetic or proteomic methods such as 16s rRNA molecular sequencing, internal transcribed spacer sequencing or LC-MS/MS methods, then an MSP for the microorganism can be established. Together, this sensitive and rapid method developed with the capacity to establish new reference MSPs from unknown microorganism isolates affirms the Biotyper platform as a robust in-house tool for microorganism identification within brewery quality control practices.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest

The authors declare no conflict of interest.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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572

573

574 **FIGURE LEGENDS**

575

576 **Fig. 1** Sample processing, mass spectrum profile (MSP) creation and microorganism identification
577 methods using MALDI Biotyper**.** ***(a)*** MSPs were created from 40 sum spectra derived from 20
578 biological replicates of microorganisms grown on agar streak plates *(left)* or broth cultures *(right)*
579 using the Bruker Biotyper 3 software; reference MSPs for brewing yeast, wild yeast and beer spoilage
580 bacteria were incorporated into the existing Biotyper MSP library (version 3.1.66). ***(b)***
581 Microorganisms were inoculated into beer samples at 10^5 cells / 100 ml and incubated; cells were
582 harvested by direct centrifugation *(left)* or membrane filtration *(right)* of 100 ml samples.
583 Microorganism protein extracts were analysed by MALDI-TOF MS and identified using Biotyper
584 analysis software. ***(c)*** Microorganism samples were harvested from brewery provided agar plates and
585 protein extracts were analysed by MALDI-TOF MS and identified using Biotyper analysis software

