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Identification and ecotyping of beverage spoiling bacteria by MALDI-TOF Mass Spectrometry

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List of Abbreviations

ABC	adenosinetriphosphate binding cassette
A	adenine
A.	*Acetobacter*
ANOSIM	analysis of similarity
a.u.	arbitrary units
BASH	bourne-again shell
BLAST	basic local alignment search tool
BLQ	Research Center Weihenstephan for Brewing and Food Quality
bp	base pair
C	cytosine
Cl	chloride
Da	Dalton
DAPC	discriminant analysis of principal components
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DSM	Deutsche Sammlung von Mikroorganismen
EDTA	ethylenediamine tetra-acetic acid disodium salt
G	guanine
g	gram
GA	Genetic Algorithm
h	hour
HCCA	alpha-cyano-4-hydroxy-cinnamic acid
HiT-MDS	high-throughput multidimensional scaling
Hz	Hertz
k	kilo
L	litre
L.	*Lactobacillus*
LIMPIC	linear MALDI-TOF MS peak indication and classification
Ln.	*Leuconostoc*
Log	logarithmic
M	molar

m	milli/meter
MALDI	matrix-assisted laser desorption/ionization
MASCAP	mass spectrometry comparative analysis package
MPI	message passing interface
Mg	magnesium
MIC	minimal inhibitory concentration
min	minute
mMRS	modified DeMan-Rogosa-Sharpe
Mn	manganese
MS	mass spectrometry
m/z	mass to charge
µ	micro
n	nano
NBB	Nachweismedium für bierschädliche Bakterien
ORF	open reading frame
OSA	orange serum agar
P.	*Pectinatus*
p	pico
p.a.	pro analysi
Pc.	*Pediococcus*
PCA	principal component analysis
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
ppm	parts per million
QC	Quick Classifier
r	ribosomal
RAPD	random amplified polymorphic DNA
Rep-PCR	repetitive element palindromic PCR
RFLP	restriction fragment length polymorphic DNA
RNA	ribonucleic acid
S	Svedberg
SNN	Supervised Neural Network
SVM	Support Vector Machine

T	thymine
TE	Tris-EDTA
Tris	tris(hydroxymethyl)aminomethane
TOF	time of flight
U	unit
UPGMA	unweighted pair group method with arithmetic mean
VTT	Technical Research Centre of Finland
v/v	volume per volume
WLN	Wallerstein laboratory nutrient
w/v	weight per volume

1 Introduction

1.1 Bacterial growth in beer and non-alcoholic beverages

Microbial growth in industrially produced beverages, such as fruit juices, soft drinks or flavored water may result in turbidity, haze and off-flavors, leading to impaired product quality as well as financial and image losses for the companies. Though beverages like juices and soft drinks provide nutrient-rich substrates, enabling growth of microorganisms, proliferation is limited due to low pH resulting from high contents of fruit acids. Still, certain microorganisms such as yeasts, molds, acetic acid bacteria and lactic acid bacteria, which are acidophilic or acid tolerant, are able to grow in these beverages [Back, 2000]. Recently, beverage industry faces new challenges as consumers increasingly ask for products from exotic fruits containing compounds, which are supposed to contribute to health [Lima-Tribst et al., 2009], or natural, fresh and minimally processed products, providing improved sensory and nutritional quality [Cheng et al., 2007; Picouet et al., 2009].

With regard to acidic fruit juices and soft drinks lactic acid bacteria, including the genera *Lactobacillus (L.)* and *Leuconostoc (Ln.),* were reported to be the most important spoilage bacteria. Lactic acid bacteria as well as acetic acid bacteria can be easily destroyed by thermal treatment and are thus less frequently found in pasteurized products, where their presence may be caused by recontamination [Lima-Tribst et al., 2009; Gressoni and Massaguer, 2003; Jay and Anderson, 2001; Juvonen et al., 2009]. In contrast to heat sensitive bacteria, spore-forming species belonging to the genus *Alicyclobacillus,* which were involved in spoilage incidents concerning various fruit juices, carbonated fruit juice drinks, lemonades and iced teas, were shown to be not only resistant to acidic environment but also showed increased resistance to thermal processing [Smit et al., 2011] and may thus be a another concern for the beverage and juice industry.

Finished beer is a microbiological rather stable product. Due to the presence of antimicrobial compounds such as ethanol, hop acids, carbon dioxide, a low pH value and a decreased oxygen concentration as well as the lack of nutrients, which have been used up by brewing yeast during fermentation [Suzuki, 2011], only a limited range of bacteria is able to grow in and thus spoil beer. In addition to beer's intrinsic hurdles, which hinder microbial growth, brewing includes processing steps such as

mashing and kettle boil, which cause thermal inactivation of vegetative cells, or filtration processes, where cells are excluded by size [Vriesekoop et al., 2012]. Still, during various stages of the brewing process, different contaminating microorganisms can be found. But only few bacteria are able to grow in beer. Amongst the most frequently reported spoilage organisms are lactic acid bacteria such as *L. brevis, L. lindneri*, *L. plantarum*, *L. casei* and *paracasei*, *L. coryniformis* and *Pediococcus (Pc.) damnosus* as well as the strictly anaerobic bacteria *Pectinatus (P.) frisingensis*, *P. cerevisiiphilus* and *Megasphaera cerevisiae* [Back, 1994; Russell and Stewart, 2003]. More recently, *L. backii* [Bohak et al., 2006], *Pc. claussenii* [Dobson et al., 2002], *P. haikarae, Megasphaera paucivorans* and *Megasphaera sueciensis* [Juvonen and Suihko, 2006] were isolated from spoiled beer. Recent data on the quantitative distribution of beer spoiling bacteria in complaints is rather scarce and data from various sources is often hard to compare as organisms are grouped differently while new species are described. It is however agreed on that *L. brevis* is the most prevalent organism associated with beer spoilage.

In contrast to the finished product beer, its precursor wort is rich in nutrients. Therefore, the brewing process itself is susceptible to growth of microorganisms [Ingledew, 1979] and the unhopped and unfermented wort is prone to contamination by a broader range of microorganisms. Microbial contaminants which are found in different stages of the brewing process originate from different sources. *Enterobacteria* can be found in wort, decreasing the fermentation rate and causing off-flavors as well as haze. Pitching yeast can carry contaminants such as wild yeasts, enteric bacteria, acetic acid bacteria as well as lactic acid bacteria resulting in super attenuation, altered flocculation and high levels of undesired aroma compounds. During fermentation lactic acid bacteria are regarded as the most important contaminants causing adverse effects such as retarded or extended fermentation and off-flavors. Together with acetic acid bacteria lactic acid bacteria are also found commonly in storage and finishing of beer and can persist in packaged beer where *Pectinatus sp.* and *Megasphaera sp.* represent another two prominent spoilage genera which can cause adverse flavor effects and haze [Hill, 2009]. As lactic acid bacteria can be practically found in all stages of the brewing process, they are rightfully recognized as one of the most important - if not the most important - group of beer spoilage bacteria.

In addition to the traditionally found spoilage bacteria, an increasing market for fruity beer-based mixed drinks, imposes new challenges on the brewing industry. These products often provide good conditions for the survival and growth of a broader range of microorganisms due to higher pH, addition of sugar or the reduction of antimicrobial agents such as hops and ethanol [Franz et al., 2009; Hutzler et al., 2007; Lima Tribst et al., 2009].

L. brevis and *Pectinatus sp.* and their effect on the brewing process and finished beer will be discussed below. These organisms are of high importance for the brewing industry due to their high prevalence and detrimental alterations of the product.

1.2 Beer spoilage caused by *Lactobacillus brevis*

L. brevis was reported to be the most frequently encountered spoilage bacteria in beer [Back, 2000]. The ability to grow in and thus spoil beer varies broadly among different isolates of *L. brevis* [Suzuki, 2011]. While some strains cause haze, sediments and acidification in a wide range of beers, other strains have been shown to possess only weak or no ability to spoil beer [Behr and Vogel, 2009; Suzuki, 2011]. Resistance against hop compounds is a prerequisite for lactic acid bacteria to grow in beer. Several hop compounds possess antibacterial activity, amongst which iso-alpha-acids play a major role [Simpson, 1993a; Sakamoto and Konings, 2003]. Due to their conversion during the wort boiling process, iso-alpha-acids show a much higher solubility in beer than their precursors and are thus the predominant bittering compounds in beer [Sakamoto and Konings, 2003]. It was demonstrated that iso-alpha-acids act as ionophores, dissipating the transmembrane pH gradient and impairing proton motive force [Behr and Vogel, 2009, Sakamoto and Konings, 2003; Simpson, 1993b].

Several resistance mechanisms of lactic acid bacteria against hop bitter compounds were proposed. HorA, a plasmid encoded multidrug transporter belonging to the ABC family, found in strongly beer spoiling *L. brevis,* was suggested to extrude hop components out of the cells [Sami et al., 1997; Sakamoto et al., 2001; Suzuki et al., 2006]. Another plasmid encoded multidrug transporter, putatively belonging to the resistance nodulation cell division family, published as horC, was shown to be associated with hop resistance and beer spoilage potential [Suzuki et al., 2005]. HitA

was suggested by Hayashi et al. [2001] to play a role in the transport of divalent cations such as Mn^{2+}. Manganese proved to be a scavenger for reactive oxygen species [Archibald and Fridovich, 1981] and is a co-factor for several enzymatic reactions at cellular level. Moreover, divalent cations, like Mn^{2+} were shown to affect the antibacterial activity of hop bitter acids [Simpson, 1993a]. Still, its role in the spoilage of beer by lactic acid bacteria is not fully understood.

Additionally, energy independent mechanisms such as alterations in the lipoteichoic acid content of the cell wall, as shown for a highly hop adapted variant of *L. brevis,* may contribute to hop resistance in beer spoiling lactic acid bacteria [Behr et al., 2006; Yasui and Yoda, 1997]. Lipoteichoic acids act as a constant reservoir of bound divalent cations [Hughes et al., 1973] and divalent cations such as Mn^{2+} were shown to be crucial for the survival of lactobacilli [Archibald and Fridovich, 1981]. Next to specific resistance mechanisms, which were presumably acquired through constant contact and thus adaptation to hop compounds, more general tolerance mechanisms may contribute to the ability of certain lactic acid bacteria to grow in the presence of hop bitter compounds. These mechanisms include the acid stress alleviating systems F_0F_1-ATPase [Sakamoto et al., 2002] as well as the arginine deiminase pathway [Behr et al., 2006] and the glutamate decarboxylase system [Schurr et al., 2013].

Moreover hop resistance is a dynamic property. While subcultivation of hop resistant strains in the absence of hop compounds can decrease hop resistance, resistance can be increased by subcultivation of strains at gradually increasing hop compound concentrations [Behr et al., 2006; Richards and Macrae, 1964]. Consequently minimal inhibitory concentrations (MIC) for hop compounds can multiply, if a strain is gradually adapted to hop compounds [Behr et al., 2006].

As bacteria, which grow in beer, need to withstand multiple stress factors such as starvation, ethanol stress and antibacterial action of hop compounds through ionophoric activity and pronounced redox reactivity [Behr et al., 2006; Behr and Vogel, 2010], it may come as no surprise that the ability to grow in beer appears to be the result of multiple complex resistance mechanisms.

1.3 Beer spoilage caused by *Pectinatus sp.*

The genus *Pectinatus* was first described as strict anaerobic, non-sporeforming, flagellated rod by Lee et al. in 1978. The strain, which became the type strain of the species *P. cerevisiiphilus*, was isolated from beer and found capable to grow therein [Lee et al., 1978]. To date two more species of the genus *Pectinatus,* which play a role in beer spoilage are described: *P. frisingensis* [Schleifer et al., 1990] and *P. haikarae* [Juvonen and Suihko, 2006]. Members of the genus *Pectinatus* produce metabolites such as the organic acids acetic, propionic and succinic acid as well as acetoin and sulphuric compounds from glucose and fructose [Juvonen and Suihko, 2006], leading to off-flavors in beer.

Reports on the prevalence of *Pectinatus* species in spoilage incidents seem to vary largely. In the early nineties a sudden increase of up to 28 % of spoilage incidents was reported. Numbers again seemed to decrease to around 5 % in 2000. The abrupt rise of incidents is generally attributed to the improvement of filling technologies, which lead to the production of virtually oxygen-free beer [Suzuki, 2011]. The oxygen content in the packaged product is recognized as one of the key factors, which influence the flavour stability of finished beer [Bamforth, 2000], as oxidation processes can lead to undesired flavour compounds. Still, the elimination of oxygen during production and filling enables and promotes the growth of *Pectinatus*. As *Pectinatus* causes particularly unpleasant off-flavours, rendering the beer undrinkable, even a small prevalence causes serious economic losses.

1.4 Identification and differentiation techniques for beverage spoiling bacteria

Rapid and reliable identification and differentiation of spoilage bacteria is crucial to ensure efficient quality control and safe products in the food industry. Conventional culture based methods for bacterial identification are rather laborious and time-consuming. Thus, there is an urgent need for fast and accurate alternative methods. In principle any method appropriate for microbial identification could be used for the identification of beverage spoiling bacteria. In practice, the most commonly applied methods are based on PCR [Gammon et al., 2007; Juvonen et al., 2008; Martínez et al., 2011; Menz et al., 2010; Russell and Stewart, 2003]. Still, PCR requires pre-

specified targets and the amount of species, which can be detected within a single run, is rather limited.

For obvious reasons, not only identification of contaminants on the species level, but also differentiation between e. g. lactic acid bacterial strains or ecotypes able to spoil beer and those, which are not, is of vital interest for the brewing industry. However, testing isolates for direct growth in beer in a “forcing test” takes several days to weeks or months [Back, 1994; Sakamoto and Konings, 2003] and is thus inapplicable if results are needed promptly. Therefore, genetic markers associated with beer spoilage bacteria such as *horA* [Sakamoto et al., 2001; Sami et al., 1997], *horC* [Iijima et al., 2006] and *hitA* [Hayashi et al., 2001] were proposed to obtain faster discrimination between spoilage and non-spoilage strains of lactic acid bacteria. According to Haakensen et al. [2008] *horA* is significantly predictive of an organism’s ability to grow in beer. Still, as approaches based on single gene markers may suffer from gene truncation yielding false-positive results [Behr et al., 2006; Haakensen et al., 2008] and it is generally agreed on that beer spoiling ability in lactic acid bacteria is based on several complex resistance mechanisms, there’s a need for reliable and alternative discrimination approaches, which are not limited to the presence or absence of a single genetic marker.

1.5 MALDI-TOF Mass Spectrometry

First described in 1985 [Karas et al.], MALDI-TOF MS is a soft ionization technique allowing the detection of large, non-volatile and labile molecules. Small amounts of sample are mixed with matrix solution and embedded in matrix during crystallization upon solvent evaporation. The matrix absorbs the UV laser radiation and transfers the energy to the sample. Every laser pulse leads to the removal of several monolayers of matrix molecules through ablation, generating ions [Hillenkamp and Karas, 2007]. The most common mass analyzer for MALDI applications is an axial TOF spectrometer. In a TOF instrument all ions are accelerated using a potential difference between the sample carrier and a nearby grid. Ions pass on into a field free tube where they travel with different velocities according to their mass to charge ratio before hitting the detector [O’Connor and Hillenkamp, 2007].

MALDI-TOF MS is currently broadly exploited for analysis of large biomolecules such as proteins and nucleic acids [Hjerno and Jensen, 2007; van den Boom and Berkenkamp, 2007].

1.5.1 Identification of bacteria by MALDI-TOF MS

MALDI-TOF MS was shown to be a rapid, reliable and powerful tool for the identification of microorganisms. It is based on the generation of spectral fingerprints, which form a characteristic protein peak pattern according to the organism analyzed [Fenselau and Demirev, 2001; Holland et al., 1996; Williams et al., 2003]. Mass spectrometry saves time and money compared to conventional phenotypic identification and outmatches PCR-based methods by its broad band applicability, which allows detection of organisms without the need to pre-specify targets [Demirev and Fenselau, 2008; Seng et al., 2009]. It's performance in clinical microbiology has been under extensive study, recently [Dieckmann et al., 2010; Hinse et al., 2011; La Scola et al., 2009; Mellmann et al., 2008; Mott et al., 2010; Seng et al., 2009; Sogawa et al., 2011; van Veen et al., 2010; Wittwer et al., 2011] while fewer investigations were conducted regarding other fields of microbiology such as environmental microbiology [Giebel et al., 2008; Munoz et al., 2011] or food microbiology [Angelakis et al., 2011; Böhme et al., 2011; Fernández-No et al., 2011; Mazzeo et al., 2006; Tanigawa et al., 2010] where until recently most studies concentrated on food-borne pathogens. Recently reports of MALDI-TOF MS as an identification tool for nonpathogenic food associated microorganisms are increasing [Andres-Barrao et al., 2013; Doan et al., 2012; Duskova et al., 2012; Ruyz-Moyano et al., 2012; Sato et al., 2012].

1.5.2 Environmental and physiological influences on MALDI-TOF MS analysis

Among the many factors which can influence MALDI-TOF MS spectra, sample preparation and microbial growth conditions have often been an issue of concern in terms of reproducibility and quality. Quality of spectra was often defined by parameters such as resolution, peak intensities, number of obtained peaks, mass range of detected signals and signal to noise ratio [Giebel et al., 2008; Mazzeo et al.,

2006; Williams et al., 2003]. Varying sample preparation methods have been employed ranging from plain cell smears, cell smears plus formic acid, solvent extractions and application of enzymes to physical methods of cell disruption using ceramic beads, heat treatment or sonication. Any extra steps of adding formic acid to cell smears or mechanical and enzymatic treatments, are supposed to enhance the release of bacterial proteins through improved cell lysis and cell wall disruption. These procedures can improve the ratio of successful and reproducible measurements and identifications for organisms, which are hard to analyze by plain cell smears such as certain gram-positive bacteria [Giebel et al., 2008; Haigh et al., 2011; Smole et al., 2002; Vargha et al., 2006] and yeast [Haigh et al., 2011]. Growth conditions like incubation time and nutrient media were often varied in studies to assess their influence on MALDI-TOF MS spectra. However results, obtained from different organisms, were not consistent. While van Veen et al. [2010] and Seibold et al. [2010] found no influence of culture conditions on the accuracy of identification on species and sub-species level, others [Alispahic et al., 2010] encountered problems with spectra acquisition and spectra patterns when cultures were grown on certain substrates. Mazzeo et al [2006] observed variability in mass spectra with varying nutrient media but not with growth time, however changes in protein profiles with growth stage were reported as well [Vargha et al., 2006].

1.5.3 Sub-species differentiation of bacteria by MALDI-TOF MS

MALDI-TOF MS has proven to be a rapid and effective tool for the identification of bacteria at the species and genus levels [Sandrin et al., 2013], It's widely used in clinical microbiology where it was shown to be well-suited for identification purposes in routine microbiology laboratories [Carbonnelle et al., 2011] and is increasingly applied for the species identification of food associated microorganisms. Some applications such as epidemiologic investigations or bacterial source tracking of environmental isolates however require a higher taxonomic resolution to the point of identification of individual strains within a single species [Sandrin et al., 2013]. Characterization of bacteria below the species level, which is also referred to as bacterial subtyping, is as well applicable in food industry. For example it allows to track foodborne pathogens through the production to determine where they enter into the system [Hyytiä-Trees et al., 2007] or to monitor the effects of any action

undertaken to eliminate contaminations. For the need of sub-species differentiation a range of genotypic methods is currently applied including random-amplified polymorphic DNA (RAPD) PCR [Plengvidhya et al., 2004], pulsed field gel electrophoresis (PFGE) [Behringer et al., 2011; Plengvidhya et al., 2004], restriction fragment length polymorphism (RFLP) [Behringer et al., 2011; Guyot et al., 2003], ribotyping [Guyot et al., 2003], multi locus sequencing typing [Behringer et al., 2011; Teh et al., 2011] and repetitive element palindromic (rep) PCR [Behringer et al., 2011; Siegrist et al., 2007]. However, none of the single subtyping methods at present completely fulfills the demands in terms of discriminatory power, robustness, portability, objectivity and throughput [Hyytiä-Trees et al., 2007]. As MALDI-TOF MS analysis of microorganisms has gained popularity due to its simple sample preparation, rapidness and high-throughput capacity, limits of taxonomic resolution have been under closer investigation recently. Amongst others, MALDI-TOF MS has been successfully applied for sub-species differentiation of *Listeria* strains [Barbuddhe et al., 2008], *Salmonella* isolates [Dieckmann et al., 2008] or *Franciscella tularensis* strains [Seibold et al., 2010] as well as for the discrimination of environmental *Escherichia coli* isolates [Siegrist et al., 2007] indicating that it's potential to discriminate microorganisms may go well beyond plain species-level identification. However it has been suggested that taxonomic resolution of MALDI-TOF MS may largely depend on the taxon under observation [Ghyselinck et al., 2011] and thus needs to be evaluated for a particular genus or species of interest.