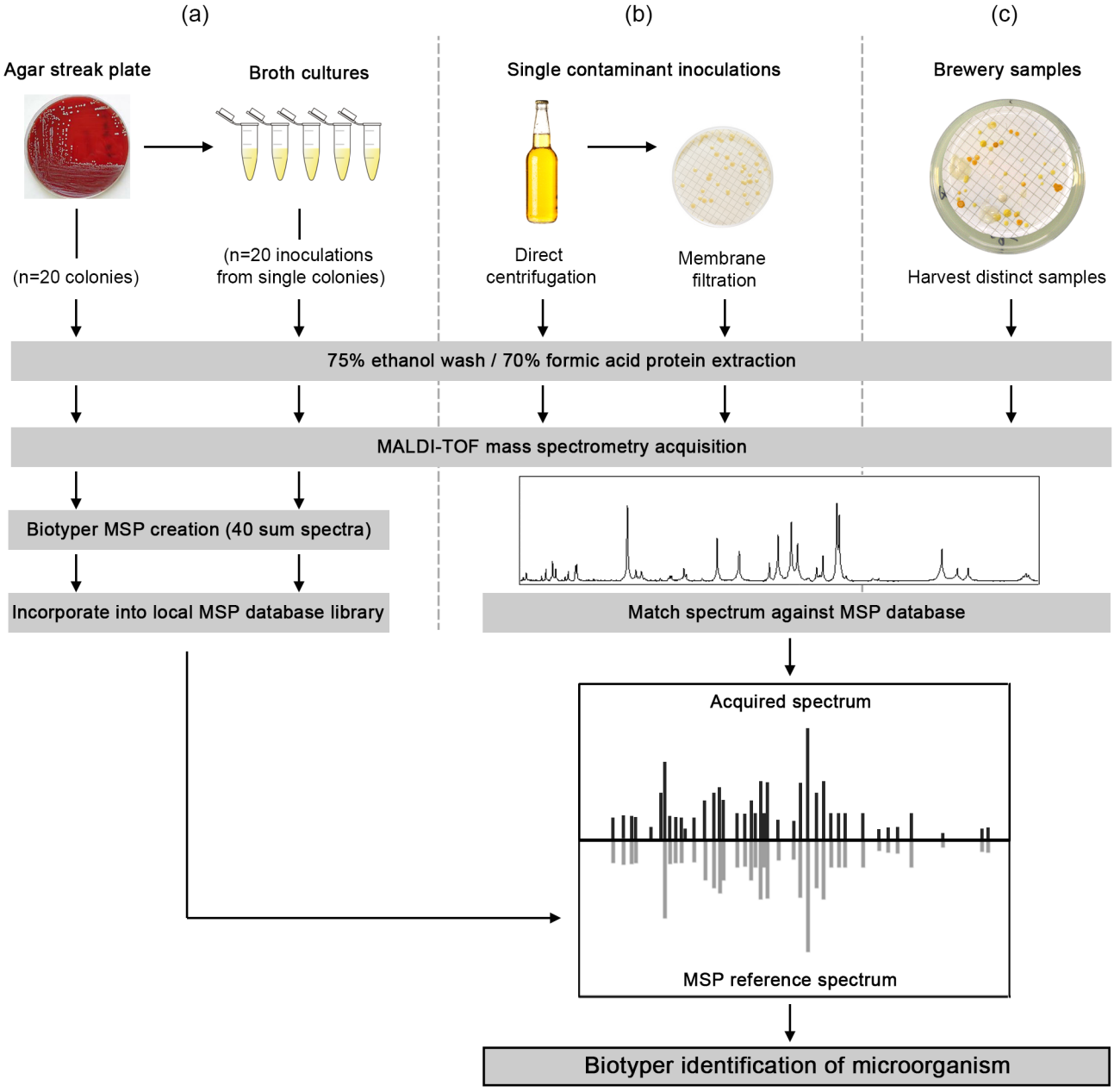
586

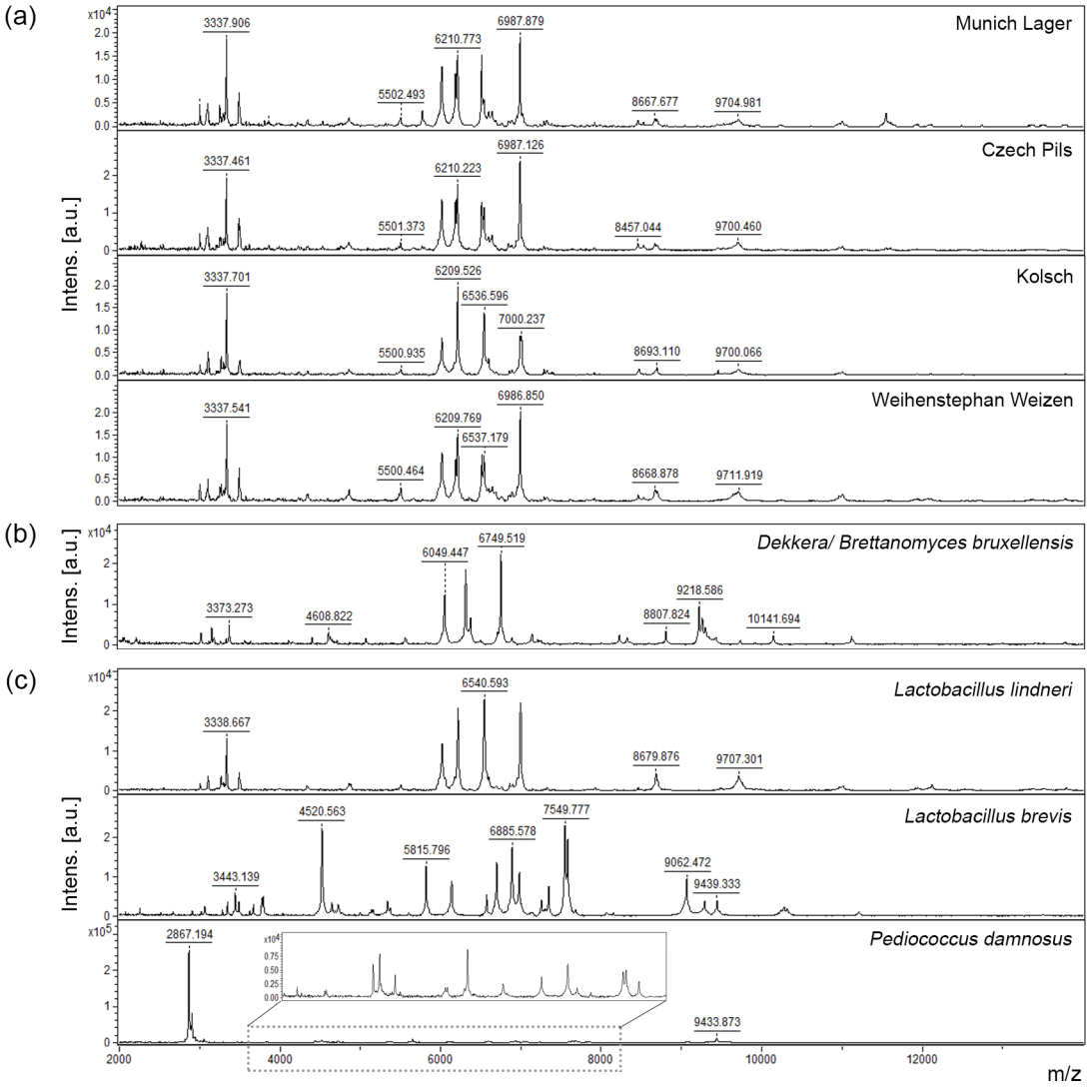
587 **Fig. 2** Representative MALDI-TOF MS spectra for yeast and bacterial strains. 40 distinct sum spectra
588 were acquired for MSP creation; representative spectra for ***(a)*** commercially-available brewing
589 yeasts, ***(b)*** wild yeast and ***(c)*** beer spoilage bacteria. *M/z* values for prominent peaks are displayed;
590 inset in *Pediococcus damnosus* spectrum represents zoomed view

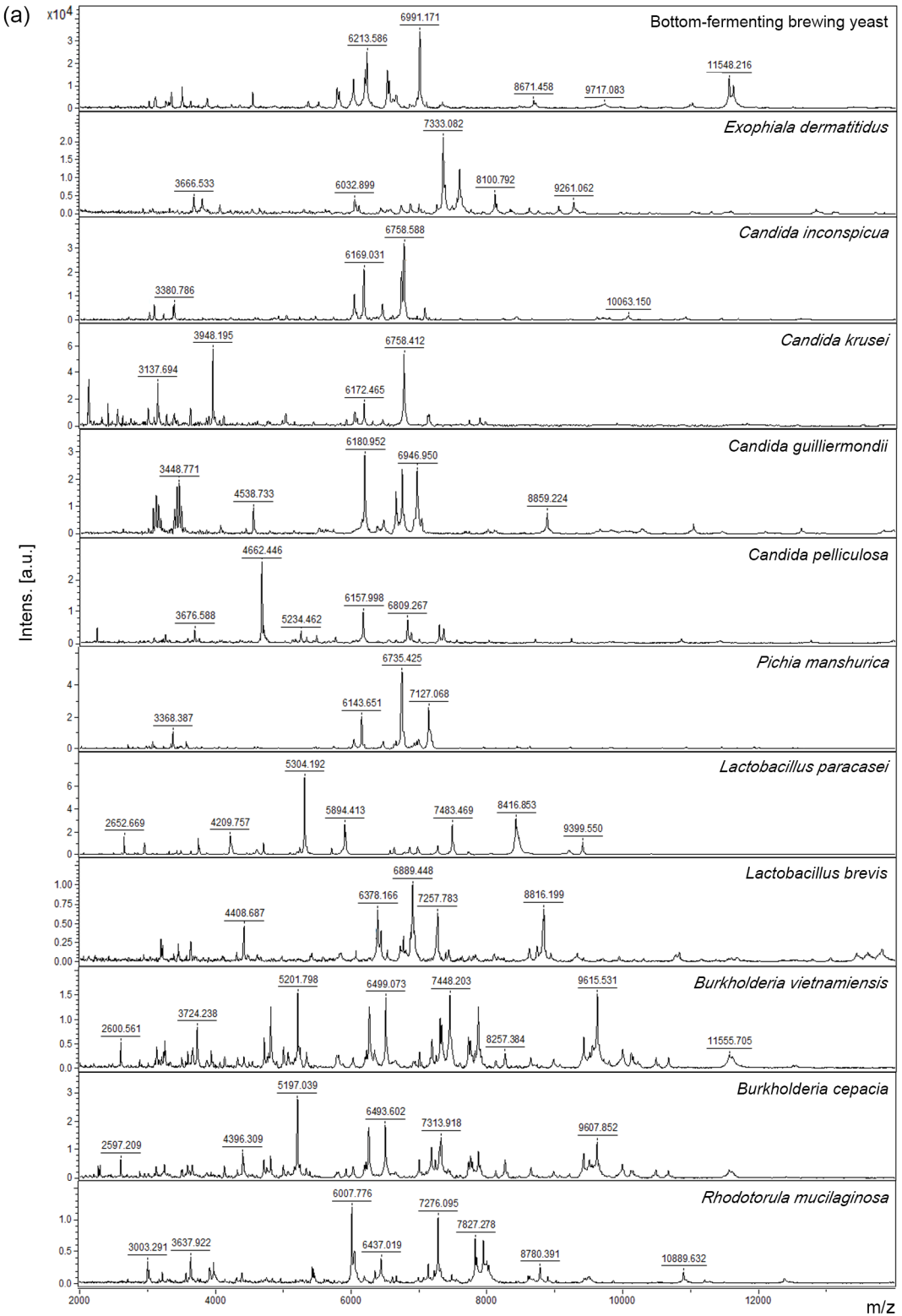
591

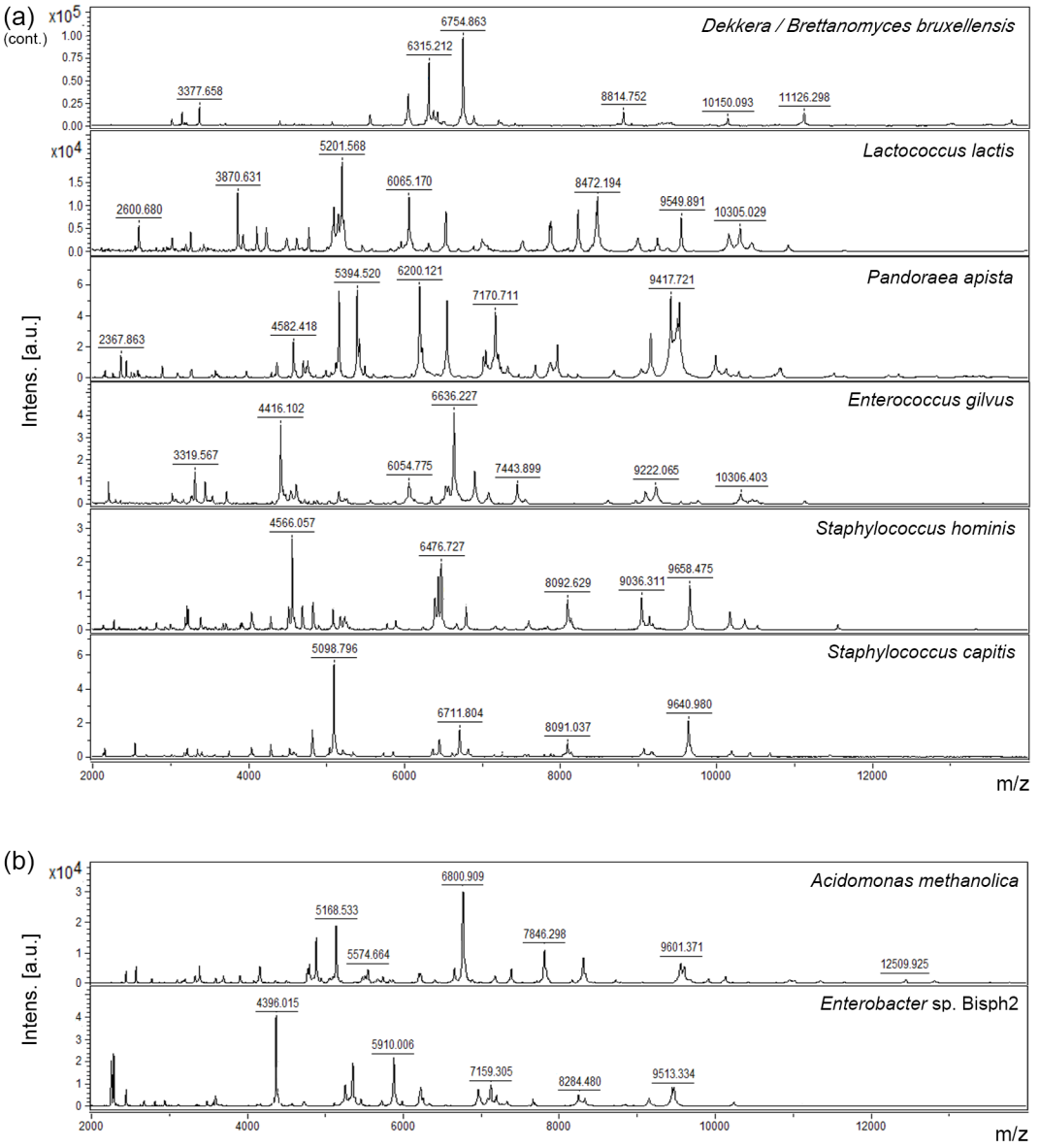
Fig. 3 Representative MALDI-TOF MS spectra for yeast and bacterial strains isolated from brewery process samples. Proteins were extracted from microorganisms grown on streak and spread agar plates, membrane filters cultivated on agar plates or agar plates exposed to the brewery environment. Sum spectra were acquired from 5 technical replicates; representative MALDI-TOF MS spectra for microorganisms are shown, ***(a)*** microorganisms identified by Biotyper ***(b)*** microorganisms without MSPs in Biotyper database putatively identified by LC-MS/MS. *M/z* values for prominent peaks are displayed

Fig. 4 Biotyper analysis of a brewery-specific isolate of *Dekkera/Brettanomyces* wild yeast. *D./B. bruxellensis* strains show slight variation; ***(a)*** Representative mass spectra of commercially-available *D./B. bruxellensis* (*upper panel*) and a Coopers Brewery isolate of *D./B. bruxellensis* (*lower panel*), boxed areas indicate m/z ranges where spectra are distinct. ***(b)*** Biotyper identification scores for 8 biological replicates (2 sum spectra per replicate) of the Coopers Brewery *D./B. bruxellensis* isolate matched against MSPs derived from the commercial strain and brewery-specific strain; two-tailed paired *t*-test, **** $p = 6.94*10^{-06}$, arithmetic mean of Biotyper scores of two technical replicates from 8 biological replicates (16 spectra)