MALDI-TOF MS has also been discussed as a tool for the detection of antibiotic resistances in bacteria such as the differentiation between methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* or the detection of β-lactamases [Hrabak et al., 2013; Russell et al., 2007] and even the differentiation of physiological states in bacteria [Salaün et al., 2010], underlying the enormous analytical potential harbored by this method.

1.6 Objectives of the work

To date MALDI-TOF MS is extensively used for the identification of microorganisms in clinical microbiology. Substantial data is available for species level identification of clinically relevant microorganisms. Most data available on the influence of

parameters such as growth conditions or sample preparation on MALDI-TOF MS' performance is also obtained from clinically relevant organisms. Microorganisms associated with food and beverage spoilage are, however, not necessarily pathogens and thus comprise different species from those encountered in clinical microbiology.

The aim of this study was to assess the applicability of MALDI-TOF MS for the identification and differentiation of bacteria found as contaminants in beverage and brewing industry. Several factors such as sample preparation and culturing conditions which might influence MALDI-TOF MS performance in terms of accuracy, reproducibility, reliability, ease of handling and time and labor input were to be investigated. Based on the acquired data, MALDI-TOF MS' applicability for the routine identification of contaminants should be evaluated and compared with established identification techniques. Furthermore its taxonomic resolution below species level should be closely examined. MALDI-TOF MS' potential to differentiate on strain level or delineate contaminants belonging to the same species into different ecotypes with regard to their spoilage ability should be determined.

2 Materials and Methods

2.1 Equipment

Table 1: Equipment used in this study

Device	Model	Manufacturer
Anaerobic chamber	WA 6200	Heraeus Instruments, Germany
Anaerobic jars	Anaerocult	Merck, Germany
Autoclave	VX-150 VE-40	Systec GmbH, Germany
Camera		Intas Science Imaging Instruments, Germany
Centrifuge	MCF-1350 Z 216 MK Z 382 K Z 383 K Z 513 K 6-16 K	Tanaka Bldg, Japan Hermle Labortechnik GmbH, Germany Sigma Laborzentrifugen GmbH, Germany
Electrophoresis chamber	A2 B2	Owl Scientific Inc. USA
FastPrep^{TM}	FastPrep-24	M.P. Biomedicals, USA
Incubator		Memmert, Germany
MALDI-TOF MS	Microflex LT	Bruker Daltonics, Germany
MALDI-TOF MS target	MSP 96 target polished steel	Bruker Daltonics, Germany
Microplate reader	SpectraFluor Sunrise	Tecan Deutschland GmbH, Germany Tecan Austria GmbH, Austria
Microscope	Axiolab E re	Carl Zeiss, Germany
pH meter	InLab 412, pH 0-14	Mettler-Toledo, Germany
Photometer	Novaspec II	Pharmacia Biotech, England
Pipettes	Eppendorf Reference Gilson pipetman	Eppendorf AG, Germany Gilson-Abomed, Germany
Power Supply	EPS 300	Pharmacia Biotech, England
Scale	SI-234 SBA 52	Denver Instrument, Germany Scaltech Instruments, Germany
Sonicator	Sonopuls HD 2200	Bandelin electronic, Germany
Stirrer	WiseStir MSH-20A Arec Heating Magnet Stir	Witeg Labortechnik GmbH, Germany Velp Scientifica, Italy

Thermocycler	Mastercycler gradient	Eppendorf AG, Germany
Ultrasonic bath	RK 103H	Bandelin electronic, Germany
UV table	UVT 28M	Herolab Laborgeräte GmbH, Germany
Vortex	Vortex Genie 2	Scientific Industries Inc.; USA
Water bath	W 19 MD 12	Thermo Haake GmbH, Germany Lauda Dr. R. Wobser GmbH & Co, Germany
Workbench	BioWizard Golden GL-200	Kojair The Oy, Finland

2.2 Consumables

Table 2: Consumables used in this study

Consumable	**Specification**	**Manufacturer**
Anaerocult	Anaerocult A Anaerocult A mini	Merck KGaA, Germany
Cuvettes	Acryl, 10x10x45 mm	Sarstedt AG & Co, Germany
Microtiter plates	Microtest plate 96 Well F	Sarstedt AG & Co, Germany
PCR-strips	Multiply-µStrip Pro 8 strip	Sarstedt AG & Co, Germany
Petri dishes	92 x 16 mm with cams	Sarstedt AG & Co, Germany
Pipette tips	ep T.I.P.S. Standard Diamond	Eppendorf AG, Germany Gilson-Abomed, Germany
Reaction tubes	Tube 50 ml, PP Tube 15 ml, PP Tube 5 ml, PP Safe seal tube 1.5 ml	Sarstedt AG & Co, Germany
Sterile filter	Filtropur S 0.2	Sarstedt AG & Co, Germany

2.3 Chemicals

Table 3: Chemicals used in this study

Chemicals	**Specification**	**Manufacturer**
100 bp Marker	Gene Ruler 100 bp Plus DNA-Ladder	Thermo Fischer Scientific Inc., USA
6x Loading Dye		Thermo Fisher Scientific Inc., USA
Acetonitrile	Rotisolv, HPLC Ultra	Carl Roth GmbH & Co KG,

	Gradient Grade	Germany
Agar	High gel concentration	BD, France
Agarose	LE-Agarose	Biozym Scientific GmbH, Germany
Alpha-cyano-4-hydroxy-cinnamic acid (HCCA)	Matrix for MALDI-TOF MS	Bruker Daltonics, Germany
Ammonium chloride	≥ 99.5 %, p.a.	Carl Roth GmbH & Co KG, Germany
Bacterial Test Standard		Bruker Daltonics, Germany
Bromokresolgreen		Merck KGaA, Germany
Calcium chloride-dihydrate	p.a.	Merck KGaA, Germany
Cysteine hydrochloride-monohydrate	≥ 98.5 %	Carl Roth GmbH & Co KG, Germany
D (+)- Glucose-monohydrate	For microbiology	Merck KGaA, Germany
Dimidium bromide		Sigma-Aldrich Chemie GmbH, Germany
Dipotassium-hydrogensulphate-trihydrate	p.a.	Merck KGaA, Germany
dNTPs	Taq DNA CORE Kit 10	MP Biomedicals LLC, USA
Ethanol	p.a.	VWR International, France
Ethylenediamine tetra-acetic acid disodium salt (EDTA)		Gerbu Biotechnik GmbH, Germany
Ferric chloride-tetrahydrate	≥ 99 %, p.a.	Fluka Chemie GmbH, Germany
Formic acid	≥ 98 %	Sigma-Aldrich Chemie GmbH, Germany
Fructose	For microbiology	Carl Roth GmbH & Co KG
Hydrochloric acid	37 %, p.a.	Merck KGaA, Germany
Iso-alpha acids	90 % in ethanol	Simon H. Steiner, Hopfen, GmbH, Germany
Lambda DNA/EcoRI+HindIII Marker 3		Thermo Fischer Scientific Inc., USA
Lysozyme	From chicken egg white, 100.000 U/mg	Serva Electrophoresis GmbH, Germany
Magnesium chloride	Taq DNA CORE Kit 10	MP Biomedicals LLC, USA
Magnesium sulphate-heptahydrate	p.a.	Merck KGaA, Germany
Maltose	For microbiology	Merck KGaA, Germany
Manganese sulphate-monohydrate	≥ 98 %	Carl Roth GmbH & Co KG, Germany
Meat extract	Granulated for microbiology	Merck KGaA, Germany
NBB		Doehler GmbH, Germany
Orange juice	100 % Orange Fair	Hermann Pfanner Getränke GmbH, Austria

Paraffin	p.a.	Sigma-Aldrich Chemie GmbH, Germany
Peptone	From meat	Merck KGaA, Germany
Potassium chloride	≥ 99 %, Cellpure	Carl Roth GmbH & Co KG, Germany
Potassium-dihydrogensulphate	≥ 99 %, p.a.	Carl Roth GmbH & Co KG, Germany
Reaction buffer	Taq DNA CORE Kit 10	MP Biomedicals LLC, USA
Resazurin-sodium salt	p.a.	Serva Electrophoresis GmbH, Germany
Taq-Polymerase	Taq DNA CORE Kit 10	MP Biomedicals LLC, USA
Trifluoroacetic acid	≥ 99.9 %, for synthesis	Carl Roth GmbH & Co KG, Germany
Tris	Ultrapure	MP Biomedicals LLC, USA
Tween 80	Polysorbate 80	Gerbu Biotechnik GmbH, Germany
Yeast extract	Granulated for microbiology	Carl Roth GmbH & Co KG

2.4 Software

Table 4: Software used in this study

Software tool	**Source/Reference**
BioNumerics 6.5	Applied Maths, Belgium
BLAST	Altschul, 1990
Clin ProTools 2.2	Bruker Daltonics, Germany
FlexAnalysis 3.3	Bruker Daltonics, Germany
FlexControl	Bruker Daltonics, Germany
MALDI Biotyper 3.0	Bruker Daltonics, Germany
Maldi Biotyper Automation Control 2.2	Bruker Daltonics, Germany
Primer-BLAST	Ye, 2012

2.5 Bacterial strains

Three strains from our culture collection which were originally isolated from beverages, *L. brevis* TMW 1.313, *Leuconostoc (Ln.) mesenteroides* TMW 1.1853 and *Pc. claussenii* TMW 2.54 were chosen for optimizing MALDI-TOF MS sample preparation procedures.

Seventeen strains of *L. brevis* from our culture collection and nineteen strains belonging to the genus *Pectinatus* and isolated from brewing environments [Suiker et al., 2007] were used to demonstrate sub-species differentiation of beverage spoiling bacteria by MALDI-TOF MS. Strains of *L. brevis* were additionally characterized with

respect to their beer spoilage potential and their tolerance to iso-alpha acids. A list of strains and their origin can be found in Table 5.

Table 5: Strains of *L. brevis* and *Pectinatus sp.* used for sub-species differentiation by MALDI-TOF MS and their origin.

Strain	Organism	Origin
TMW 1.100	*L. brevis*	sourdough
TMW 1.1205	*L. brevis*	sourdough
TMW 1.1326	*L. brevis*	silage
TMW 1.1369	*L. brevis*	plant fermentation
TMW 1.1370	*L. brevis*	plant fermentation
TMW 1.1371	*L. brevis*	plant fermentation
TMW 1.230	*L. brevis*	beer
TMW 1.240	*L. brevis*	beer
TMW 1.302	*L. brevis*	beer
TMW 1.313	*L. brevis*	beer
TMW 1.315	*L. brevis*	beer
TMW 1.317	*L. brevis*	beer
TMW 1.465	*L. brevis*	soft drink (brewery)
TMW 1.485	*L. brevis*	beer
TMW 1.6 (DSM 20054)	*L. brevis*	faeces
TMW 1.841	*L. brevis*	sourdough
TMW 1.939	*L. brevis*	sourdough
TMW 2.1487	*P. frisingensis*	brewery 1
TMW 2.1488	*P. frisingensis*	brewery 2
TMW 2.1489	*P. frisingensis*	brewery 2
TMW 2.1490	*P. frisingensis*	brewery 3
TMW 2.1491	*P. frisingensis*	brewery 4
TMW 2.1492	*P. frisingensis*	VTT
TMW 2.1493	*P. frisingensis*	VTT
TMW 2.1494	*P. cerevisiiphilus*	VTT
TMW 2.1495	*P. frisingensis*	VTT
TMW 2.1496	*P. haikarae*	VTT
TMW 2.1497	*P. frisingensis*	brewery 5
TMW 2.1498	*P. frisingensis*	brewery 5
TMW 2.1499	*P. frisingensis*	brewery 5
TMW 2.1500	*P. frisingensis*	brewery 5
TMW 2.1501	*P. frisingensis*	brewery 6
TMW 2.1502	*P. frisingensis*	brewery 7
TMW 2.1503	*P. frisingensis*	brewery 7
TMW 2.1504	*P. frisingensis*	brewery 7
TMW 2.1505	*P. frisingensis*	brewery

671 isolates derived from breweries and beverage producing industries and collected at the Research Center Weihenstephan for Brewing and Food Quality (BLQ) were

identified by MALDI-TOF MS. 100 of these isolates, all belonging to the species *L. brevis,* were characterized according to their beer-spoilage potential. Isolates were additionally checked for the presence of marker genes representative for a strong or weak beer spoilage potential, which were previously established at the Lehrstuhl für Technische Mikrobiologie in AiF project 16292. Strains and their origins are listed in Table 6.

Table 6: Brewery isolates of *L. brevis* used for MALDI-TOF MS and marker gene detection and their origin

Isolate	**Organism**	**Brewery of origin**
15	*L. brevis*	W
16	*L. brevis*	W
22	*L. brevis*	C
23	*L. brevis*	V
24	*L. brevis*	P
25	*L. brevis*	V
26	*L. brevis*	V
30	*L. brevis*	T
39	*L. brevis*	U
53	*L. brevis*	P
56	*L. brevis*	T
59	*L. brevis*	T
66	*L. brevis*	S
69	*L. brevis*	S
70	*L. brevis*	E
78	*L. brevis*	K
79	*L. brevis*	E
88	*L. brevis*	E
89	*L. brevis*	E
90	*L. brevis*	E
92	*L. brevis*	G
93	*L. brevis*	G
101	*L. brevis*	E
102	*L. brevis*	B
106	*L. brevis*	Q
112	*L. brevis*	P
114	*L. brevis*	G
117	*L. brevis*	E
119	*L. brevis*	-
120	*L. brevis*	-
121	*L. brevis*	P

122	*L. brevis*	G
124	*L. brevis*	A
132	*L. brevis*	O
137	*L. brevis*	E
141	*L. brevis*	N
154	*L. brevis*	M
155	*L. brevis*	M
159	*L. brevis*	K
161	*L. brevis*	M
162	*L. brevis*	M
163	*L. brevis*	M
164	*L. brevis*	M
165	*L. brevis*	E
166	*L. brevis*	M
174	*L. brevis*	M
201	*L. brevis*	L
211	*L. brevis*	E
213	*L. brevis*	I
215	*L. brevis*	J
218	*L. brevis*	-
220	*L. brevis*	-
222	*L. brevis*	I
232	*L. brevis*	K
233	*L. brevis*	J
234	*L. brevis*	J
241	*L. brevis*	-
243	*L. brevis*	B
246	*L. brevis*	B
250	*L. brevis*	I
272	*L. brevis*	-
274	*L. brevis*	E
275	*L. brevis*	-
277	*L. brevis*	E
284	*L. brevis*	E
285	*L. brevis*	E
286	*L. brevis*	E
307	*L. brevis*	-
342	*L. brevis*	E
343	*L. brevis*	-
350	*L. brevis*	H
354	*L. brevis*	G
355	*L. brevis*	G
356	*L. brevis*	E
357	*L. brevis*	E
360	*L. brevis*	E

366	*L. brevis*	G
368	*L. brevis*	E
369	*L. brevis*	E
370	*L. brevis*	E
371	*L. brevis*	E
381	*L. brevis*	E
385	*L. brevis*	E
386	*L. brevis*	E
387	*L. brevis*	E
388	*L. brevis*	-
390	*L. brevis*	E
391	*L. brevis*	E
392	*L. brevis*	E
394	*L. brevis*	F
415	*L. brevis*	E
416	*L. brevis*	E
417	*L. brevis*	E
418	*L. brevis*	E
548	*L. brevis*	-
550	*L. brevis*	D
680	*L. brevis*	A
706	*L. brevis*	C
750	*L. brevis*	B
751	*L. brevis*	A

2.6 Nutrient media and growth conditions

All strains and isolates were routinely streaked on modified DeMan-Rogosa-Sharpe Agar (mMRS) [Stolz et al., 1997] and – if not required and indicated else – incubated aerobic at 30 °C. Isolates belonging to the genera *Pectinatus* and *Megasphaera* were handled in an anaerobic chamber to ensure strictly anaerobic conditions. Strains recovered from cryopreservation were sub-cultured at least three times on the required medium before any experiments were conducted.

L. brevis TMW 1.313, *Lc. mesenteroides* TMW 1.1853 and *Pc claussenii* TMW 2.54 were grown on four different nutrient media widely used for quality control purposes in the beverage industry to assess the influence of growth medium on MALDI-TOF MS analysis. The compositions of mMRS agar, Orange Serum (OS) agar [Anonymous, 1978] and Wallerstein Laboratory Nutrient (WLN) agar [Green and Grey, 1950] can be found in Table 7. Nachweismedium für bierschädliche Bakterien

(NBB) was obtained from Doehler (Germany). Additionally availability of oxygen and growth time was varied to investigate its impact on MALDI-TOF MS spectra. Anaerobicity was achieved by the use of Merck's Anaerocult A system, while aerobic cultivation referred to incubation under atmospheric oxygen pressure. An overview of the varied growth parameters can be found in Table 8.

Table 7: Composition of mMRS, OSA and WLN nutrient media [g per L]

Modified DeMan-Rogosa-Sharpe Agar		Orange Serum Agar		Wallerstein Laboratory Nutrient Agar	
Peptone	10 g	Peptone	10 g	Peptone	5 g
Yeast extract	5 g	Yeast extract	3 g	Yeast extract	4 g
Meat extract	5 g	Glucose	4 g	Glucose	50 g
Dipotassium-hydrogen-phosphate	3.3 g	Dipotassium-hydrogen-phosphate	3 g	Potassium-dihydrogen-phosphate	0.55 g
Potassium-dihydrogen-phosphate	2.6 g	Orange Serum	200 ml	Potassium-chloride	0.425 g
Ammonium-chloride	3 g	Agar	15 g	Calcium-chloride	0.125 g
Tween 80	1 g	pH	5.5	Magnesium-sulphate	0.2 g
Cysteine-hydrochloride	0.5 g			Manganese-sulphate	0.0025 g
Magnesium-sulphate	0.1 g			Ferric-chloride	0.0025 g
Manganese-sulphate	0.02 g			Bromokresol-green	0.02 g
Glucose	5 g			Agar	15 g
Maltose	10 g			pH	5.5
Fructose	5 g				
Agar	15 g				
pH	6.2				

Table 8: Overview of the varying growth parameters used for MALDI-TOF MS

Nutrient media	Growth time	Oxygen availability
Strains grown under atmospheric oxygen pressure for 48 h on	Strains grown anaerobic in mMRS broth for	Strains grown on mMRS agar for 48 h under
• mMRS agar • NBB agar • OS agar • WLN agar	• 6 h • 12 h • 24 h • 48 h	• Atmospheric oxygen pressure • anaerobic

For the determination of the minimimal inhibitory (MIC) concentration of alpha-acids, a variant of mMRS was used where magnesium and manganese content as well as pH value were adjusted to meet concentrations typically found in beer. This broth was prepared without cysteine at a concentration of 98 mg/L magnesium and 0.16 mg/L manganese and pH was adjusted to 4.3 [Schurr et al., 2013].

2.7 Primer

Table 9: Primers used in this study

Primer	Sequence (5' – 3')	Purpose	Reference
probe 1_F probe1_R	AGT GCA GCC GAA AGT TTA ACT GGG G ACA GCC ACG AGC CAT TGA GCG	Multiplex PCR	AiF project 16292
probe 2_F probe 2_R	TTC ATT CGT GGA ATC CTC GCG CC TCG GGT GAG CTA GCT TTG TCC GAT T	Multiplex PCR	AiF project 16292
probe 3_F probe 3_R	ACA CCG TAC GGG GGA TTG GCT GGG CGC GTG ATT TGT TCG GC	Multiplex PCR	AiF project 16292
probe 4_F probe 4_R	TGC CAA AGA CTG TTT CTC AAC CGC C ACG ACC AAT TTG CCA CAA AAC GCC	Multiplex PCR	AiF project 16292
probe 5_F probe 5_R	CGT TAA CCA TGC GCT TTA GTC CGC ATG CAG CCA AGC CAC CAC CC	Multiplex PCR	AiF project 16292
probe 6_F probe 6_R	CGT CTC AAA GGG TGG CAG AGT CG CAT CGT CCA GCC CTG CGG TG	Multiplex PCR	AiF project 16292
probe 7_F probe 7_R	TTT GTC CCA AGC TAC TTC ATC TGG C TGG GCC ATC CCC TGA GTC GT	Multiplex PCR	AiF project 16292
probe 8_F probe 8_R	CGG TGA CGG GAG GTG TCG TG TGA GCC CAC CAG CCA CTT GC	Multiplex PCR	AiF project 16292
probe 9_F probe 9_R	GCG CCG GAT GGG TTA CGG TT CGG CTG ACC CAC TGG	Multiplex PCR	AiF project 16292

	ACC AC		
probe 10_F probe10_R	GCT GGT TAC GCC AAT ATT TCA CGC C CCG CAA AAA CAT GTA GGT AGC CCC C	Multiplex PCR	AiF project 16292
probe 11_F probe 11_R	GCC AGC ACC TAA CTG GTC CCC CGC GTC AGC TGG GTT GGA GA	Multiplex PCR	AiF project 16292
probe 12_F probe12_R	GAC GCT TCG TTA CAA CAG ATG GCC T GAT TGG CCG GTG CTT GGT CTC T	Multiplex PCR	AiF project 16292
probe 13_F probe 13_R	AAC ATC CTT TCG TAA TTG CT AAA TAA TGG TGT GAC GAT GC	Multiplex PCR	AiF project 16292
probe 14_F probe 14_R	CCC TCT GCC TTT AAG AAG AC TGA TTG CAT TAA GTC ATT TAG C	Multiplex PCR	AiF project 16292
M13V	GTT TTC CCA GTC ACG AC	RAPD PCR	Brosius, 1981
pRH45II_F pRH45II_R	TAT CCG TGC AGT ATC ACG CC GTG AGT CAG GCC GAA GAA CA	Plasmid-specific PCR	This work
pLB925A04_F pLB925A04_R	CGT CCG CGA ATG GGA AAA TC AGT AGG GGA CTT CAT CGG CT	Plasmid-specific PCR	This work
horA_F horA_R	AAT CTT AAC CCT GCC GGT GG TGG ATT CGA GTG GTT GAG CC	horA-specific PCR	Popovic, 2013
616V	AGA GTT TGA TYM TGG CTC AG	16S rDNA sequence analysis	Brosius, 1981
609R	ACT ACY VGG GTA TCT AAK CC	16S rDNA sequence analysis	Brosius, 1981

2.8 Determination of growth kinetics

Growth kinetic curves were recorded on a Sunrise microplate reader at a wavelength of 590 nm. 250 µl of fresh mMRS broth were inoculated at 1 % with a turbid culture in stationary phase (48 h), overlayed with 75 µl of paraffin oil to prevent desiccation and incubated at 30 °C. Absorbance was read every 30 min for 54 h. All experiments were done in triplicate.

2.9 Evaluation of beer-spoiling ability

Beer-spoiling ability was evaluated based on the detection of metabolic activity by resazurin as described previously [Preissler et al., 2010]. In short, strains were pre-cultured in mMRS broth, harvested in late stationary phase and inoculated into a total volume of 200 µl of four different beers (wheat beer with 14 international bitterness units, lager beer with 21 international bitterness units and two different pilsner beers with 32 and 40 international bitterness units) at a final OD of 0.05 (λ=590 nm). After three days and 30 days of incubation at 30 °C, respectively, 5 µl of Tris buffer (0.5 M, pH 8.8) containing resazurin (4.9 mM) were added. Samples were visually inspected for color changes from blue to pink indicating metabolic activity after an hour of incubation. Three biological replicates of each sample were tested and tests were done in duplicate.

2.10 Determination of tolerance to iso-alpha acids

Tolerance to hop bitter compounds was assessed by determination of the MIC for iso-alpha-acids. Strains were pre-cultured in mMRS broth till late stationary phase. MIC values themselves were determined in mMRS broth reaching a pH of 4.3, a magnesium concentration of 98 mg/L and a manganese concentration of 0.16 mg/L, supplemented with varying concentrations of iso-alpha-acids in ethanol. Strains were pre-cultured for 48 hours and inoculated at 0.1 % into the broth containing gradually increasing concentrations of iso-alpha acids from 2 to 20 µM by steps of two. After three days of incubation at 30 °C Tris buffer (0.5 M, pH 8.8) containing resazurin was added to reach a final resazurin concentration of 10.5 mM. Again, positive samples were detected visually by a color change from blue to pink. Two biological replicates of each strain were tested and tests were done in duplicate.

2.11 Plasmid curing

Plasmid curing of *L. brevis* was done by repeatedly sub-culturing organisms in mMRS broth at 37 °C. Fresh broth was inoculated at 1 % every 24 h over a period of four weeks.

2.12 DNA isolation

Strains were cultured overnight in mMRS, harvested at 6000 g for 10 min, washed in TE-buffer, re-suspended in TE-buffer containing 5 mg/ml lysozyme and incubated at 37 °C for one hour. All further steps of DNA extraction were done in accordance with the instructions of the e.Z.N.A Bacterial DNA kit.

2.13 Random-amplified polymorphic DNA (RAPD) PCR

RAPD PCR was carried out using the M13V primer in a total reaction volume of 50 µl containing 50 pmol of the primer, 0.2 mM each dNTP, 3.5 mM $MgCl_2$, 1.25 U Taq polymerase, reaction buffer and 1 µl of isolated DNA. Amplification was done as follows on an eppendorf Mastercycler gradient: 3 cycles at 94 °C for 3 min, 40 °C for 5 min and 72 °C for 5 min followed by 32 cycles at 94 °C for 1 min, 60 °C for 2 min and 72 °C for 3 min. PCR products were mixed with 6x Loading Dye and separated by gel electrophoresis on a 1.2 % (w/v) agarose gel. Lambda DNA/EcoRI + HindIII Marker 3 was used as a molecular weight size marker.

RAPD pattern were analyzed using BioNumerics 6.5. Pearson product-moment correlation was applied to calculate similarity coefficients and Cluster analysis was performed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

2.14 16S rDNA sequence analysis

Verification of species identity was, if required, performed by 16S rDNA sequencing using primers 616V and 609R for PCR in a total reaction volume of 50 µl containing 5 pmol of each primer, 0.1 mM each dNTP, 2.5 mM $MgCl_2$, 0.75 U Taq polymerase, reaction buffer and 1 µl of isolated DNA. Amplification was carried out, applying the following parameters: 94 °C for 2 min, 32 cycles of 94 °C for 45 sec, 52 °C for 90 sec and 72 °C for 2 min followed by a final elongation step of 72 °C for 5 min on an eppendorf Mastercycler gradient. Amplified fragment DNA was purified using Omega bio-tek's cycle pure kit and purified DNA was sequenced at GATC Biotech (Germany). Obtained sequences were submitted to the Basic Local Alignment Search Tool (BLAST) for comparison to published bacterial 16S rDNA sequences.

2.15 horA and plasmid-specific PCR

Primers specific for the horA gene as well as the two *L. brevis* plasmids pLB925A05 and pRH45II can be found in Table 9. Amplification was carried out as described for 16S rDNA sequencing. Amplified PCR fragments were separated on a 1.2 % agarose gel (w/v) using a 100 bp DNA ladder as a molecular weight size marker.

2.16 Multiplex-PCR

Amplification of marker genes which were identified to be specific for beer-spoiling or non-beer-spoiling variants of *L. brevis* in AiF project 16292 was done by multiplex-PCR.

Table 10: Marker genes used for multiplex-PCR and their predicted function. Marker genes amplified by primer mixes 1-4 were associated with a high beer-spoilage potential whereas target sequences of primer mix 5 were associated with a low beer-spoilage potential.

Predicted function of gene	Abbreviation	Size of amplicon	Mix
Competence/damage-inducible protein CinA	probe 1	200 bp	1
LysrR	probe 2	400 bp	1
Sensor kinase protein	probe 3	600 bp	1
Major facilitator superfamily protein	probe 4	300 bp	2
Phosphotransferase system PTS sorbose-specific IIC subunit	probe 5	500 bp	2
GY family cell surface protein	probe 6	700 bp	2
ArsR	probe 7	200 bp	3
Membrane-associated phospholipid phosphatase	probe 8	400 bp	3
Cell wall surface anchor	probe 9	675 bp	3
TetR	probe 10	353 bp	4
Polygalacturonase	probe 11	500 bp	4
Undecaprenyl-phosphate-4-deoxy-4-formamido-L-arabinose-transferase	probe 12	768 bp	4
Aldo/keto-reductase of diketogulonate reductase family	probe 13	850 bp	5
ABC-type multidrug transport system, ATPase component	probe 14	700 bp	5

Multiplex-PCR was carried out in a total reaction volume of 50 µl containing 5 pmol of each primer, 0.1 mM each dNTP, 2.5 mM $MgCl_2$, 0.75 U Taq polymerase, reaction buffer and 1 µl of isolated DNA. Amplification was done as follows: 94 °C for 2 min, followed by 32 cycles of 94 °C for 45 sec, 60 °C for 45 sec for primer mixes 1-4 and 55 °C for 45 sec for primer mix 5 and 72 °C for 2 min followed by 72 °C for another 5 min. Bands were visualized on a 1.2 % (w/v) agarose gel using a 100 bp DNA ladder as molecular weight size marker.

2.17 MALDI-TOF MS analysis

2.17.1 Sample preparation

Six different sample preparation methods were investigated, including direct transfer methods such as cell smears (CS) and cell smears plus ”on-target extraction” by formic acid (CS/FA) as well as cell extraction procedures. For cell smears single colonies were picked from mMRS plates, directly smeared onto a stainless steel target using a toothpick and overlaid with matrix solution containing 10 mg/ml HCCA in acetonitrile, water and trifluoroacetic acid (50:47.5:2.5, v/v). In addition a variant of cell smears which included the application of 1 µl of formic acid directly onto the deposited and air-dried cell material on the target before adding matrix was tested (“on-target extraction”).

Plain cell extraction procedure (CE), cell extraction procedure plus sonication for 30 seconds at 20 kHz (S), cell extraction procedure plus enzymatic treatment with lysozyme at a concentration of 100 mg/ml for 30 min at 37 °C (L) and a combination of enzymatic treatment and sonication as described above (S/L) were performed as well. One full 1 µl loop of biomass of the cultivated strains was suspended in 300 µl of de-ionized water to carry out cell extraction. For liquid cultures cells were harvested from 1 ml of broth after 48 h of culturing by centrifugation, washed with de-ionized water and re-suspended in 300 µl de-ionized water. Bacteria were inactivated by ethanol (70 %) and proteins were extracted using formic acid, de-ionized water and acetonitrile (35:15:50, v/v). Samples were centrifuged to spin down cell debris. Subsequently 1 µl of the obtained supernatant was applied onto a stainless steel target plate, overlaid with 1 µl of matrix solution and air-dried. An overview of sample preparation techniques can be found in Table 11.

All samples were spotted in triplicates. Any mechanical or enzymatic treatment of the cells was applied immediately after suspension of the cells in de-ionized water. Cells treated with lysozyme were washed twice with de-ionized water before they were subjected to the extraction procedure. Cultures in broth were used for sonication and enzymatic treatment. To allow for better comparison between direct transfer methods and cell extraction procedures the latter was carried out using colonies grown on agar plates as well as cultures in broth.

In addition, the application of cell suspensions instead of cell extracts was investigated. Therefore, bacterial isolates on agar plates were processed as described above for cell extraction, but without removal of cell debris by centrifugation.

Table 11: Overview of the different MALDI-TOF MS sample preparation methods

Direct transfer methods	**Cell smears (CS)**	**Cell smears+ "on-target" extraction (CS/FA)**		
Deposition of cell material on target	●	●		
Addition of formic acid		●		
Addition of HCCA	●	●		
Extraction procedures	**Cell extraction (CE)**	**Sonication (S)**	**Lysozyme (L)**	**Sonication + Lysozyme (S/L)**
Suspension of cells	●	●	●	●
Lysozyme treatment			●	●
Sonication		●		●
Inactivation with ethanol	●	●	●	●
Extraction using formic acid and acetonitrile	●	●	●	●
Deposition of extract on target	●	●	●	●
Addition of HCCA	●	●	●	●

2.17.2 Spectra acquisition

Mass spectra were acquired automatically on a Microflex LT MALDI-TOF mass spectrometer equipped with a nitrogen laser (λ= 337 nm). Biotyper Automation Control 2.0 was used to control operation in linear positive ion detection mode. For each sample the mass spectra of 240 laser shots were accumulated in a mass range window from 2000 to 20000 Dalton. Bacterial Test Standard was used for external mass calibration. Samples for the generation of reference database entries (reference spectra) were prepared from one single biological replicate which was spotted eight times on the target and each spot was measured three times. All other samples were prepared from one single biological replicate, which was spotted three times on the target and each spot was measured once. This procedure was applied for the generation of spectra for comparison of different sample preparation techniques, spectra used for species- level identification and test spectra generated for sub-species differentiation of *L. brevis* and *Pectinatus sp.*

2.17.3 Data processing

2.17.3.1 Identification of bacterial samples by MALDI-TOF MS

Sample identification was carried out using MALDI BioTyper 3.0 Software featuring a database of 4111 microbial reference spectra provided by the manufacturer. The database was supplemented with another 87 reference entries of bacteria commonly encountered as spoilage organisms in the brewing and beverage industry, belonging mainly to the genera *Acetobacter, Lactobacillus, Leuconostoc, Megasphaera, Pectinatus* and *Pediococcus*. The reliability of identification was judged based on the log-score which takes into account the similarity between the spectrum of the unknown organism and the reference spectrum, the similarity between the reference spectrum and the spectrum of the unknown organism and the correlation of intensities of the matching peaks. The resulting score between 0 and 1000 is transformed into a log-score between 0 and 3. A log-score >2.0 indicated a successful identification of a sample on the species level, in accordance with the manufacturer’s instructions. Log-scores between 1.7 and 2.0 were interpreted as identification of the respective genus. No second hit, representing another species or

genus, respectively, within the database exceeding a log-score of 2.0 for species identification or 1.7 for genus level identification was tolerated.

2.17.3.2 Principal Component Analysis (PCA) of MALDI-TOF MS spectra

PCAs were generated in Clin ProTools 2.2.

2.17.3.3 Peak counting and generation of MALDI-TOF MS sum spectra based on LIMPIC

To identify the preparation technique yielding good quality spectra with a maximum information content, the total numbers of reproducible peaks within replicate raw spectra were determined. Therefore a computational method based on LIMPIC (linear MALDI-TOF-MS peak indication and classification) preprocessing, where peak picking parameters were set to a signal to noise ratio of 3 and a peak width of 0.5 Da [Mantini et al., 2007], was applied. A total number of 30 single spectra per strain and method, acquired from ten biological and three technical replicates, each, were merged to one spectrum using LIMPIC. In order to allow only peaks present in at least 80 percent of the single spectra to be included in the sum spectrum, the minimum accepted peak detection rate, referring to the ratio between the number of spectra containing a certain peak and the total number of analyzed spectra, was set to 0.8 [Mantini et al., 2007]. All peaks with an intensity of at least 1000 arbitrary units (a.u.), reaching an effective range of up to approx. 5×10^4 a.u., in the mass range between 3000 and 12000 Da were counted and assigned to five intervals based on their intensity.

2.17.3.4 Classification of MALDI-TOF MS spectra according to environmental conditions

Clin ProTools 2.2, a bioinformatics software, tool originally developed to search for biomarkers in clinical applications [Ketterlinus et al., 2005], was used to detect stable biomarkers which arose from different environmental conditions during culturing. A total of 30 spectra per strain and condition were acquired under varying

environmental conditions from ten biological replicates and three technical replicates each. Spectra were consequently randomly split into two data sets but without separating the three technical replicates derived from one biological replicate. All three technical replicates were used exclusively for the same set. The first set was used to create a reference class and a prediction model for the respective condition. Thirty percent of spectra, referring to 9 spectra per strain and condition (total of 270 spectra) were excluded from model generation and used for validation. Classification of this second set of spectra was performed to check the capability of the obtained class prediction. Four different algorithms incorporated in ClinProTools 2.2 namely Genetic Algorithm (GA), Support Vector Machine Algorithm (SVM), Supervised Neural Network Algorithm (SNN) and QuickClassifier Algorithm (QC) were used for model generation.

2.17.3.5 Sub-typing of bacterial strains by MALDI-TOF MS

MALDI-TOF MS raw data were exported using FlexAnalysis 3.3 as .dat-files. All consecutive processing steps were carried out, using an in-house software application based on MASCAP [Mantini et al., 2010], implemented in octave [Eaton and Rawlings, 2003] and run in parallel on an open sharedroot computer cluster (ATIX; http://opensharedroot.org). A message passing interface (MPI) was applied for job control [Gabriel et al., 2004] and software pipelines were constructed by the use of BASH (http://www.gnu.org/software/bash) scripts.
Peak processing and detection was carried out according to Mantini et al. [2010]. The distance tolerance limit for peak alignment and clustering was set to 600 ppm. For the creation of sum spectra out of the single test spectra, a peak detection rate of 0.4 was applied in order to include only peaks at least present in 40 % of all single spectra in the resulting sum spectrum.

2.17.3.6 Identification of biomarkers in MALDI-TOF MS spectra

Test spectra of *L. brevis* strains were checked for potential biomarkers to delineate strongly beer spoiling strains. The minimum accepted peak detection rate, was set to 0.4 in order to allow only peaks present in at least 40 percent of the single spectra to

be included for biomarker detection. To ensure that only peaks showing reasonably stable intensities were considered as potential biomarkers, minimal signal intensity of peaks possibly serving as biomarkers was limited to 50 % of the average peak intensity of the respective spectra. The maximum standard deviation for signal intensity was set to 20 %. No neighboring peaks were allowed within a mass range of 1000 ppm and potential biomarkers were tolerated to be detectable in no more than 10 % of the spectra acquired from the respectively opposite group to assure exclusivity of biomarkers.

2.17.3.7 Analysis of similarity (ANOSIM)

ANOSIM was applied to test for significant differences between groups. It compares the ranks of distances between groups to the ranks of distances within groups based on their means, where the resulting R test statistic reflects no separation of groups (R=0) or separation of groups (R=1). R values equal to or larger than 0.75 are considered to represent a good separation of groups [Clarke and Green, 1988; Ramette, 2007].

2.17.3.8 Comparative analysis of the beer spoilage potential of *Lactobacillus brevis* isolates

Classification of the isolates was primarily based on a growth assay using resazurin for faster detection. Growth in the different beers was assessed as follows: growth in pilsner beer I after three days (factor 1.5), growth in pilsner beer I after 30 days (factor 1.2) and growth in pilsner beer II after 30 days (factor 1.5) were given more weight than growth in wheat beer or lager beer at any point of time (factor 1). Similarities were calculated based on the Minkowski distance and visualized using high-throughput multidimensional scaling (HiT-MDS) [Strickert et al. 2007]. Isolates were grouped according to their spoilage potential and differences between groups were tested for their statistical significance by ANOSIM.

Likewise, similarity matrices were calculated based on the presence of gene markers or MALDI-TOF MS spectra using the mean Manhattan distance and were again visualized by HiT-MDS.

Additionally, Discriminant Analysis of Principal Components (DAPC) was done for MALDI-TOF MS spectra of the *L. brevis* isolates. In contrast to PCA, which focuses on total variance, thus describing global diversity and neglecting differences between groups, DAPC optimizes variance between groups while minimizing variance within groups. Therefore, synthetic variables are sought, which optimally reflect differences between groups, while minimizing variation within groups [Jombart, 2013]. To employ DAPC the adegenet package for R was used. Intensities of single test spectra were log-transformed to reduce the influence of intensity variations on the DAPC groups.

2.17.4 Validation of MALDI-TOF MS for fast and reliable identification of industrial bacterial samples

To confirm validity of MALDI-TOF MS identification results for industrial bacterial samples, 671 samples from the brewing industry, which were collected at the BLQ, were analyzed and identified by MALDI-TOF MS. Beverage, air, swab, yeast, wort and water samples as well as colonies from membrane-filters on agar, were additionally routinely identified at BLQ. BLQ used the commercially available Biotecon foodproof beer screening kit (Biotecon, Germany) to distinguish the following organisms: *L. backii, L. brevis, L. buchneri/parabuchneri, L. casei/paracasei, L. collinoides, L. coryniformis/pentosus/plantarum/paraplantarum, L. harbinensis/perolens, L. lindneri, L. rossiae, Megasphaera cerevisiae, Megasphaera sueciensis/paucivorans, Pectinatus sp., Pediococcus claussenii, Pediococcus damnosus, Pediococcus inopinatus* and *Pediococcus parvulus/pentosaceus/acidilactici*. Species separated by slashes cannot be differentiated and are identified as a group by the PCR kit. Differentiation between *L. perolens* and *L. harbinensis* based on the PCR kit is currently validated and has therefore not been taken into account. Organisms, which were not detectable and thus identifiable using the PCR kit, were identified by 16S rDNA sequencing.

Anonymized liquid samples, which had been pre-enriched for PCR at the BLQ, were streaked on mMRS agar [Stolz et al., 1993] prior to MALDI-TOF MS analysis, samples on agar plates were directly subjected to MALDI-TOF MS.