(a)
(b)
(c)
Agar streak plate
Broth cultures
Single contaminant inoculations
Brewery samples
(n=20 colonies)
(n=20 inoculations from single colonies)
Direct centrifugation
Membrane filtration
Harvest distinct samples
75% ethanol wash / 70% formic acid protein extraction
MALDI-TOF mass spectrometry acquisition
Biotyper MSP creation (40 sum spectra)
Incorporate into local MSP database library
Match spectrum against MSP database
Acquired spectrum
MSP reference spectrum
Biotyper identification of microorganism


(a)
Munich Lager
3337.906
6210.773
6987.879
5502.493
8667.677
9704.981
Czech Pils
3337.461
6210.223
6987.126
5501.373
8457.044
9700.460
Kolsch
3337.701
6209.526
6536.596
7000.237
5500.935
8693.110
9700.066
Weihenstephan Weizen
3337.541
6209.769
6537.179
6986.850
5500.464
8668.878
9711.919
Intens. [a.u.]
(b)
Dekkera/ Brettanomyces bruxellensis
3373.273
4608.822
6049.447
6749.519
8807.824
9218.586
10141.694
(c)
Lactobacillus lindneri
3338.667
6540.593
8679.876
9707.301
Lactobacillus brevis
3443.139
4520.563
5815.796
6885.578
7549.777
9062.472
9439.333
Pediococcus damnosus
2867.194
9433.873
2000
4000
6000
8000
10000
12000
m/z