Samples were processed as described above for cell smear preparation technique. In rare occasions direct application of whole cells did not yield spectra of sufficient

quality to allow identification of the isolates. These samples were processed according to the simple extraction protocol before analysis was repeated.

3 Results

3.1 Evaluation of beer-spoilage ability of *Lactobacillus brevis*

Growth of strains TMW 1.100, 1.1205, 1.1326, 1.1369, 1.1370, 1.1371, 1.230, 1.240, 1.302, 1.313, 1.315, 1.317, 1.465, 1.485, 1.6, 1.841 and 1.939 in four different beers was determined based on the detection of metabolic activity by resazurin. Samples were checked after 3 and 30 days of incubation at 30 °C respectively (Table 12). Strain TMW 1.6 was unable to grow in any of the tested beers. Strains TMW 1.1369 and 1.939 were positive for metabolic activity in wheat beer only after 30 days and showed no signs of activity in lager beer or pilsner beer. Strains TMW 1.100, 1.1205, 1.1326, 1.1369, 1.1371 and 1.841 showed growth in wheat beer after three days but were unable to grow in any of the other beers within 30 days. Strains only capable of growing in wheat beer were classified as weak spoilage organisms. Strains TMW 1.1370 and 1.302 showed growth in wheat beer after three days as well as in lager beer only after 30 days of incubation. Thus, they were regarded as strains with a moderate potential to spoil beer. All strains, which were at least capable to grow in lager beer within three days and were positive for growth in at least one of the two pilsner beers after 30 days, were considered to possess a high spoilage potential.

Table 12: Growth of *L. brevis* strains in four different beers. Results are shown after 3 days/30 days of incubation.

strain	wheat beer	lager beer	pilsner beer I	pilsner beer II
TMW 1.100	+/+	-/-	-/-	-/-
TMW 1.1205	+/+	-/-	-/-	-/-
TMW 1.1326	+/+	-/-	-/-	-/-
TMW 1.1369	-/+	-/-	-/-	-/-
TMW 1.1370	+/+	-/+	-/-	-/-
TMW 1.1371	+/+	-/-	-/-	-/-
TMW 1.230	+/+	+/+	-/+	-/+
TMW 1.240	+/+	+/+	-/+	-/+
TMW 1.302	+/+	-/+	-/-	-/-
TMW 1.313	+/+	+/+	+/+	-/+
TMW 1.315	+/+	+/+	-/+	-/+
TMW 1.317	+/+	+/+	-/+	-/+
TMW 1.465	+/+	+/+	-/+	-/+
TMW 1.485	+/+	+/+	-/+	-/+
TMW 1.6	-/-	-/-	-/-	-/-
TMW 1.841	+/+	-/-	-/-	-/-
TMW 1.939	-/+	-/-	-/-	-/-

3.2 Determination of *Lactobacillus brevis'* tolerance to iso-alpha acids

Minimal inhibitory concentrations of iso-alpha acids were determined based on the detection of metabolic activity by resazurin in mMRS supplemented with gradually increasing concentrations of iso-alpha acids by steps of two. Mean values calculated from two biological replicates analyzed in duplicate can be found in Table 13.

Table 13: Minimal inhibitory concentrations of iso-alpha acids [µM] of *L. brevis* strains

strain	mean value	standard deviation
TMW 1.100	9.0	1.4
TMW 1.1205	8.5	0.7
TMW 1.1326	5.0	1.4
TMW 1.1369	2.0	-
TMW 1.1370	7.0	1.4
TMW 1.1371	4.0	-
TMW 1.230	16.5	0.7
TMW 1.240	15.5	3.5
TMW 1.302	5.0	1.4
TMW 1.313	>20.0	-
TMW 1.315	17.5	3.5
TMW 1.317	>20.0	-
TMW 1.465	>20.0	-
TMW 1.485	7.5	0.7
TMW 1.6	4.7	1.4
TMW 1.841	6.0	2.8
TMW 1.939	7.0	1.4

3.3 Computer-assisted analysis of RAPD-PCR patterns of *Lactobacillus brevis*

RAPD-PCR based on the M13V-primer was employed to discriminate 17 *L. brevis* strains and provide a genotypical reference method to compare similarities of strains. Using UPGMA for clustering, four groups at a similarity level of 75 % were identified. Group one consisted of two subgroups at a similarity level of ≥ 85 %. The first subgroup included strains TMW 1.1326, 1.1369 and 1.6 whereas the second subgroup was comprised of strains TMW 1.100, 1.1205, 1.841 and 1.939 (Figure 1).

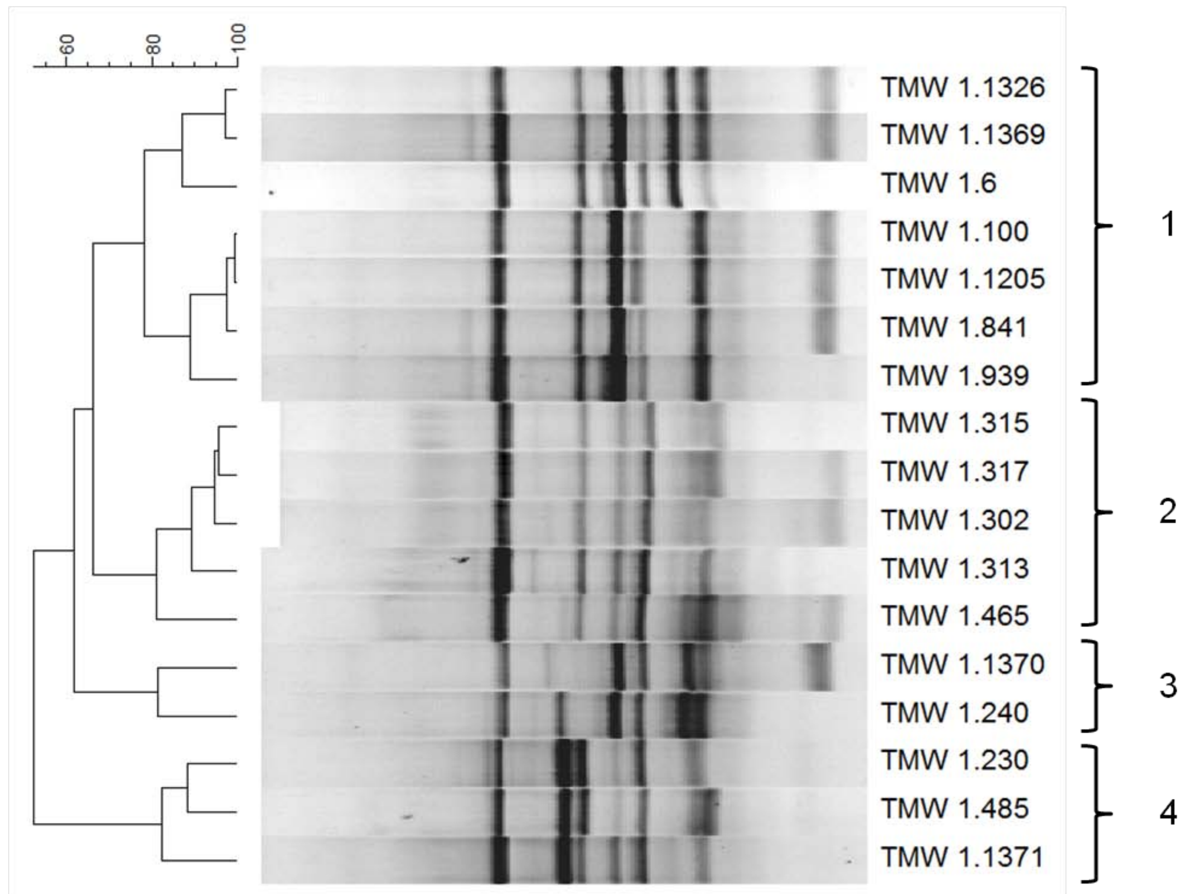

Figure 1: Cluster analysis of the RAPD pattern of seventeen *L. brevis* strains using UPGMA.

All strains found in group one were thus derived from non-brewing environments. Group two consisted of strains TMW 1.315, 1.317, 1.302, 1.313 and 1.465, all of them being brewery isolates. Group three included only two strains, TMW 1.1370 and 1.240. Group four was comprised of the three strains TMW 1.230, 1.485 and 1.1371. Groups three and four combined both brewing and non-brewing isolates of *L. brevis*.

3.4 MALDI-TOF MS

3.4.1 Optimization of MALDI-TOF MS sample preparation

Three different organisms, *L. brevis, Ln. mesenteroides* and *Pc. claussenii*, all originating from beverages, were used to assess the influence of sample preparation techniques on MALDI-TOF MS spectra. Different sample preparations were investigated including direct transfer techniques as well as extraction procedures. In

addition to plain direct transfer, where whole cells are applied onto the stainless steel target and directly overlaid with matrix, an “on-target extraction” was carried out requiring the addition of formic acid before matrix is applied. Next to a standard extraction procedure, the influence of mechanical or enzymatical treatment or a combination of both on the acquired spectra, was tested. Comparison of spectra was based on the total amount of reproducible peaks found within spectra derived from a particular preparation method and their intensities which were calculated by LIMPIC processing with subsequent peak counting and intensity classification. Table 14 shows the resulting numbers of peaks for all strains and sample preparation techniques.

Table 14: Number of reproducible peaks obtained from different sample preparation techniques. CS refers to plain cell smears, CS/FA to “on-target extraction”, CE/B to cell extraction from cultures grown in broth, CE/P to cell extraction from colonies grown on plates, S to cell extraction plus sonication as a pre-treatment, L to cell extraction plus lysozyme treatment and S/L to cell extraction plus both, lysozyme and ultrasonic treatment. Peak classes are sorted by normalized intensity.

Pc. claussenii	**CS**	**CS/FA**	**CE/B**	**CE/P**	**S**	**L**	**S/L**
Total ≥ 1000	**52**	**70**	**65**	**63**	**55**	**48**	**53**
> 1000 – 3000	23	38	33	33	33	25	30
> 3000 – 6000	18	13	20	17	12	11	11
> 6000 – 10000	5	9	4	6	3	4	4
> 10000 – 15000	5	6	3	1	4	3	3
> 15000	1	4	5	6	3	5	5
L. brevis							
Total ≥ 1000	**45**	**40**	**53**	**50**	**51**	**33**	**33**
> 1000 – 3000	17	13	22	29	25	22	19
> 3000 – 6000	15	15	19	12	19	5	8
> 6000 – 10000	13	12	7	5	3	3	2
> 10000 – 15000	0	0	3	3	4	1	2
> 15000	0	0	2	1	0	2	2
Ln. mesenteroides							
Total ≥ 1000	**65**	**70**	**54**	**62**	**59**	**46**	**55**
> 1000 – 3000	28	31	25	31	34	23	31
> 3000 – 6000	16	18	15	14	11	11	10
> 6000 – 10000	8	7	6	7	7	5	5
> 10000 – 15000	8	10	5	8	5	3	4
> 15000	5	4	3	2	2	4	5

Spectra acquired from *Pc. claussenii* and *Ln. mesenteroides* showed the maximum amount of reproducible peaks when samples were subjected to “on-target extraction”. This technique however performed inferior when *L. brevis* was analyzed where it did not only result in a decrease in the total amount of peaks compared to other sample preparation techniques but also impaired automatic spectra acquisition, necessitating manual user interaction to ensure successful measurements.

Neither the application of lysozyme nor sonication enhanced performance with regard to the number of reproducible peaks obtained. Compared to cell extractions without any additional mechanical or enzymatical treatments, lysozyme lead to a decrease in total numbers of peaks for all three strains. Cells of other organisms treated with lysozyme were reported to show peaks with higher intensities [Giebel et al., 2008]). These findings were not found to be applicable for *L. brevis*, *Ln. mesenteroides* and *Pc. claussenii* as no shift of signals to higher intensities and thus no beneficial effects of lysozyme on spectra were observed. In contrast, intense signals deriving from the single or double charged molecule ions of the enzyme may interfere with the detection of less intensive peaks which result from the bacterial protein subset accessible to MALDI-TOF MS and may thus adversely affect spectra. Use of sonication slightly increased the amount of peaks found in spectra of *Ln. mesenteroides* compared to simple cell extraction. No such effect could be confirmed for *L. brevis* and *Pc. claussenii,* where numbers of peaks tended to decrease when cells were subjected to sonication. The combination of both sonication and lysozyme treatment did not perform superior to the simple cell extraction procedure either.

As the use of cell suspensions instead of cell extracts was shown to improve signal to noise ratio of peaks [Šedo et al., 2011], analysis of cell suspensions was conducted, to assess the influence on the acquired protein peak pattern. The number of reproducibly found peaks as well as peak pattern remained largely constant for *Ln. mesenteroides* and *Pc. claussenii.* For *L. brevis* however a clear increase in the total number of reproducible peaks was observed (Figure 2).

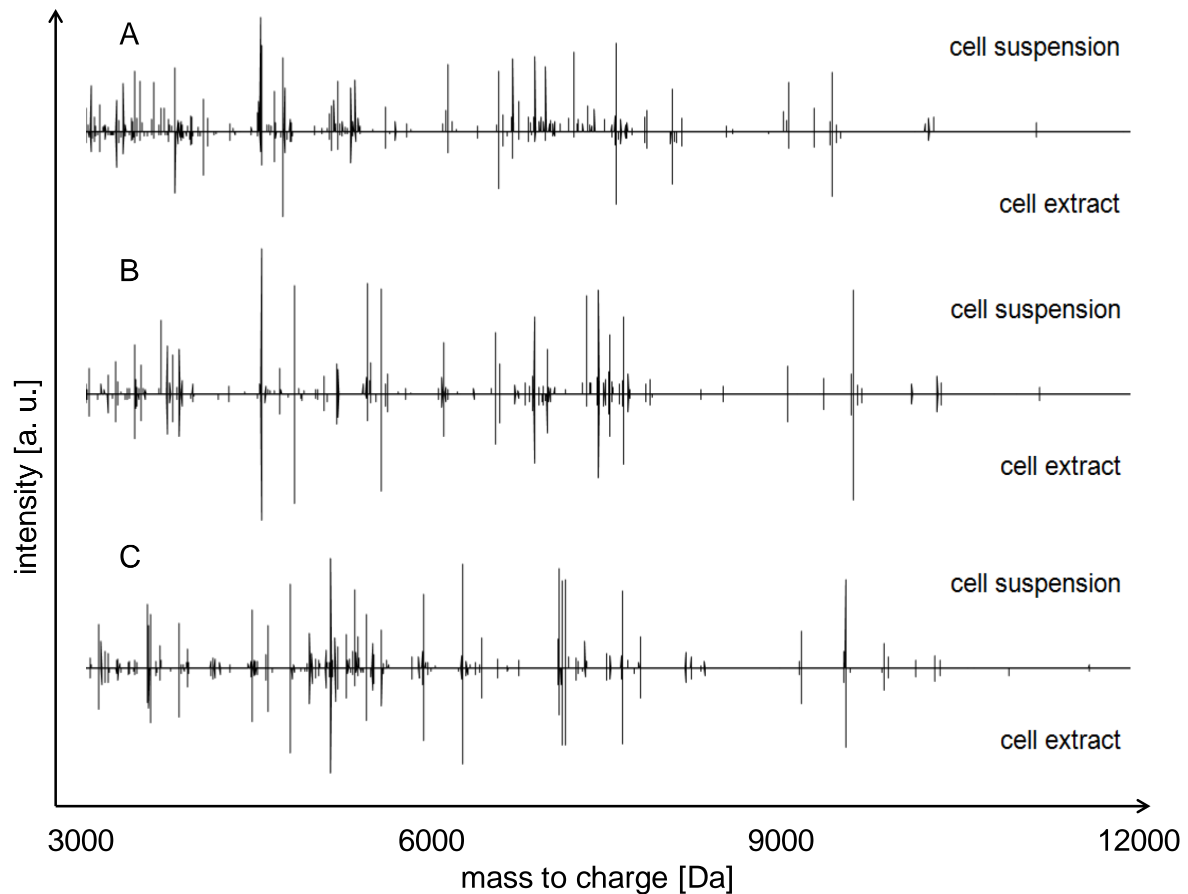

Figure 2: Pattern of reproducible peaks in the mass range from 3000 to 12000 Da for *L. brevis* (A), *Pc. claussenii* (B) and *Ln. mesenteroides* (C) as obtained from cell suspensions (regular) or cell extracts (inversely).

Based on the obtained results from different sample preparation techniques, if not indicated else, simple protein extraction without any mechanical or enzymatical treatment was applied for all subsequent analysis. As MALDI-TOF MS spectra have been shown to be less susceptible to variances due to growth stage if organisms are grown on agar plates instead of broth [Salaün et al., 2010; Vargha et al., 2006], and cells grown in broth did not perform better in any of the conducted experiments, strains were grown on agar plates. Solemnly the influence of growth time on MALDI-TOF MS spectra was assessed using cells grown in broth.

3.4.2 Influence of growth conditions on MALDI-TOF MS spectra

Nutrient media (MRS, NBB, OS and WLN agar), growth time (6 h, 12 h, 24 h and 48 h) and availability of oxygen (aerobic and anaerobic) were varied to check the influence of growth conditions on MALDI-TOF MS spectra. At first, reliability of identification of microorganisms was tested under varying growth parameters. Acquired spectra showed only minor differences in their protein peak pattern and were thus reliably identified on the species level regardless of the growth conditions used, when spectra were compared to a database containing a reference spectrum of the same strain grown under standardized conditions (mMRS agar, 48 h, aerobic). The average of the achieved log-scores varied among different culture conditions, but was, however, not found to be considerably higher, when strains were grown under the same standardized conditions, which were used for the generation of the reference spectra.

Table 15: Average, minimum and maximum log-scores obtained from spectra generated under varying culture conditions. Log-scores from 0 to 1.699 indicate no reliable species identification, log-scores from 1.700 to 1.999 indicate probable genus identification, log-scores from 2.000 to 2.299 indicate secure genus and probable species identification and values equal or greater than 2.300 indicate highly probable species identification.

L. brevis	**Average log-score**	**Minimum log-score**	**Maximum log-score**
Nutrient media			
MRS agar	2.717	2.620	2.803
NBB agar	2.671	2.567	2.789
OS agar	2.546	2.371	2.628
WLN agar	2.653	2.442	2.795
Growth time			
6 h	2.378	2.306	2.515
12 h	2.359	2.262	2.447
24 h	2.420	2.253	2.510
48 h	2.472	2.359	2.576
Oxygen availability			
aerobic	2.717	2.620	2.803
anaerobic	2.716	2.625	2.777
Ln. mesenteroides			
Nutrient media			
MRS agar	2.650	2.143	2.792

NBB agar	2.492	2.222	2.544
OS agar	2.423	2.255	2.544
WLN agar	2.520	2.155	2.708
Growth time			
6 h	2.465	2.209	2.698
12 h	2.567	2.189	2.761
24 h	2.644	2.163	2.791
48 h	2.506	2.088	2.659
Oxygen availability			
aerobic	2.675	2.369	2.836
anaerobic	2.704	2.443	2.818
Pc. claussenii			
Nutrient media			
MRS agar	2.644	2.520	2.746
NBB agar	2.597	2.425	2.663
OS agar	2.504	2.402	2.616
WLN agar	2.600	2.374	2.713
Growth time			
6 h	2.540	2.346	2.702
12 h	2.590	2.422	2.731
24 h	2.607	2.473	2.738
48 h	2.560	2.416	2.697
Oxygen availability			
aerobic	2.644	2.520	2.746
anaerobic	2.628	2.526	2.706

As species level identification proved to be insusceptible to the applied growth conditions, it was assumed that the majority of peaks was stably found under all conditions. To check this hypothesis, sum spectra were calculated similarly to those used for comparison of sample preparations except for the pdr being lowered to 0.6 instead of 0.8 and no intensity threshold was applied. The resulting spectra, which represented 30 single measurements per strain and condition, were checked for common and distinguishing peaks (Table 16, Table 17, Table 18). Spectra showed up to 53 protein signals which were only observed under a certain condition. Spectra were found to have between 67 and 91 peaks in common, if four conditions were compared to each other (growth medium and growth time). If only two conditions (availability oxygen) were compared, number of common peaks was naturally higher, ranging from 115 to 139 depending on the strain under investigation. While peaks found in all spectra obtained from a certain strain under varying growth conditions

exhibited maximum intensities between 17408 and 49381arbitrary units [a.u.], peaks which were distinctively present only in spectra acquired from organisms grown under specific conditions generally showed maximum peak intensities which were about one order of magnitude lower.

Table 16: Number of common and discriminating peaks obtained from strains grown on different media. Peaks were present in at least 60 percent of all spectra acquired from one strain and condition, within a mass range from 3000 to 12000 Dalton.