(a)
x10^4
Bottom-fermenting brewing yeast
6991.171
6213.586
11548.216
8671.458
9717.083
Exophiala dermatitidus
7333.082
3666.533
6032.899
8100.792
9261.062
Candida inconspicua
6758.588
6169.031
3380.786
10063.150
Candida krusei
3948.195
6758.412
3137.694
6172.465
Candida guilliermondii
6180.952
6946.950
3448.771
4538.733
8859.224
Candida pelliculosa
4662.446
6157.998
6809.267
3676.588
5234.462
Pichia manshurica
6735.425
7127.068
6143.651
3368.387
Lactobacillus paracasei
5304.192
5894.413
7483.469
8416.853
2652.669
4209.757
9399.550
Lactobacillus brevis
6889.448
6378.166
7257.783
8816.199
4408.687
Burkholderia vietnamiensis
5201.798
6499.073
7448.203
9615.531
3724.238
2600.561
8257.384
11555.705
Burkholderia cepacia
5197.039
6493.602
7313.918
9607.852
2597.209
4396.309
Rhodotorula mucilaginosa
6007.776
7276.095
7827.278
3003.291
3637.922
6437.019
8780.391
10889.632
Intens. [a.u.]
2000
4000
6000
8000
10000
12000
m/z


(a)
(cont.)
Dekkera / Brettanomyces bruxellensis
Lactococcus lactis
Pandoraea apista
Enterococcus gilvus
Staphylococcus hominis
Staphylococcus capitis
Intens. [a.u.]
m/z
(b)
Acidomonas methanolica
Enterobacter sp. Bisph2

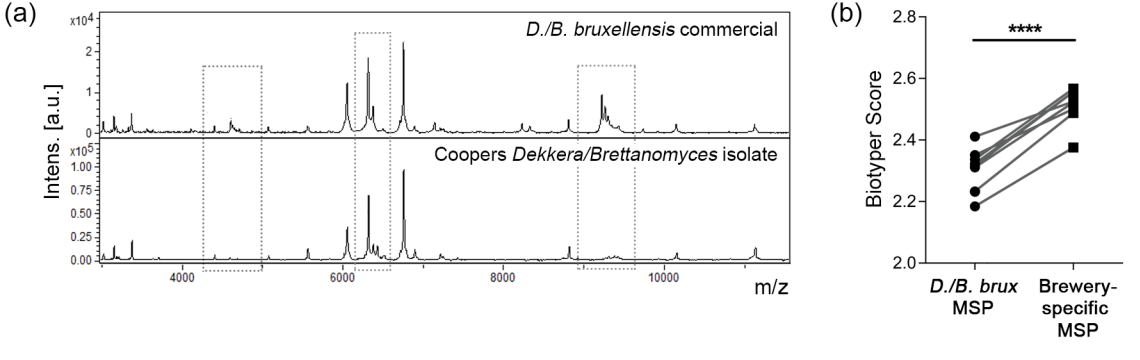

(a)
x10^4
2
1
0
D./B. bruxellensis commercial
Intens. [a.u.]
x10^5
1.00
0.75
0.50
0.25
0.00
Coopers Dekkera/Brettanomyces isolate
4000
6000
8000
10000
m/z
(b)
2.8
2.6
2.4
2.2
2.0
****
Biotyper Score
D./B. brux MSP
Brewery-specific MSP

Table 1: Definitions of Biotyper identification scores

Score	Identification status
2.300 - 3.000	Highly probable species identification
2.000 - 2.299	Secure genus identification, probable species identification
1.700 - 1.999	Probable genus identification
0.000 - 1.699	Not reliable identification

Table 2: Identification of beer spoilage microorganisms from inoculated beer samples using MALDI Biotyper database and analysis

	Inoculated strain	Identification Performance [a]		Score for Detected Species [b]
Brewing yeast (bottom-fermenting)	Munich Lager	Direct	(4 / 4)	**2.515 / 2.595 / 2.624 / 2.499**
		Filter	(3 / 4)	**2.369 / 2.422 / 2.377 /** Czech Pils (1)
	Czech Pils	Direct	(4 / 4)	**2.493 / 2.525 / 2.501 / 2.416**
		Filter	(0 / 4)	Detected as Munich Lager (4)
Brewing yeast (top-fermenting)	Weihenstephan Weizen	Direct	(4 / 4)	**2.170 / 2.081 / 2.152 / 2.205**
		Filter	(4 / 4)	**2.080 / 2.243 / 2.132 / 2.120**
	Kölsch	Direct	(4 / 4)	**2.516 / 2.533 / 2.557 / 2.518**
		Filter	(4 / 4)	**2.463 / 2.386 / 2.492 / 2.471**
Wild yeast	*Dekkera/Brettanomyces bruxellensis*	Direct	(4 / 4)	**2.294 / 2.212 / 2.202 / 2.302**
		Filter	(4 / 4)	**2.195 / 2.179 / 2.190 / 2.120**
Spoilage bacteria	*Lactobacillus lindneri*	Direct	(4 / 4)	**2.610 / 2.520 / 2.535 / 2.570**
		Filter	(4 / 4)	**2.400 / 2.252 / 2.209 / 2.298**
	Lactobacillus brevis	Direct	(4 / 4)	**2.531 / 2.477 / 2.516 / 2.521**
		Filter	(4 / 4)	**2.318 / 2.096 / 2.359 / 2.044**
	Pediococcus damnosus	Direct	(4 / 4)	**2.698 / 2.607 / 2.644 / 2.537**
		Filter	(4 / 4)	**2.248 / 2.309 / 2.332 / 2.273**

[a] Four technical replicate sum spectra were acquired from a single sample; successful identification was attributed if correctly matched to respective MSP; threshold for score was defined as >1.7

[b] Scores for identifications of four spectra. If inoculated strain was not top scoring identification, the top scoring microorganism is stated; bold, correct identification; plain text, incorrect identification. Refer to Table 1 for definition of score values.

Table 3: Identification of beer spoilage microorganisms from brewery process samples using MALDI Biotyper database and analysis

Plate #	Source	Plate / sample description		Biotyper Identification [a]	Identification Performance [b]	Score [c] MIN	Score [c] MAX
1	Membrane filter	2 sample types;	pink/green peaks	Bottom-fermenting brewing yeast	5 / 5	2.438	2.526
	Unpasteurized bottle		small black colony	*Exophiala dermatitidus*	5 / 5	2.036	2.166
2	Membrane filter	3 sample types;	pink/green peaks	Bottom-fermenting brewing yeast	5 / 5	2.339	2.416
	Unpasteurized bottle		pink structured growth	*Candida inconspicua*	5 / 5	2.187	2.427
			flat pink colony	*Pichia manshurica*	5 / 5	1.940	2.032
3	Spread plate	1 sample type;	single white colony	*Lactobacillus paracasei*	5 / 5	2.180	2.215
4	Membrane filter	2 sample types;	pink/green peaks	Bottom-fermenting brewing yeast	5 / 5	2.150	2.205
	Bright beer tank		small green colonies	No ID *	-	-	-
5	Membrane filter	4 sample types;	pink/green peaks	Bottom-fermenting brewing yeast	5 / 5	2.343	2.484
	Bright beer tank		pink coral structured growth	*Burkholderia vietnamiensis*	5 / 5	1.858	2.034
			green viscous growth	No ID *	-	-	-
			pink sporous colony	*Candida krusei*	5 / 5	2.479	2.511
6	Spread plate	2 sample types;	single pink colony	*Rhodotorula mucilaginosa*	5 / 5	1.863	2.137
	Fermenter vessel		many small white colonies	Bottom-fermenting brewing yeast	5 / 5	2.655	2.690
7	Streak plate (isolate)	1 sample type;	white peaks	*Dekkera/Brettanomyces bruxellensis*	5 / 5	2.282	2.434
8	Bright beer tank	1 sample type;	white peaks	Bottom-fermenting brewing yeast	5 / 5	2.373	2.503
9	Fermenter vessel	1 sample type;	black colonies	*Exophiala dermatitidus*	5 / 5	1.842	2.095
10	Membrane filter	3 sample types;	pink/green peaks	Bottom-fermenting brewing yeast	5 / 5	2.491	2.588
	Bright beer tank		viscous growth	*Burkholderia vietnamiensis*	5 / 5	2.129	2.250
			single brown colony	*Exophiala dermatitidus*	5 / 5	1.718	1.909
11	Membrane filter	3 sample types;	pink flat colony	*Pichia manshurica*	5 / 5	1.751	1.852
	Bright beer tank		coral-like growth	*Burkholderia vietnamiensis*	5 / 5	1.991	2.234
			green viscous growth	No ID *	-	-	-
12	Membrane filter	3 sample types;	green viscous growth	No ID *	-	-	-
	Bright beer tank		pink/green peaks	Bottom-fermenting brewing yeast	5 / 5	2.407	2.489
			viscous growth	*Burkholderia cepacia*	5 / 5	2.136	2.316
13	Membrane filter	3 sample types;	green viscous growth	No ID *	-	-	-
	Bright beer tank		pink/green peaks	Bottom-fermenting brewing yeast	5 / 5	2.427	2.503
			small brown colony	*Exophiala dermatitidus*	5 / 5	2.148	2.240
14	Bright beer tank	1 sample type;	small flat yellow colonies	*Lactococcus lactis*	5 / 5	1.836	2.022