L. brevis	**MRS**	**NBB**	**OS**	**WLN**
Number of common peaks	91			
Maximum intensity of common peaks [a.u.]	23207			
Number of discriminating peaks	15	18	15	5
Maximum intensity of discriminating peaks [a.u.]	2059	2546	2195	2494
Ln. mesenteroides				
Number of common peaks	67			
Maximum intensity of common peaks [a.u.]	34569			
Number of discriminating peaks	15	12	11	13
Maximum intensity of discriminating peaks [a.u.]	2414	1106	2678	1144
Pc. claussenii				
Number of common peaks	69			
Maximum intensity of common peaks [a.u.]	49381			
Number of discriminating peaks	14	6	12	4
Maximum intensity of discriminating peaks [a.u]	1540	1368	4058	1534

Table 17: Number of common and discriminating peaks obtained from strains harvested in different growth phases. Peaks were present in at least 60 percent of all spectra acquired from one strain and condition, within a mass range from 3000 to 12000 Dalton.

L. brevis	**6 h**	**12 h**	**24 h**	**48 h**
Number of common peaks	71			
Maximum intensity of common peaks [a.u.]	17408			
Number of discriminating	33	33	47	43

peaks				
Maximum intensity of discriminating peaks [a.u.]	2459	1723	1598	1859
Ln. mesenteroides				
Number of common peaks	71			
Maximum intensity of common peaks [a.u.]	31489			
Number of discriminating peaks	43	17	53	42
Maximum intensity of discriminating peaks [a.u.]	2369	1371	1754	2553
Pc. claussenii				
Number of common peaks	74			
Maximum intensity of common peaks [a.u.]	23627			
Number of discriminating peaks	43	20	21	33
Maximum intensity of discriminating peaks [a.u]	1942	1195	1135	2152

Table 18: Number of common and discriminating peaks obtained from strains grown under varying oxygen availabiliy. Peaks were present in at least 60 percent of all spectra acquired from one strain and condition, within a mass range from 3000 to 12000 Dalton.

L. brevis	**aerobic**	**anaerobic**
Number of common peaks	139	
Maximum intensity of common peaks [a.u.]	26292	
Number of discriminating peaks	34	35
Maximum intensity of discriminating peaks [a.u.]	2817	2985
Ln. mesenteroides		
Number of common peaks	124	
Maximum intensity of common peaks [a.u.]	26987	
Number of discriminating peaks	22	38
Maximum intensity of discriminating peaks [a.u.]	1250	9871
Pc. claussenii		
Number of common peaks	115	
Maximum intensity of common peaks [a.u.]	27854	
Number of discriminating peaks	17	24
Maximum intensity of discriminating peaks [a.u]	1494	3515

In a second approach, spectra were examined in more detail to elucidate if adaptation to different growth conditions was reflected consistently in MALDI-TOF MS fingerprints. Four different algorithms incorporated in ClinProTools 2.2, Genetic

Algorithm (GA), Support Vector Machine Algorithm (SVM), Supervised Neural Network Algorithm (SNN) and QuickClassifier Algorithm (QC) were used to investigate whether spectra could be reliably assigned to the applied culture conditions. Classification of spectra according to the respective growth condition is shown in Figure 3 for all tested algorithms.

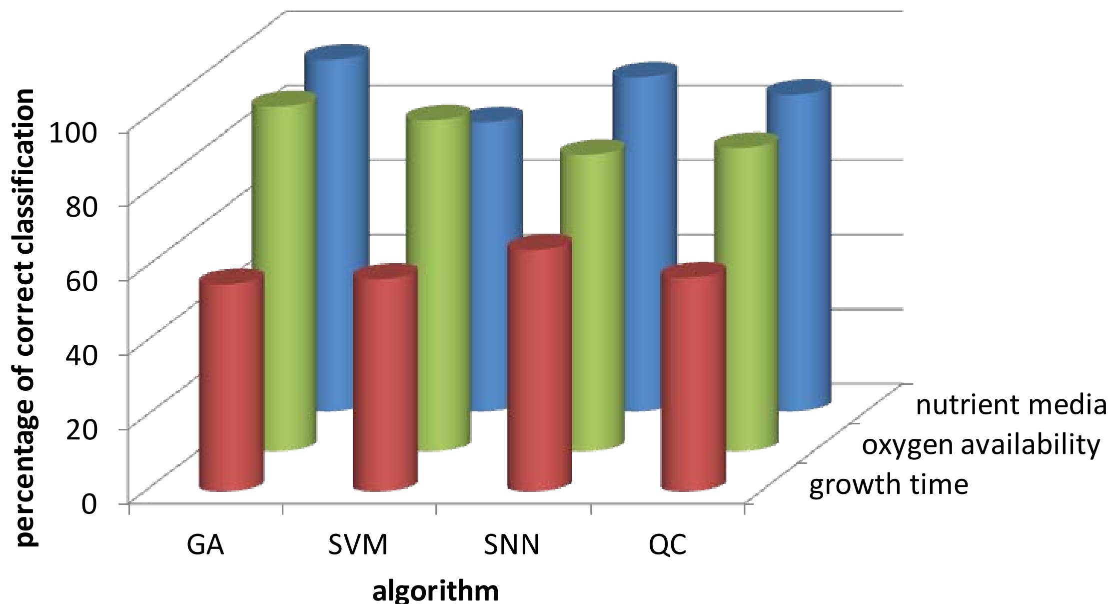

Figure 3: Percentage of correct classifications of single spectra of the three tested strains as achieved for growth time oxygen availability and nutrient media using four different algorithms for data processing: Genetic Algorithm (GA), Support Vector Machine Algorithm (SVM), Supervised Neural Network Algorithm (SNN) and QuickClassifier Algorithm (QC).

GA correctly assigned 94.4 % of spectra to the medium used for culturing strains while SVM (77.6 %), SNN (89.7 %) and QC (85.0 %) performed slightly inferior. The prevailing oxygen availability was successfully predicted in 92.6% of all spectra by GA, 88.9 % by SVM, 79.6 % by SNN and 81.5 % by QC. Percentages of correct classification were significantly lower when growth time had to be predicted, ranging from 55.6 % (GA) to 64.8 % (SNN). Overall, the pattern recognition model applying GA was considered to be the most capable and accurate one among the tested approaches to predict growth conditions of microorganisms based on their protein profiles. It delivered the maximum amount of correct classifications for the type of

nutrient media and the availability of oxygen. Still results derived from GA were least efficient in determining growth time. Closer examination of the data showed that for *L. brevis* and *Pc. claussenii* 88 % of all misclassifications of growth time resulting from GA occurred either between the points 6 h (lag phase/early exponential phase) and 12 h (exponential phase) or 24 h (beginning stationary phase) and 48 h (late stationary phase) demonstrating a certain potential to differentiate between early growth phases (exponential) and late growth phases (stationary). Results from *Ln. mesenteroides* however showed misidentifications among the points 12 h, 24 h and 48 h (Table 19).

Table 19: Itemization of misidentifications as obtained from Genetic Algorithm for varying growth times. Numbers are expressed as a percentage.

L. brevis	predicted as			
Actual growth time	6 h	12 h	24 h	48 h
6 h	22	78		
12 h	33	67		
24 h			0	100
48 h	11		89	0
Ln. mesenteroides	predicted as			
Actual growth time	6 h	12 h	24 h	48 h
6 h	100			
12 h		0	100	
24 h			100	
48 h		78		22
Pc. claussenii	predicted as			
Actual growth time	6 h	12 h	24 h	48 h
6 h	22	78		
12 h		44	56	
24 h			100	
48 h				100

3.4.3 Sub-species differentiation of beer-spoiling bacteria by MALDI-TOF MS

3.4.3.1 Subtyping of *Lactobacillus brevis* strains

Seventeen strains of *L. brevis* were analyzed by MALDI-TOF MS. To investigate whether MALDI-TOF MS allows the differentiation of *L. brevis* strains a reference database entry of each strain was created based on 24 spectra measured from one biological replicate. Four more biological replicates were analyzed in triplicate each to generate a test set of twelve spectra per strain, adding up to a total of 204 test

spectra. Test spectra were used to run a database search against the deposited 17 reference entries. Out of 204 spectra 185 were correctly assigned to their respective reference entry (90.7 %). Strains TMW 1.1369, 1.1371, 1.230, 1.240, 1.302, 1.313, 1.317, 1.465, 1.485, 1.6, 1.841 and 1.939 were correctly matched at every single measurement. All test spectra of strain TMW 1.1205 were constantly misclassified as strain TMW 1.100 representing 63.2 % of all misclassifications. Among the twelve test spectra of strain 1.315, four were falsely assigned to the reference spectrum of strain 1.317. One spectrum with each of the strains TMW 1.100, 1.1326 and 1.1370 was falsely classified as TMW 1.1205, 1.841 and 1.100, respectively. As visualized in the gel view in Figure 4, test spectra of different *L. brevis* strains in general show a high similarity in peak pattern, which provides the basis for identification purposes on the species level. Still, a low number of highly reproducible peaks were found to exclusively appear in spectra of only one strain or a group of strains, which allowed differentiation of the strains. Strains TMW 1.6, 1.1371, 1.313, 1.230, 1.465, 1.317, 1.315, 1.240, 1.939, 1.1369 and 1.1326 showed strain specific signals which were visually detectable at 2902.9, 3620.3, 5188.4, 6312.6, 4869.2, 2778.3, 2934.7, 5589.9, 6912.2, 4015.6 and 10993.7 m/z respectively and are indicated by white arrows in Figure 4.

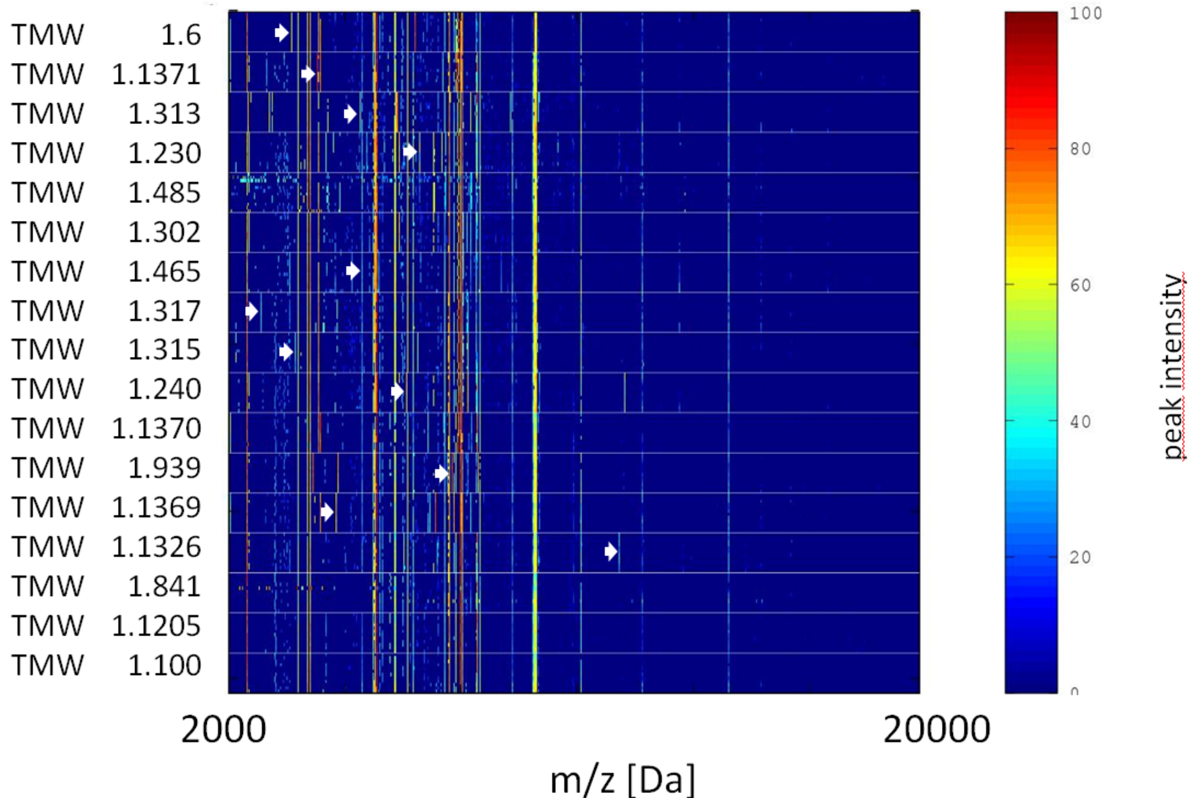

Figure 4: Side by side gel view of the twelve MALDI-TOF MS test spectra acquired per strain in the mass range from 2000 to 20000 Da. Colour coding refers to the intensity of peaks from red (maximum intensity) to blue (minimum intensity). White arrows indicate strain specific signals and continuous lines show signals which were detectable in all spectra.

Additionally cluster analysis was performed on the reference database spectra to visualize similarities between spectra of different strains of *L. brevis*. Cluster analysis split the strains into two groups (Figure 5). One of the groups consisted solemnly of strains isolated from non-brewing environments (TMW 1.939, 1.1369, 1.1326, 1841, 1.1205 and 1.100). The other group was comprised of two subgroups. The first subgroup included all strains isolated from brewery environments (TMW 1.313, 1.230, 1.485, 1.302, 1.465, 1.317, 1.315 and 1.240) plus strain TMW 1.1370. The second subgroup consisted of only two strains derived from non-brewing isolates.

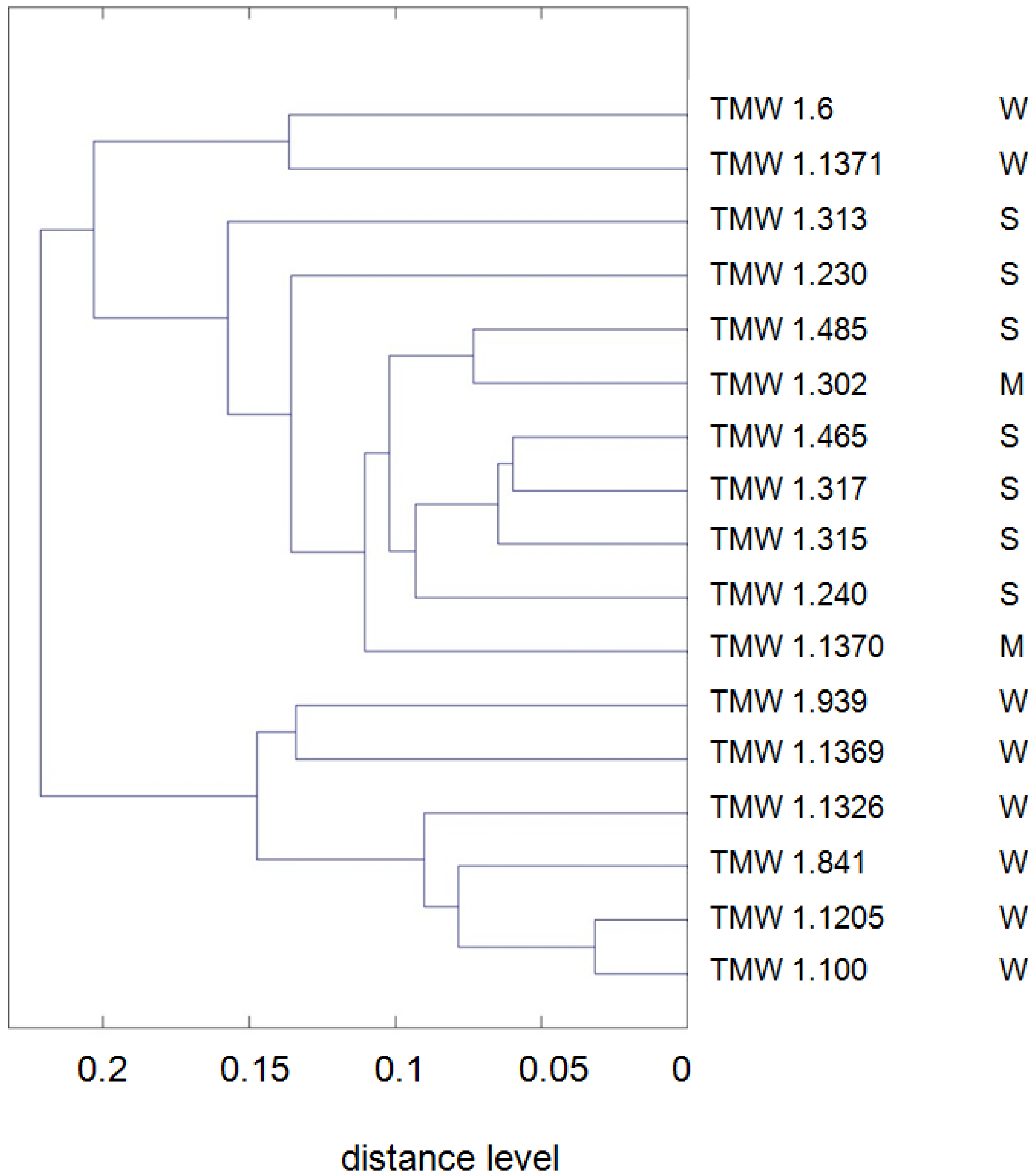

Figure 5: Cluster analysis of the MALDI-TOF MS reference database spectra of seventeen *L. brevis* strains were coded according to their beer spoilage potential. W refers to strains showing a weak spoilage potential, M represents strains with a moderate spoilage potential and S indicates a strong spoilage potential. Distance level indicates similarity of spectra ranging from 0 (identical spectra) to 1 (maximum variability).

However, applying the previously described criteria (see section 2.17.3.6) for biomarker detection, no biomarkers were discovered neither for strains representing

a weak beer spoilage potential nor for strains that showed a strong beer spoilage potential.

3.4.3.2 Subtyping of *Pectinatus* sp. isolates

Nineteen isolates of *Pectinatu*s sp., TMW 2.1487-2.1505, were analyzed by MALDI-TOF MS and results were compared to rep-PCR profiles of the isolates as published previously by Suiker et al. [2007].

Table 20: Rep-PCR profiles of *Pectinatus* sp. isolates [adapted from Suiker et al., 2007]

strain	origin	rep-PCR profile
TMW 2.1487 (140)	brewery 1	4a2
TMW 2.1488 (160)	brewery 2	4b2
TMW 2.1489 (169)	brewery 2	4b2
TMW 2.1490 (173)	brewery 3	4b1
TMW 2.1491 (175)	brewery 4	4a2
TMW 2.1492 (225)	VTT	4a2
TMW 2.1493 (227)	VTT	4d1
TMW 2.1494 (228)	VTT	2
TMW 2.1495 (232)	VTT	3b1
TMW 2.1496 (233)	VTT	1
TMW 2.1497 (237)	brewery 5	4c2
TMW 2.1498 (238)	brewery 5	4b1
TMW 2.1499 (239)	brewery 5	4c2
TMW 2.1500 (240)	brewery 5	4c2
TMW 2.1501 (256)	brewery 6	4b2
TMW 2.1502 (259)	brewery 7	4a2
TMW 2.1503 (260)	brewery 7	4a1
TMW 2.1504 (264)	brewery 7	4a1
TMW 2.1505 (389)	-	-

Reference and test spectra were acquired as described for *L. brevis* in section 3.4.3.1. Sub-species level differentiation was again carried out by means of an in-house software application. Reference entries were created for all 19 isolates using the reference spectra data sets. Subsequently twelve single test spectra per strain were compared to the generated reference entries. Out of 228 test spectra, 136 could be assigned to the correct reference spectrum (60 %). The number of correct sub-species classifications dropped with an increasing number of total analyzed

isolates from 73 % (nine isolates analyzed) to 60 % (nineteen isolates analyzed). This effect is probably due to the inclusion of several highly similar isolates (e.g.TMW 2.1497, 2.1499 and 2.1500) which originated from the same brewery, shared a rep-PCR fingerprint type at a similarity of approximately 85 % and are thus likely to promote misidentifications.

To better allow for technical as well as biological variability, for each isolate the twelve test spectra were merged to a sum spectrum, which was subsequently compared to the deposited reference spectra, resulting in the correct assignment of sixteen out of nineteen isolates, with strains TMW 2.1497, 2.1500 and 2.1505 representing the misidentifications. Test spectra of strain TMW 2.1497 were falsely assigned to the reference spectrum of strain TMW 2.1500, TMW 2.1500 was assigned to TMW 2.1499 and TMW 2.1505 was classified as TMW 2.1488. Misidentification among these isolates indicates a high degree of similarity of the acquired spectra.

To further investigate the relatedness of the analyzed isolates, reference spectra were subjected to Cluster Analysis as shown in Figure 6. High similarities between reference spectra of isolates TMW 2.1488, 2.1489 and 2.1505 (distance level of approximately 0.1) and TMW 2.1497, 2.1499 and 2.1500 (distance level smaller than 0.1) respectively, resulting from Cluster Analysis are also supporting close relationship between the isolates.

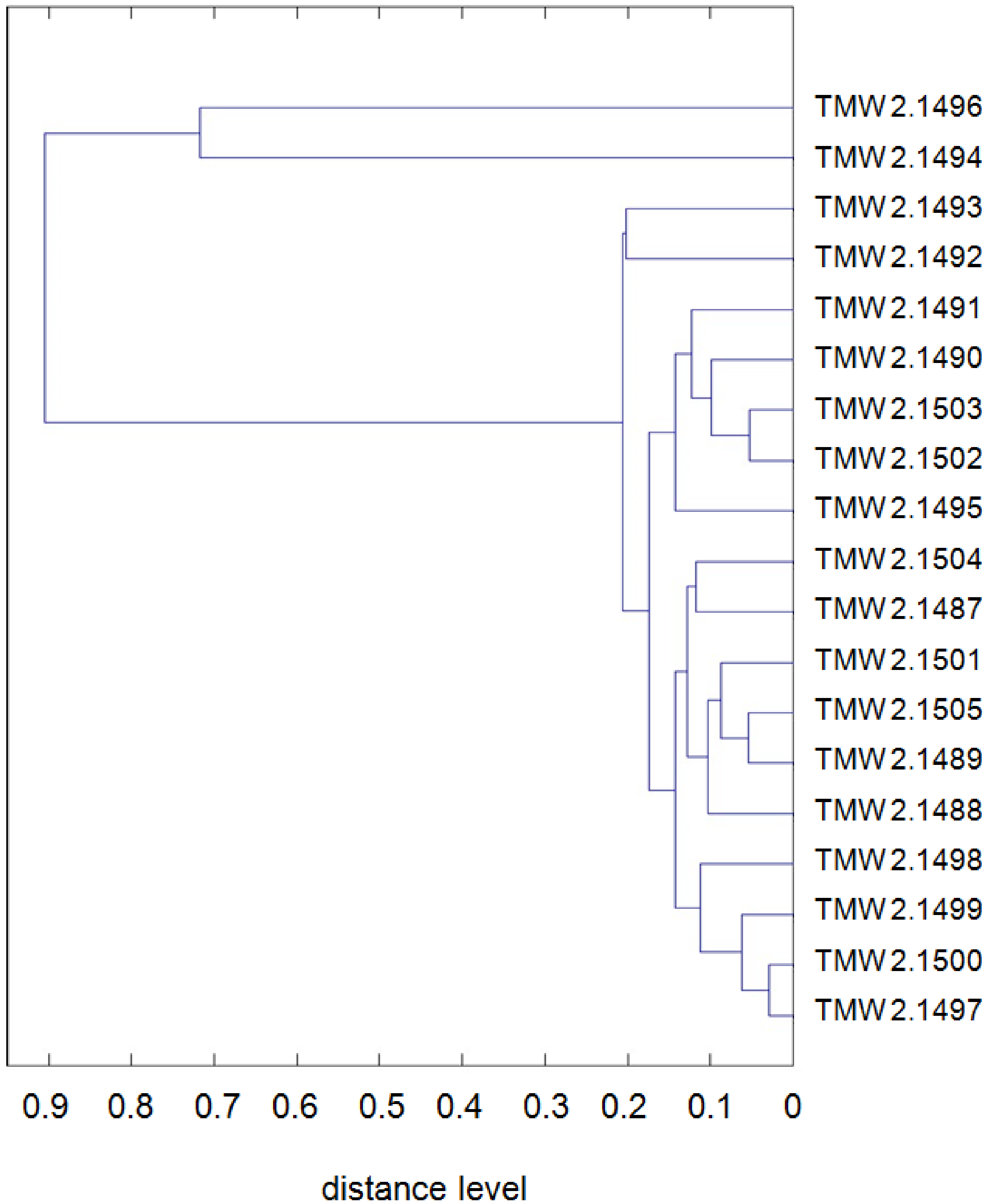

Figure 6: Cluster analysis of strains TMW 2.1494 (*P. cerevisiiphilus*), TMW 2.1496 (*P. haikarae*), TMW 2.1487- 2.1493, 2.1595 and 2.1497- 2.1505 (*P. frisingensis*), y-axis indicating similarity of spectra from 0 (identical spectra) to 1 (maximum variability).

As expected, strains TMW 2.1494 (*P. cerevisiiphilus*) and 2.1496 (*P. haikarae*) show a very low level of similarity compared to each other as well as to all other analyzed

isolates (*P. frisingensis*). The examined *P. frisingensis* strains formed four sub-cluster with strains TMW 2.1492 and 2.1493 showing higher variability to each other and the rest of the *P. frisingensis* isolates, which – with the exception of strain TMW 2.1495 – consisted of recent brewery isolates only and were split into three sub-clusters. One of those sub-clusters harbored exclusively isolates from a single brewery (isolates TMW 2.1497, 2.1498, 2.1499 and 2.150; brewery 5). Compared with the previously published rep-PCR data [Suiker et al., 2007], two fingerprint types are represented in this sub-cluster. The second sub-cluster consisted of isolates TMW 2.1488, 2.1489 (both brewery 2), 2.1505 and 2.1401 (brewery 6) as well as TMW 2.1487 (brewery 1) and TMW 2.1504 (brewery 7). Thus, the second sub-cluster consisted of isolates which formed three rep-PCR fingerprint types. The third sub-cluster was formed by isolates TMW 2.1502 and 2.1503 (both brewery 7), TMW 2.1490 (brewery 3) and 2.1491 (brewery 4) and isolate 2.1495 (VTT). With the exception of isolates TMW 2.1502 and 2.1491, each of these isolates represented a separate fingerprint type of the rep-PCR data.

Isolates, which were involved in misclassifications (TMW 2.1488 and 2.1505 as well as TMW 2.1497, 2.1499 and 2.1500) using the database approach, showed reference spectra with a variability of less than 0.1 in the cluster analysis (see Figure 6). The high similarity of reference spectra likely plays a major role for the impaired classification of these isolates. Still, isolates TMW 2.1502 and 2.1503, which showed comparable similarities to each other as isolates TMW 2.1488 and 2.1505, were distinguishable. Applying an analysis of similarity, the test spectra of isolates TMW 2.1502 and 2.1503 showed remarkable low variation of the rank of distances within groups (data not shown). Keeping variation in replicate spectra as low as possible may thus support the differentiation of isolates, which show highly similar spectra.

3.4.4 Identification of isolates from the brewing and beverage industry

671 bacterial isolates, which were collected at the Research Centre Weihenstephan for Brewing and Food Quality (BLQ) were analyzed by MALDI-TOF MS. Results of identification were additionally compared to results obtained at the BLQ by means of a PCR kit (Biotecon foodproof beer screening kit, Biotecon, Germany) or 16S rDNA sequencing to prove validity of MALDI-TOF MS for rapid identification of industrial

bacterial samples. Frequency of obtained identification results is presented in Table 21.

Samples, which were only identified as a certain group by the PCR kit and identified to the species level by MALDI-TOF MS, were interpreted as concordant, if the respective species identified by MALDI-TOF MS was contained within the group-identification received from the PCR kit. 591 samples were found to deliver concordant results from both identification at the BLQ and MALDI-TOF MS (88.1 % of all samples). Among the residual samples, 44 (6.6 % of all samples) could only be identified to the species level by either 16S rDNA sequencing or MALDI-TOF MS. These samples included mainly organisms belonging to the genera *Acetobacter*, *Enterobacter*, *Klebsiella* and *Serratia*. With the exception of three samples, which were identified as *Enterobacter cloacae, Enterobacter durans* and *Kluyvera ascorbata* by MALDI-TOF MS and as *Aerococcus* sp*., Citrobacter* sp*.* and *Klebsiella* sp*.* at the BLQ, these samples showed concordant genus level identification.

Table 21: Identifications obtained from MALDI-TOF MS and their frequency

Genus	**Species**	**Frequency of identification**
Acetobacter	sp.	5
Acetobacter	*fabarum*	11
Acetobacter	*indoniensis*	3
Acetobacter	*malorum*	1
Acetobacter	*orientalis*	1
Acetobacter	*pasteurianus*	1
Aeromonas	*eucrenophila*	1
Bacillus	sp.	1
Bacillus	*licheniformis*	1
Bacillus	*pumilus*	1
Bacillus	*thuringiensis*	1
Citrobacter	*freundii*	3
Enterobacter	sp.	4
Enterobacter	*asburiae*	5
Enterobacter	*cloacae*	5
Enterococus	sp.	3
Enterococcus	*casseliflavus*	3
Enterococcus	*durans*	1
Enterococcus	*faecalis*	1
Enterococcus	*faecium*	2
Enterococcus	*gallinarum*	1
Enterococcus	*italicus*	2
Enterococcus	*mundtii*	2

Escherichia	*coli*	1
Hafnia	*alvei*	2
Klebsiella	*oxytoca*	7
Kluyvera	sp.	1
Kluyvera	*ascorbata*	8
Lactobacillus	*acetotolerans*	1
Lactobacillus	*backii*	49
Lactobacillus	*brevis*	293
Lactobacillus	*buchneri*	1
Lactobacillus	*casei*	1
Lactobacillus	*curvatus*	1
Lactobacillus	*dextrinicus*	2
Lactobacillus	*fermentum*	5
Lactobacillus	*harbinensis*	23
Lactobacillus	*lindneri*	4
Lactobacillus	*parabuchneri*	9
Lactobacillus	*paracasei*	88
Lactobacillus	*plantarum*	24
Lactobacillus	*rossiae*	1
Lactobacillus	*sakei*	2
Lactococcus	sp.	1
Lactococcus	*lactis*	2
Leuconostoc	*citreum*	1
Leuconostoc	*mesenteroides*	7
Micrococcus	*luteus*	2
Megasphaera	*cerevisiae*	8
Paenibacillus	sp.	2
Pectinatus	*frisingensis*	17
Pediococcus	*damnosus*	28
Propionibacterium	*acidipropionici*	1
Raoultella	*ornithinolytica*	4
Rhizobium	*radiobacter*	1
Serratia	sp.	1
Serratia	*liquefaciens*	1
Serratia	*marcescens*	1
Staphylococcus	*capitis*	1
Staphylococcus	*epidermidis*	1
Staphylococcus	*haemolyticus*	1
Staphylococcus	*hominis*	2
Staphylococcus	*pasteuri*	3
Staphylococcus	*warneri*	1
Streptococcus	*parasanguinis*	2
Weissella	sp.	1
Weissella	*cibaria*	3

Among the residual 36 non-compliances in species identification, 12 (1.8 % of all samples) were caused by isolates belonging to the genus *Acetobacter (A.)*. Again, these isolates were concordantly identified to the genus level. In eleven cases,

identification by MALDI-TOF MS resulted in *A. fabarum* while rDNA sequence analysis favored *A. lovaniensis*. The second hit for MALDI-TOF MS identification was *A. lovaniensis,* however, with significantly lower log-scores which were < 2.0. 16S rDNA sequencing could not help to clarify their species affiliation, as obtained sequences showed high similarities (≥ 99 %) to deposited sequences from more than one *Acetobacter* species.

The residual 24 non-compliances (3.5 % of all samples) involved 22 different species (Table 22). For each of these mismatched pairs, all available MALDI-TOF MS reference spectra in the database of the two respective species were compared to each other to determine possible similarities in protein profiles. Achieved log-scores generally ranged from 0.2 to 1.0, indicating very low similarity among the spectra. As samples were processed and analyzed in two different laboratories and were occasionally found to contain more than one species, it is possible that in some cases one of the species was identified in the first laboratory, while the second laboratory identified another species. Part of the samples was re-analyzed in both laboratories and obtained results were in agreement.

Table 22: Non-compliances in species-level identification obtained from MALDI-TOF MS and results obtained from the PCR kit or 16S rDNA sequencing at the BLQ.

MALDI-TOF MS	**PCR kit/ 16S rDNA sequencing**
Enterococcus italicus	*Enterobacter cloacae*
Kluyvera ascorbata	*Buttianxella agrestis*
Lactobacillus backii	*Pediococcus damnosus*
Lactobacillus backii	*Lactobacillus buchneri*
Lactobacillus backii	*Lactobacillus perolens*
Lactobacillus brevis	*Lactobacillus collinoides*
Lactobacillus brevis	*Leuconostoc mesenteroides*
Lactobacillus brevis	*Lactobacillus buchneri*
Lactobacillus brevis	*Hafnia alvei*
Lactobacillus fermentum	*Lactobacillus reuteri*
Lactobacillus parabuchneri	*Lactobacillus brevis*
Lactobacillus sakei	*Lactobacillus casei*
Lactobacillus sakei	*Bacillus thuringiensis*
Leuconostoc mesenteroides	*Streptococcus pneumoniae*
Raoulthella ornithinolytica	*Lactobacillus perolens*
Raoulthella ornithinolytica	*Lactobacillus brevis*
Staphylococcus haemolyticus	*Lactobacillus brevis*
Weissella cibaria	*Leuconostoc mesenteroides*

Part of the bacteria could only be detected in groups using the PCR kit, MALDI-TOF MS however delivered single species identifications. 88 samples were identified as *L. casei/paracasei* by the commercial PCR kit. Only one of these samples was identified as *L. casei* by MALDI-TOF MS. The residual 87 samples were identified as *L. paracasei* based on their peptide mass fingerprints.

23 samples were identified as *L. harbinensis/perolens* applying the PCR kit. With samples belonging to one of these two species it was observed that comparison of acquired MALDI-TOF MS spectra with the database generally yielded two hits with a log-score greater than 2.0, being *L. harbinensis* and *L. perolens*. This effect may be due to the inclusion of *L. perolens* DSM 12745 as a reference strain for the species *L. perolens* in the MALDI Biotyper 3.0 database. The reference spectrum of *L. perolens* DSM 12745 yields a remarkable high log-score (> 2.6) when compared to the reference spectrum of *L. harbinensis*, while it yields a lower log-score when compared to the reference spectrum of *L. perolens* type strain DSM 12744 (< 2.0) showing, that the spectrum obtained from *L. perolens* DSM 12745 is more similar to the reference spectrum of the type strain of the species *L. harbinensis* (DSM 16991) than to its own species' type strain (DSM 12744). Removal of the respective reference spectra resulted in all 23 isolates being unambiguously identified as *L. harbinensis* by MALDI-TOF MS. Samples which were randomly chosen for 16S rDNA sequencing to confirm results were in accordance with the identification achieved by MALDI-TOF MS, showing sequence similarities of 100 % to published *L. harbinensis* sequences whereas similarities to *L. perolens* sequences did not exceed 98 %.

Eight samples which were identified as *L. buchneri/parabuchneri* by PCR were assigned to the species *L. parabuchneri* by MALDI-TOF MS. As the PCR kit applied was unable to distinguish between *L. buchneri* and *L. parabuchneri*, results from MALDI-TOF MS were confirmed by 16S rDNA sequencing, verifying MALDI-TOF MS' capability to distinguish between the species *L. buchneri* and *L. parabuchneri.*

All 24 samples, which were matched to the *L. coryniformis* group by PCR were identified as *L. plantarum* by MALDI-TOF MS. Four of these samples achieved log-scores > 2.0 also for *L. paraplantarum* indicating a certain unability to distinguish between these two species. 16S rDNA sequencing was performed but could not help to clarify results, as obtained sequences showed 99 % similarity to published 16S rRNA gene sequences of both *L. plantarum* and *L. paraplantarum.*

3.4.5 Comparative analysis of the beer-spoilage potential of *Lactobacillus brevis* brewery isolates by MALDI-TOF MS and gene marker analysis

The data presented in this chapter was collected by Sabine Popovic in the course of her master thesis under the guidance of the author.

One hundred isolates of *Lactobacillus brevis,* which were recently found in brewing environments, were examined with regard to their potential to spoil beer. Results from physiological tests, genome-based markers and proteomic data were gathered, evaluated and compared to each other. A physiological resazurin assay was done as described above and its results are presented in Figure 7.

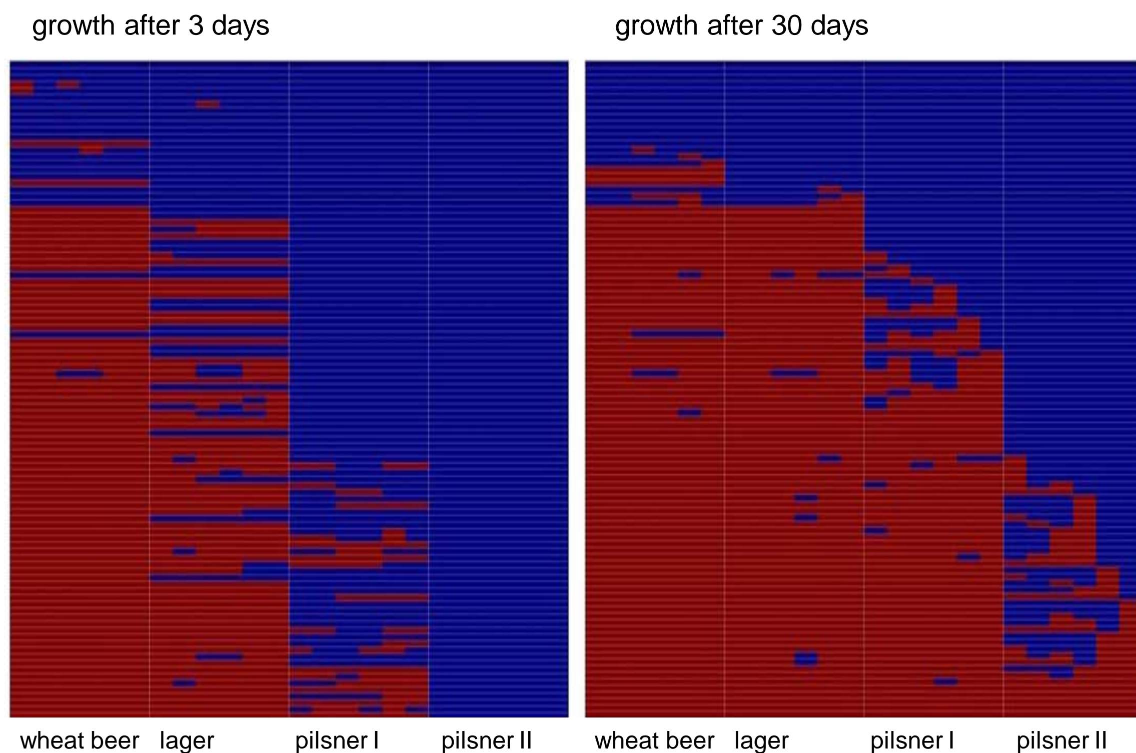

Figure 7: Growth of 100 *Lactobacillus brevis* strains in four different beers as assessed by a resazurin assay. Red fields indicate positive growth behavior, blue fields indicate no growth in the respective beer. Isolates are listed vertically and results from biological and technical replicates are shown side by side [adapted from Popovic, 2013].

Isolates were assigned to four different groups, non-beer spoilers (NB), weak beer spoilers (WB), beer spoilers (B) and strong beer spoilers (SB), based on their growth in beer. Generally, non-beer spoilers did not grow in any of the beers, weak beer

spoilers grew in wheat beer and at the utmost in lager beer after 30 days. Beer spoilers were able to grow in pilsner I and strong beer spoilers were even able to proliferate in the highly hopped pilsner II. Metabolic activity in different beers was weighted and HiT-MDS was applied to visualize the obtained results in Figure 8.