15	Membrane filter Bright beer tank	3 sample types;	pink/green peaks pink flat growth green sporous growth	Bottom-fermenting brewing yeast *Pichia manshurica* *Pandoraea apista*	5 / 5 5 / 5 5 / 5	2.501 1.862 1.854	2.621 1.991 2.139
16	Yeast tank	1 sample type;	large beige colony	*Candida guilliermondii*	5 / 5	2.048	2.187
17	Unpasteurized bottle	1 sample type;	small flat yellow colonies	*Lactococcus lactis*	5 / 5	1.753	1.988
18	Spread plate (tank)	1 sample type;	green flat colonies	*Lactobacillus brevis*	5 / 5	2.337	2.440
19	Spread plate Keg	2 sample types;	few large white colonies many small white/blue colonies	*Dekkera/Brettanomyces bruxellensis* *Enterococcus gilvus*	5 / 5 5 / 5	2.200 2.112	2.242 2.252
20	Spread plate (wort)	1 sample type;	large beige colony	*Candida guilliermondii*	5 / 5	1.882	1.901
21	Spread plate (tank)	1 sample type;	white surface colonies	*Lactobacillus brevis*	5 / 5	2.067	2.201
22	Spread plate (tank)	3 sample types;	white surface colonies discs growing into agar colony growth underneath agar	*Lactobacillus brevis* *Lactobacillus brevis* *Lactobacillus brevis*	5 / 5 5 / 5 5 / 5	2.086 2.056 2.011	2.313 2.166 2.153
23	Yeast tank	1 sample type;	white peaks	*Candida pelliculosa*	5 / 5	2.012	2.169
24	Yeast tank	1 sample type;	beige colonies	*Candida guilliermondii*	5 / 5	1.976	2.175
25	Spread plate (tank)	1 sample type;	green flat colonies	*Lactobacillus brevis*	5 / 5	1.984	2.164
26	Keg	1 sample type;	few small blue colonies	*Staphylococcus hominis*	5 / 5	2.305	2.360
27	Keg	1 sample type;	few small blue colonies	*Staphylococcus capitis*	5 / 5	2.323	2.404
28	Bright beer tank	1 sample type;	pink/green peaks	Bottom-fermenting brewing yeast	5 / 5	2.409	2.601
29	Bright beer tank	1 sample type;	small flat yellow colonies	*Lactococcus lactis*	5 / 5	1.992	2.055

[a] Microorganism identification is defined as the best matched organism when identified against Biotyper MSP database of 5643 entries

[b] Five technical replicate sum spectra were acquired per sample; performance is defined as the number of spectra matched to the MSP of the identified microorganism in [(a)] as the top scoring identification (out of 5 acquisitions).

[c] Scores for identified microorganism; threshold for score was defined as >1.7; minimum and maximum scores attained are stated.

* Microorganisms without Biotyper identification putatively identified by LC-MS/MS; refer to text and Fig. S1 and S2.

Applied Microbiology and Biotechnology

SUPPLEMENTARY MATERIAL

Identification of beer spoilage microorganisms using the MALDI Biotyper platform

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SUPPLEMENTARY MATERIAL

Identification of beer spoilage microorganisms using the MALDI Biotyper platform

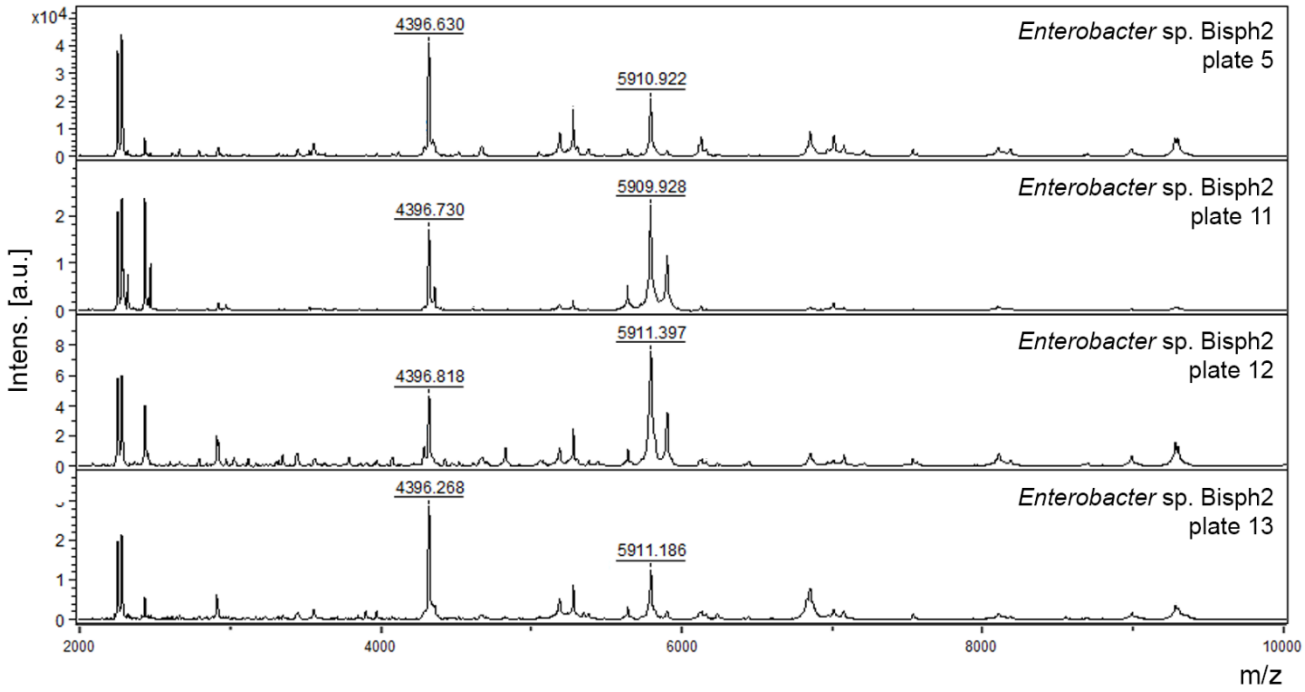

Fig. S1 Representative MALDI-TOF MS spectra for samples dominantly containing *Enterobacter* sp. *Bisph2*. Proteins were extracted from four phenotypically similar microorganism samples harvested from four independent membrane filter agar plates sourced from brewery processes. Sum spectra were acquired from 5 technical replicates; representative MALDI-TOF MS spectra for microorganisms are shown. *M/z* values for prominent peaks are displayed

SUPPLEMENTARY MATERIAL

Identification of beer spoilage microorganisms using the MALDI Biotyper platform

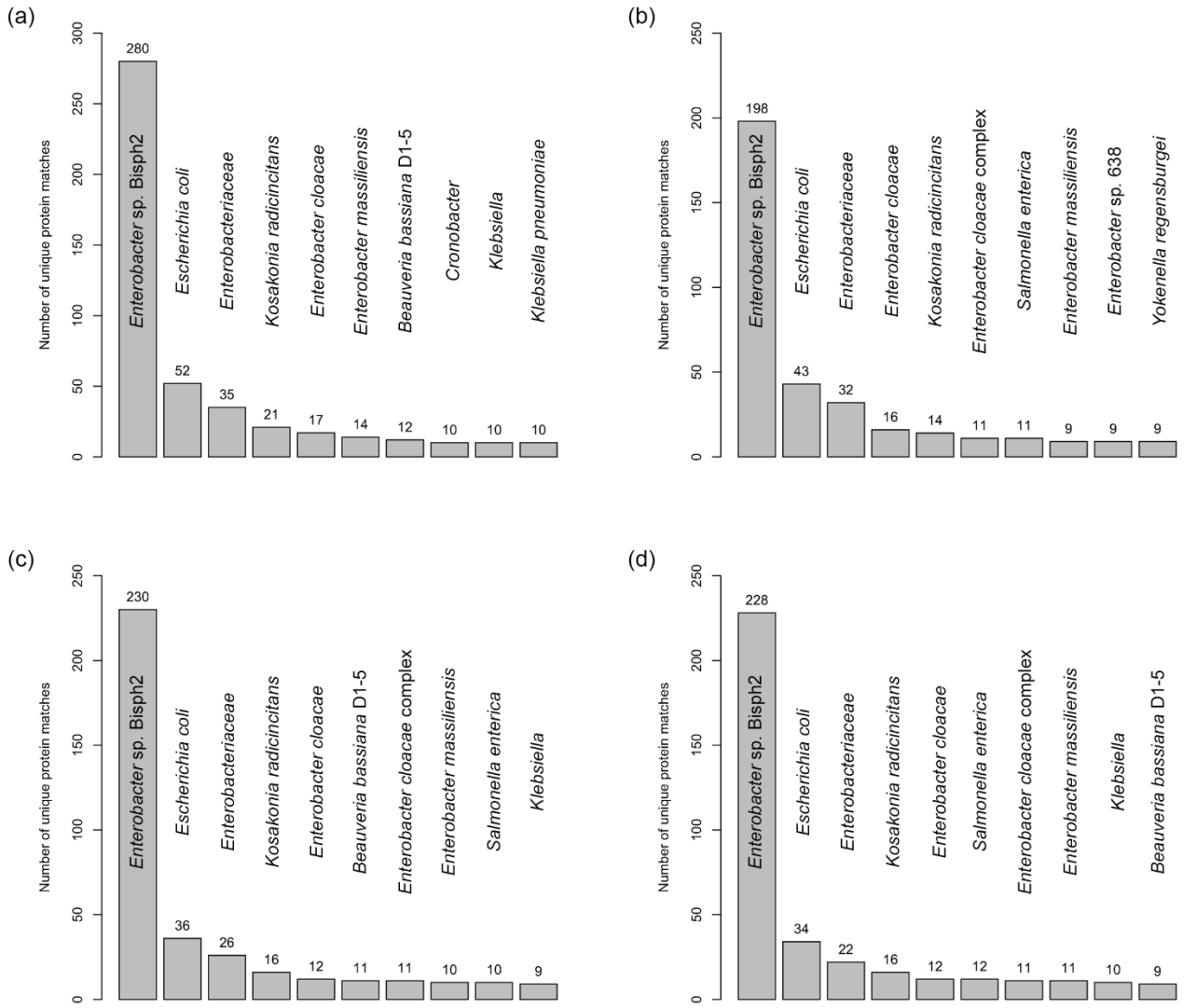

Fig. S2 Identification of *Enterobacter* sp. *Bisph2* as dominant microorganism in Biotyper-unidentified samples by LC-MS/MS. Top scoring protein within a protein family (proteins indistinguishable by acquired MS/MS data) was exported and corresponding microorganisms ranked according to their total number of appearance within the protein list. Top 10 assigned microorganisms per sample shown. Microorganisms sampled from ***(a)*** Plate 5, ***(b)*** Plate 11, ***(c)*** Plate 12 and ***(d)*** Plate 13. Identification of additional microorganisms with high number of top scoring protein hits (e.g. *Escherichia coli*) indicates a mixture of microorganism in the original sample and possible explanation for the failure of identification by Biotyper