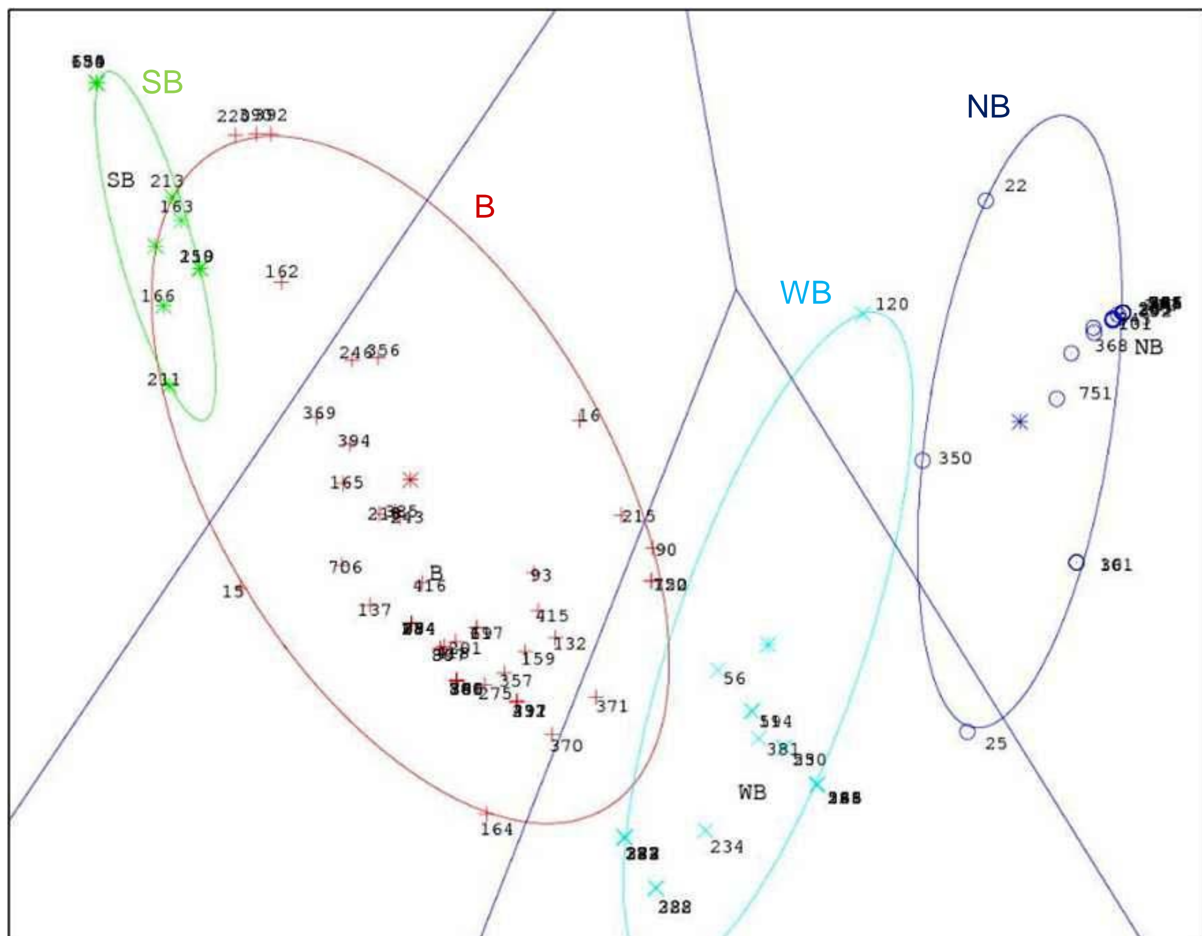

Figure 8: Hit-MDS of 100 *L. brevis* isolates based on their growth potential in beer evaluated by metabolic activity in different beers. Green asterisks represent strongly beer spoiling isolates (SB), red crosses indicate beer spoiling isolates (B), teal x's represent weak beer spoiling isolates (WB) and blue circles indicate no spoilage potential (NB) [adapted from Popovic, 2013].

ANOSIM was used to test for significant differences between the four groups. R values greater than 0.75, which are generally interpreted as well separated [Ramette, 2007], were achieved for the pair-wise comparison between groups B and NB (0.99), NB and SB (1.0), NB and WB (0.96) as well as SB and WB (1.0). R values between 0.5 and 0.75 for the pair-wise comparison between groups B and SB (0.74) as well

as B and WB (0.63) indicate separated but overlapping groups [Ramette, 2007], supporting the statistical significance of the performed grouping.

In addition isolates were compared based on the presence or absence of genes which were identified as markers for beer spoiling (probe 1, probe 2, probe 3, probe 4, probe 5, probe 6, probe 7, probe 8, probe 9, probe 10, probe 11, probe 12 and horA) or non-beer spoiling (probe 13 and probe 14) strains. Isolates were labeled according to the respective results from the metabolic resazurin assay as B, NB, SB and WB and results were again visualized by Hit-MDS. Figure 9 shows that the resulting ovals, which include all isolates assigned to a certain group according to their metabolic activity in beer, overlapped, if the distribution was based on the presence of marker genes. No oval was obtained for the group of strong beer spoilers as only two different configurations of marker genes were observed within this group. Results from the physiological test did not correlate well with the presence and absence of the chosen marker genes in terms of beer spoilage potential.

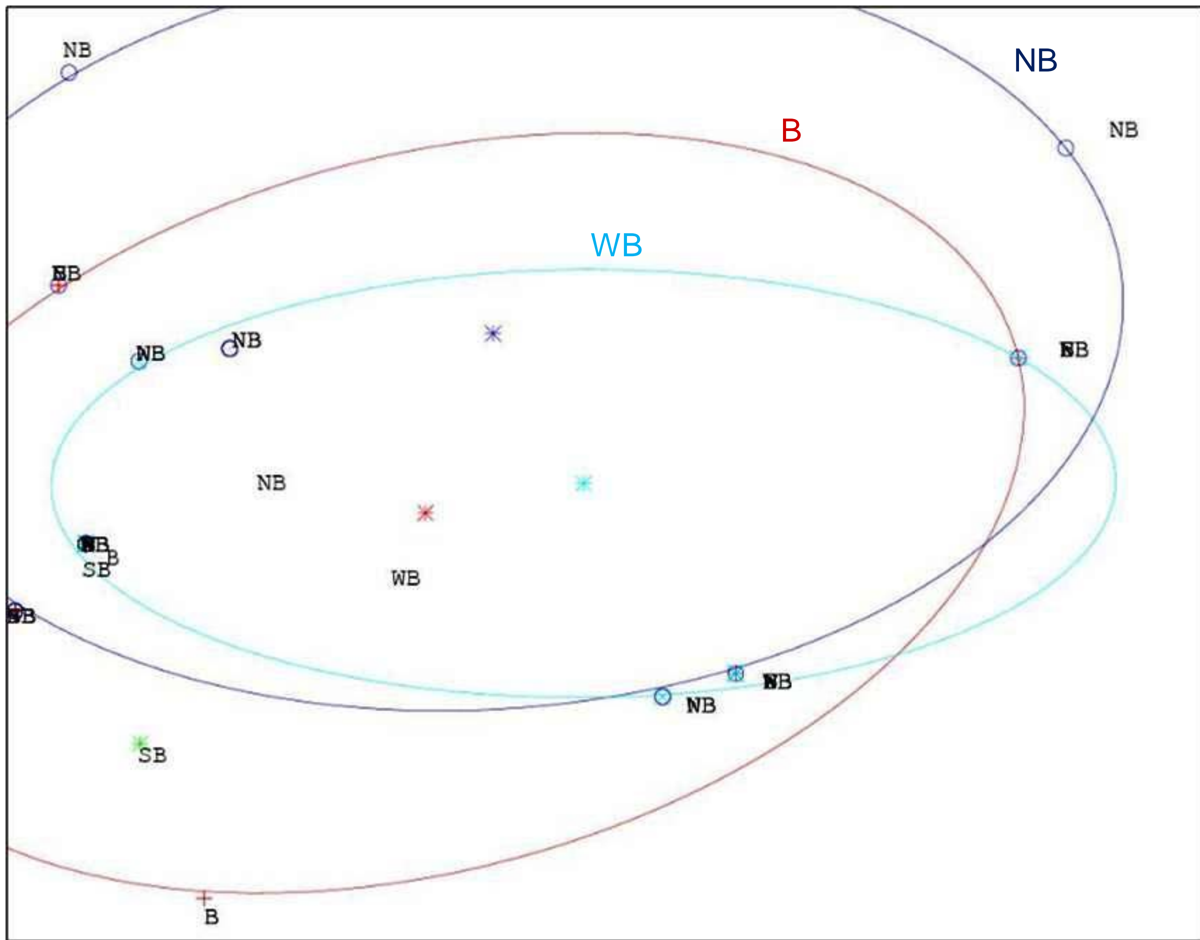

Figure 9: Distribution of 100 *L. brevis* isolates based on the presence of genetic markers associated with beer spoilage and visualized by HiT-MDS. Green asterisks represent strongly beer spoiling isolates (SB), red crosses indicate beer spoiling isolates (B), teal x's represent weak beer spoiling isolates (WB) and blue circles indicate no spoilage potential (NB). Overlapping symbols show isolates classified into different groups of beer spoilage potential which exhibited identical sets of marker genes [adapted from Popovic, 2013].

In a similar approach, isolates were compared to each other based on twelve MALDI-TOF MS test spectra per isolate, which were merged to sum spectra. The resulting Hit-MDS in Figure 10 shows that mass spectra overlap among different spoilage groups as determined by a physiological growth test. It also becomes clear that isolates included in the group of non-beer spoiling (blue) show the largest spread, especially compared to the group of strongly beer spoiling isolates (green). This may be partially due to the fact that 23 isolates were classified as non-beer spoiling whereas only nine isolates were classified as strongly beer spoiling. However, 49

isolates were classified as beer spoilers (red) and this group still shows a lower spread than the non-beer spoilers.

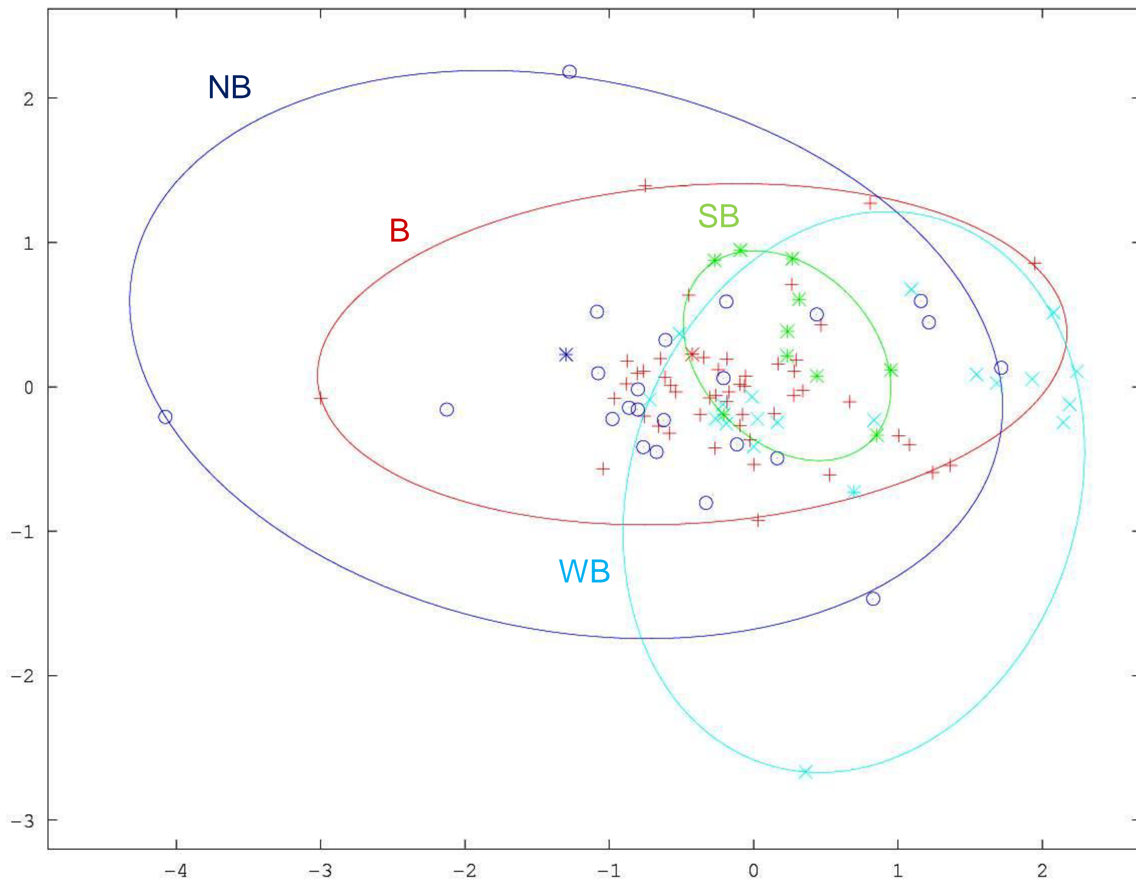

Figure 10: Hit-MDS of 100 *L. brevis* isolates based on their MALDI-TOF MS test spectra. Green asterisks represent strongly beer spoiling isolates (SB), red crosses indicate beer spoiling isolates (B), teal x's represent weak beer spoiling isolates (WB) and blue circles indicate no spoilage potential (NB) [adapted from Popovich, 2013].

Spread within groups was generally rather high. Therefore DAPC, which allows to focus more on variances between groups and minimizes variances within groups was employed, too. The results in Figure 11 show that most MALDI-TOF MS spectra of *L. brevis* isolates belonging to the same spoilage group scatter around the centre of the respective group, still, outliers are observed which are positioned closer to the centre of one of the neighboring groups.

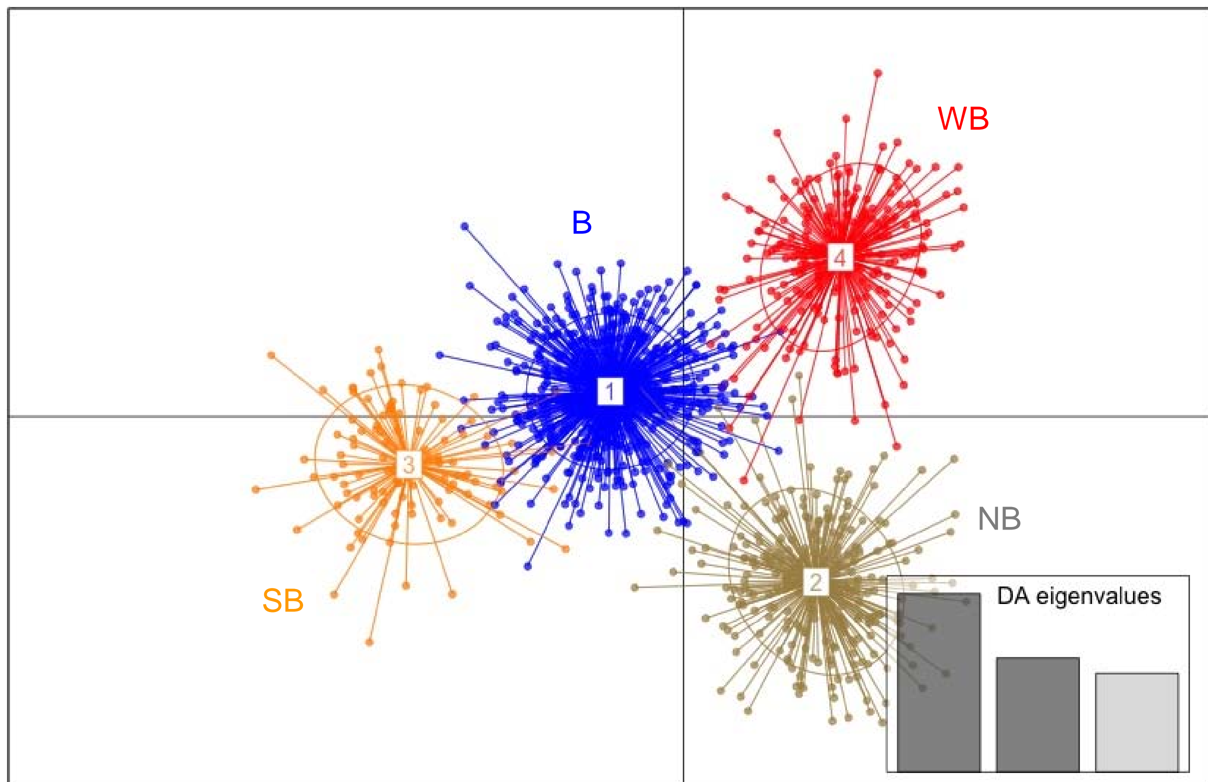

Figure 11: Discriminant Analysis of Principal Components of MALDI-TOF MS spectra from *L. brevis* isolates. Blue dots represent spectra derived from strains classified as beer spoilers, olive dots represent spectra from strains classified as non-beer spoilers, orange dots represent spectra of strong beer spoilers and red dots represent spectra of weak beer spoilers.

Additionally, MALDI-TOF MS ability to trace isolates was tested. Therefore 24 reference spectra per isolate were merged to a reference database entry as described in section 3.4.3.1. The residual twelve test spectra were subsequently compared to the reference database entries as single spectra. Only 42 % of all test spectra were correctly assigned to their respective reference database entry. However if compared based on their origin, 74 % of test spectra were assigned to a reference spectrum of an isolate derived from the same brewery as the test spectra. If spectra were compared with regard to the isolate's beer spoilage potential determined in the physiological test, 71 % of test spectra were matched to a reference spectrum of an isolate within the same group.

3.4.6 Effect of plasmid curing on MALDI-TOF MS spectra of *Lactobacillus brevis* TMW 1.313

It is generally agreed on that the presence of plasmid-encoded transport proteins such as horA is associated with the hop resistance of beer-spoiling lactic acid bacteria [Sakamoto et al., 2001]. Additonally, the copy number of certain plasmids, carrying resistance genes, was reported to be associated with beer spoilage potential of *L. brevis* [Sami et al., 1997]. Plasmid curing may thus alter the growth behavior of *L. brevis* strains in beer.

Published sequences of plasmids from *L. brevis* were compared to the genome of *L. brevis* TMW 1.313. *L. brevis* TMW 1.313 showed sequence fragments identical to the sequence of pRH45II (23381 bp), the largest of which had a size of 7731 bp. For plasmid pLB925 A04 (65037 bp), a 29308 bp segment was found within the genome of *L. brevis* TMW 1.313 indicating the presence of plasmids in this strain. To check the influence of plasmids on MALDI-TOF MS spectra of *L. brevis*, strain TMW 1.313 was subjected to plasmid curing by repeated sub-cultivation at elevated temperatures. Subsequently colonies showing different morphologies were picked and checked for their growth in beer. A variant of strain TMW 1.313, referred to as TMW 1.313 PC, which did not show metabolic activity in any of the tested beers after three days was chosen for MALDI-TOF MS analysis. TMW 1.313 PC formed smooth colonies on agar while the original TMW 1.313 formed rough colonies with a serrated border. Additionally primers were designed specific for the sequence fragments of plasmids pRH45II and pLB925 A04 found in the genome of *L. brevis* TMW 1.313. PCR yielded amplicons with both primer pairs in strain TMW 1.313 but neither in TMW 1.313 PC nor in TMW 1.6, which was used as a negative control.

Five biological replicates of TMW 1.313 as well as TMW 1.313 PC were analyzed and spectra were merged to a sum spectrum for each of the variants to show only reproducibly obtained peaks. The resulting spectra, which exhibit only minor differences in peak patterns are plotted in Figure 12.

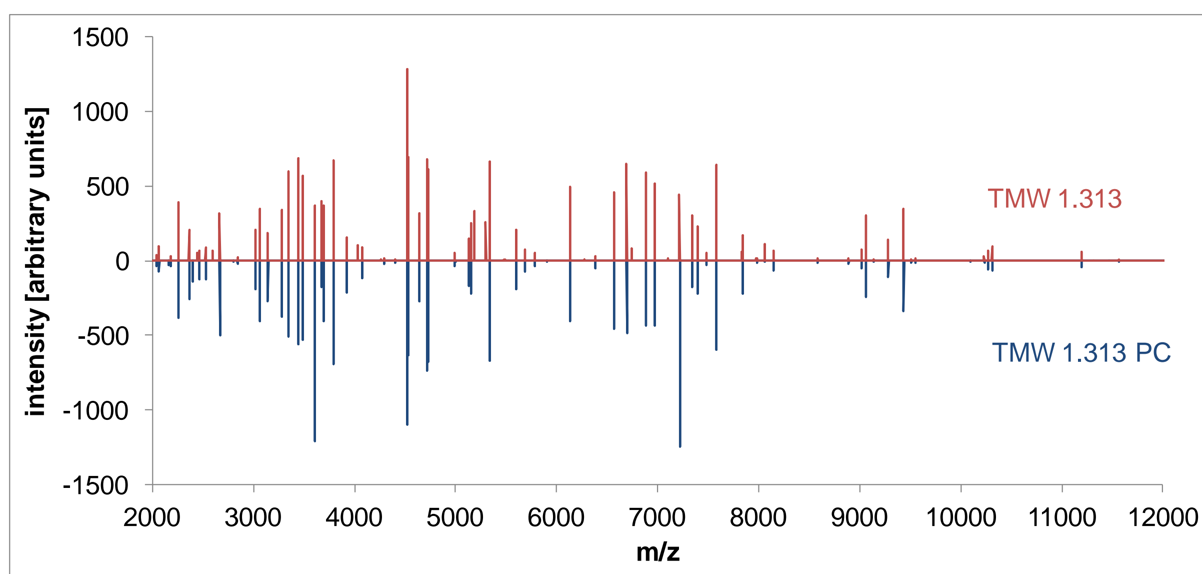

Figure 12: Spectra of *L. brevis* TMW 1.313 (red) and the non beer spoiling variant *L. brevis* TMW 1.313 PC (blue).

A detailed view of the mass range from 5100 to 5400 Da (see Figure 13), however, clearly demonstrates visible differences in the presence of peaks between the two variants of TMW 1.313. The spectra of TMW 1.313 show peaks at 5190 Da and 5300 Da which are absent in the spectra of TMW 1.313 PC. These peaks were neither detectable in spectra of any of the other *L. brevis* strains which were examined in this study, when grown on MRS plates. Spectra obtained from strains TMW 1.315 and 1.317 however showed comparable signals within a mass range of 500 ppm when grown in beer. A database search of the detected masses against protein masses calculated from genome data of *L. brevis,* accepting a maximum mass deviation of 600 ppm, revealed three matches for the signal at 5190 Da and one match for the signal at 5300 Da. Taking into account possible double and threefold charged ions as well as posttranslational modifications, the signal at 5190 Da could correspond to a methylated 30S ribosomal protein, a methylated protein belonging to UPF0297 or a methylated URI-like endonuclease. The signal at 5300 Da yielded only one match relating it to a putative uncharacterized protein.

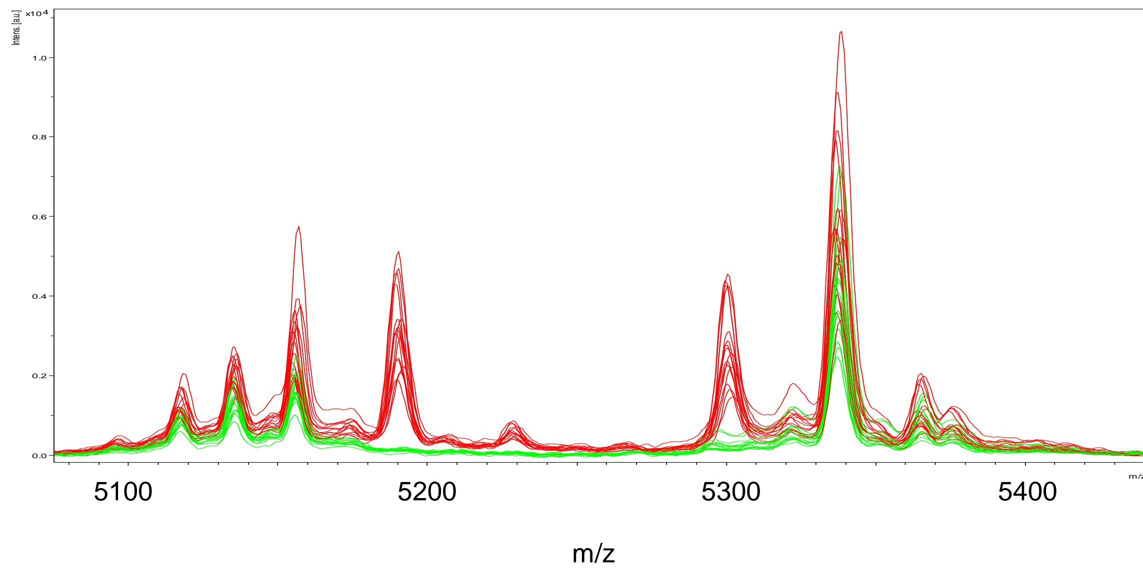

Figure 13: Mass spectra of *L. brevis* TMW 1.313 (red) and *L. brevis* TMW 1.313 PC (green) within the mass range from 5100 to 5400 Da.

4 Discussion

Over the last years, MALDI-TOF MS has become a renowned identification tool for bacteria. First broadly applied in clinical microbiology it has recently succeeded to expand into other fields of microbiology such as food, industrial or environmental microbiology or even the prevention of biological warfare. To date MALDI-TOF MS is not only applied for the identification of isolates on the species level, but its taxonomic limits are constantly challenged in bacterial source tracking or the differentiation of bacteria harboring certain resistance mechanisms to antibiotics from susceptible organisms of the same species. Increased taxonomic resolution requires higher discriminatory power and resolution of the method. Thus, the higher the desired taxonomic resolution, the more attention must be paid to factors influencing the accuracy of the results. Spectra quality and reproducibility must be assured through thorough and standardized cultivation of organisms and sample preparation. While simple cell smears proved to deliver sufficiently reliable results for numerous routine applications, more laborious procedures are required for the differentiation of strains or biotypes. Another concern to assure a high level of accuracy of the analysis and the results thereof is the availability of high quality reference databases, which contain a broad range of reference organisms relevant for the desired application.

These issues were tackled in the present thesis. It demonstrates that beverage and beer spoiling bacteria are reliably identified by MALDI-TOF MS once an appropriate and accurate reference database is established. Through the use of improved bioinformatic tools, even a higher taxonomic resolution, namely the differentiation of ecotypes within the species *L. brevis* that show different growth behavior in beer can be achieved. At the same time, this thesis also reaches the current limits of subtyping by MALDI-TOF MS, showing that the differentiation of highly similar isolates is still a challenge and in the future might be overcome by improvements in both instrumentation and software tools.

4.1 Optimization of sample preparation for MALDI-TOF MS

As sample preparation is known to be a crucial factor influencing spectra quality, different preparation methods were tested to ensure obtained spectra were of good

quality. Good quality was defined by a high number of reproducible peaks showing at least a certain minimum intensity of 1000 a.u., while intensity maxima reached up to 30000 a.u.. Cell smears were found to be a fast and convenient way of preparation for *Ln. mesenteroides* and *Pc. claussenii*, resulting in a high number of distinguishable peaks as reported before for analysis of intact cells [Vargha et al., 2006]. However, cell smears proved to be less applicable for the analysis of *L. brevis* because of their lack of reliability to constantly deliver spectra when operating in automatic acquisition mode. Though the addition of formic acid increased the number of successful measurements for *L. brevis,* which is in accordance with the results from Haigh et al. [2011] obtained for other gram positive bacteria, the total amount of peaks was lower than the total amount of peaks delivered by the extraction procedure. Lysozyme treatment was discussed as an enhancer for spectral quality by increasing the amount of peaks found in the higher mass range when *Arthrobacter* strains were analyzed [Vargha et al., 2006] and increasing the complexity and spectral range of mass fingerprints from gram positive isolates of the family *Enterobacteriaceae* [Smole et al., 2002]. It was also shown to elevate peak intensities in spectra acquired from *Enterococcus* isolates, while at the same time, broadening the mass ranges over which peaks occurred [Giebel et al., 2008]. In this study, none of the strains tested showed any beneficial effects on spectral pattern after lysozyme treatment. There was no significant shift to peaks of higher intensities and neither did lysozyme broaden the mass range over which reproducible peaks were detected. In contrast, application of the enzyme decreased the total amount of peaks and induced the occurrence of two intense additional peaks resulting from the single and double charged ions of lysozyme. Enzyme dependent signals are not only indistinguishable from those of bacterial proteins as reported by Vargha et al. [2006] but may also interfere with the acquisition of less intense signals at the same or very similar mass to charge ratio derived from the bacterial sample.

All spectra obtained by any of the tested sample preparation procedures were successfully assigned to the correct species when matched against the Bruker database containing 4111 reference entries of microbial strains. Mechanical and enzymatical sample treatments turned out to be rather laborious and time-consuming without offering any additional value in terms of spectral information. Cell smears plus ”on-target extraction” by formic acid proved to be fast and practicable but less universally applicable than cell extraction procedure which was shown to be of

special avail for the analysis of *L. brevis,* where the number of peaks detected could be additionally increased if cell suspensions, containing cell debris, instead of cell extracts were prepared. The observed effect could be a result of the tough cell wall composition of beer-spoiling strains of *L. brevis* which renders them largely resistant to lysis [Behr et al., 2007]. Strain TMW 1.313 was shown to exhibit strong beer spoiling potential. Thus insufficient release of proteins into the supernatant may account for discrepancies in peak pattern when measuring cell extracts in contrast to cell suspensions, where proteins attached to cell debris could still be accessible to ionization. The analysis of *L. brevis* strains may therefore benefit from the usage of cell suspensions instead of cell extracts.

Cell extraction procedures were found to present a universally applicable way to approach the analysis of beverage spoiling bacteria. The increased reliability of spectra acquisition combined with the higher amount of information retrieved from spectra may compensate for the considerable time and effort required, in particular when microorganisms need to be analyzed, which derive from an environment where they were exposed to stress factors such as hop or acid stress in beer.

4.2 Influence of culture conditions on MALDI-TOF MS spectra

Different culture conditions such as time, availability of oxygen and composition of nutrient media were varied to assess their influence on MALDI-TOF MS spectra. This is of interest for routine application since quality control procedures may differ vastly in practice. Strains were grown on four different media frequently used for detection of contaminants in brewing and beverage industry, incubated in the presence and absence of oxygen and harvested in different growth stages. None of the changes in culture conditions was able to hamper identification on species level, which complies with priorly published data, where accuracy of identification was not affected by culture conditions for clinically relevant bacteria [Seibold et al., 2010; van Veen et al., 2010]. In practice this could allow fast identification of spoilage bacteria without the need to rigorously obey a standard protocol for cultivation or additional subculturing. However, it was reported before that discernable differences in bacterial profiles appeared when isolates were grown on different nutrient media [Mazzeo et al., 2006] or collected in different growth stages [Vargha et al., 2006] prior to measurement.

Closer examination of the spectra obtained from different growth media showed that for each tested strain, a set of more than 60 reproducible peaks was found. Common peaks exhibited high intensities, whereas not only the number of peaks unique for spectra resulting from varying growth media was smaller, ranging from 4 to 18, dependent on strain and media, but also the intensities affiliated with these peaks were about one order of magnitude lower than those of signals found independent of growth medium. Similar results were obtained for varying growth time and the presence or absence of oxygen. It can be concluded that though spectra may differ under varying culture conditions, a significant set of signals is reproducibly detected under all tested conditions, resulting in stable peak patterns that allow reliable identification of samples, which is in accordance with the findings of Mazzeo et al. [2006], Valentine et al. [2005] and Welker and Moore [2011]. This marked stability of spectra for a particular species is usually attributed to the fact that ribosomal and cell structure proteins account for the majority of peaks in mass spectra of microbial samples [Welker and Moore, 2011].

As it has been recently shown that MALDI-TOF MS has promising potential to differentiate microbial samples beyond species level such as sub-species classification [Dieckmann et al., 2008; Seibold et al., 2010; Tanigawa et al., 2010] or bacterial source tracking [Siegrist et al., 2007], spectra were subjected to further analysis to elucidate if changes in MALDI-TOF MS fingerprints due to different growth conditions were reflected sufficiently consistent to assign spectra to the respective culture conditions used. Since typing of microorganisms requires to focus on discriminating features of strains instead of focusing on the similarities of strains to assign them to a given species for identification purposes [Welker and Moore, 2011], there’s a need for alternative approaches from pattern recognition algorithms, which serve identification of microbial samples [Seibold et al., 2010]. In this study, ClinProTools, a bioinformatics software tool, which was primarily developed for the detection of biomarkers in clinical applications, was used to classify spectra based on changing environmental conditions. Within this software package, Genetic Algorithm function proved to be the most capable option for the prediction of class affiliation, correctly assigning more than 90 percent of all spectra to the respective condition when growth medium or oxygen availability were varied. However, numbers of correct classifications dropped significantly when growth time was changed, with the majority of misidentifications occurring between the points 6 h and 12 h or 24 h and

48 h, respectively, for *L. brevis* and *Pc. claussenii,* indicating a certain potential to differentiate between early and late growth phase. Results from *Ln. mesenteroides* showed that only point 6 h could be unambiguously distinguished. Subsequent growth curve analysis revealed that this strain grew faster under the standardized conditions than *L. brevis* and *Pc. claussenii* and thus entered stationary phase earlier, which might account for interferences in discriminating spectra measured at points 12 h, 24 h and 48 h. The particular choice of measuring points, resulting in a lack of clear cutting points between the growth phases for part of the tested strains, may have contributed to misclassifications among neighboring time points. Nevertheless, it can't be precluded that variations due to growth time are more elusive for the tested strains than variations due to other growth conditions under investigation as has been shown before for other bacteria [Mazzeo et al., 2006].

It was demonstrated that variations in culture conditions did not hamper identification as provoked changes in peak pattern are too small to have an impairing influence on results. Nevertheless, the findings of these experiments do not only underline the capability, robustness and convenience of MALDI-TOF MS as a rapid tool for the identification of unknown bacterial samples in food microbiology but also reveal its potential to stably detect minor variations in peptide mass fingerprints, which could be employed for differentiation of isolates beyond species level.

4.3 Sub-species differentiation of beer-spoiling bacteria by MALDI-TOF MS

MALDI-TOF MS' potential to differentiate beer-spoiling bacteria below species level was investigated using *L. brevis* and *Pectinatus sp.* as model organisms. Seventeen strains of *L.brevis*, exhibiting different growth behavior in beer, were examined as well as seventeen brewery isolates of the genus *Pectinatus*.

4.3.1 Subtyping of *L. brevis* strains

L. brevis strains were characterized with regard to their ability to grow in and thus spoil beer and their tolerance to hop derived iso-alpha-acids. MALDI-TOF MS spectra were acquired from all strains, checked for strain-specific differences in protein

fingerprints and inspected for the presence of biomarkers to distinguish between strains with differing spoilage potential.

Based on the results of a growth test, which detects metabolic activity by resazurin reduction in four different beers, strains were assigned to weak, moderate or strong beer spoilage potential. Fifteen strains were either classified as weak or strong beer spoilers while two strains (TMW 1.1370 and 1.302) represented a moderate risk when present in beer as they showed only slow growth in lager beer. Weak spoilage potential was related to a MIC of iso-alpha-acids of less than 10 µM whereas strong spoilage potential was generally associated with a MIC of at least 15 µM. TMW 1.1370 and 1.302 which were considered to pose a moderate threat to beer based on the results of the growth assay, showed MIC values of 7 and 5 µM respectively which is within the range of MIC for iso-alpha-acids of weak spoilage strains. It has been reported before that the ability of *Lactobacillus* isolates to tolerate the antimicrobial effects of hop-compounds is a multi-factorial trait depending on the presence and absence of other selective pressures such as ethanol or the availability of nutrients [Haakensen et al., 2009]. Thus determination of MIC values for iso-alpha-acids alone may not be sufficient for the evaluation of a strains ability to grow in beer but could provide additional information to assess inter-strain variations of *L. brevis,* given that all strains are pre-cultured under standardized conditions and treated accordingly.

A database search based on MALDI-TOF MS reference and single test spectra of the 17 *L. brevis* strains successfully assigned 90 % out of 204 test spectra to the correct strain. Among the 19 falsely classified spectra, 12 spectra were derived from strain TMW 1.1205, which were constantly mismatched to the reference spectrum of strain TMW 1.100. Both, MALDI-TOF MS spectra and RAPD-PCR pattern of these two strains showed remarkable similarity to each other (> 95 %) as demonstrated in Figure 1 and Figure 5, which suggests close relationship between the two strains. Moreover, TMW 1.100 and 1.1205 exhibited identical growth behavior in beer and similar MIC values for iso-alpha-acids (9.0 +/- 1.4 µM and 8.5 +/- 0.7 µM respectively). Thus the two strains, which were both isolated from sourdough, do not only show genotypical similarity and similarity in their MALDI-TOF MS protein fingerprints but also in their physiological behavior. Another four test spectra from strain TMW 1.315 were mismatched to the reference spectrum of strain TMW 1.317, which again show high similarity to each other in terms of RAPD-PCR pattern and

MALDI-TOF MS spectra (> 90 %), similar MIC values for iso-alpha-acids (17.5 +/- 3.5 µM and > 20 µM) and identical growth behavior in beer. These results indicate that strains, which show highly similar MALDI-TOF MS spectra and are thus prone to misclassification, might also show similar behavior in terms of hop-resistance and growth in beer as well as genotypical similarity as assessed by RAPD-PCR. The remaining three misclassifications affected one single spectrum of each of the strains TMW 1.100, 1.1326 and 1.1370. With the exception of strain TMW 1.1370, which was once assigned to TMW 1.100, mismatching never occurred concerning strains with different beer spoilage potential and not in a single case, a strain exhibiting a high potential to spoil beer was matched to a reference showing only a weak potential to spoil beer or vice versa. Additionally, cluster analysis showed a certain tendency to group strains according to their ability to grow in beer when based on MALDI-TOF MS profiles. These findings could be attributed to the fact that though MALDI-TOF MS spectra of *L. brevis* in general show a remarkable similarity, spectra acquired from strongly beer spoiling strains are more similar to each other than spectra acquired from strains that show a weak spoilage potential. This may come as no surprise since beer spoiling strains share a common habitat to which they are adapted. Strains that show a low potential to spoil beer come from a variety of habitats such as sourdough, silage or other plant fermentations like the strains used in this study. Beer spoiling strains may thus form a unique “ecotype” adapted to the harsh conditions predominant in brewing environments, whereas strains with a low potential to spoil beer show a higher variability in their spectral peak pattern.

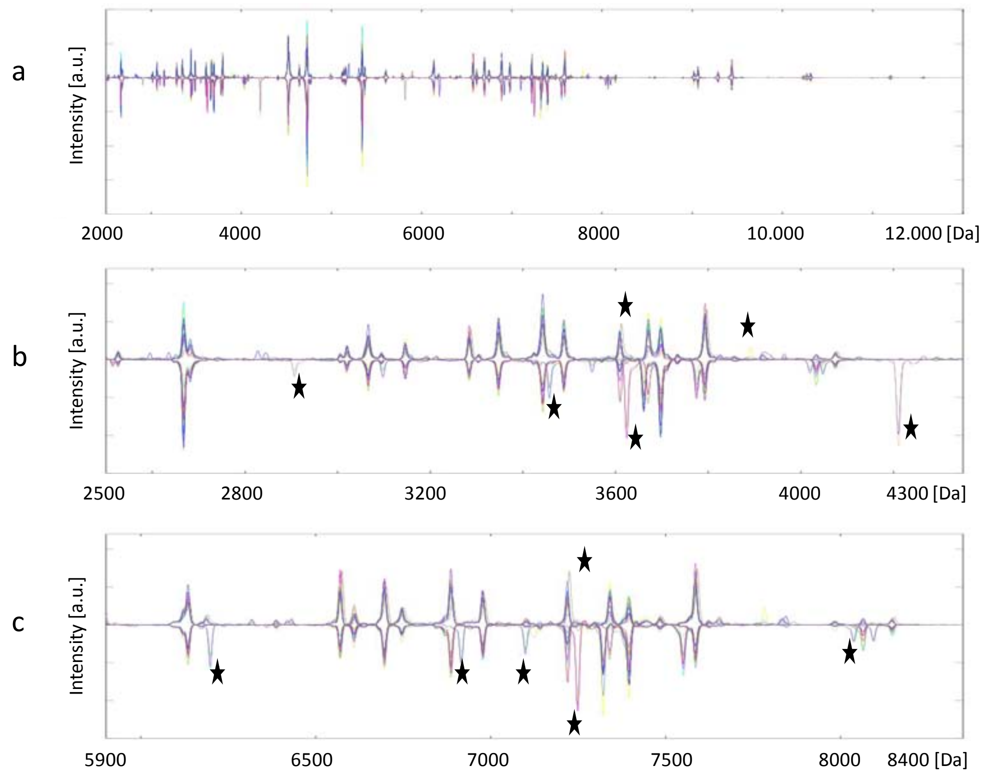

Figure 14: Inversed view of two representative test spectra per *L. brevis* strain. (A) Overview of the mass range from 2000 to 12000 Da. (B) Enlargement showing the mass range from 2500 to 4300 Da. (C) Enlargement showing the mass range from 5900 to 8400 Da. Asterisks indicate the position of reproducible signals which are not shared among all strains. Mass spectra of beer spoiling strains are displayed regularly, spectra of strains with a low beer spoilage potential are displayed inversely.

Figure 14 displays MALDI-TOF MS spectra derived from strains showing a strong beer spoilage potential regularly, whereas spectra from strains showing only a weak beer spoilage potential are illustrated inversely. As indicated by asterisks, more reproducible signals specific for one or more strains, but not shared among all strains, occur among strains with a low spoilage potential. However, no single biomarkers representative either for strong or weak beer spoilage potential were detected. The ability of *L. brevis* strains to grow in beer could not be linked to one marker exclusively present in one of the groups. Still, if MALDI-TOF MS allows differentiation of strains with known physiological traits based on their protein fingerprints and thus enables source-tracking of isolates, it represents a valuable tool for the characterization of beer spoiling bacteria.

Though members of a single species tend to yield highly similar MALDI-TOF MS spectra [Sandrin et al., 2013], MALDI-TOF MS has been successfully applied for the sub-species differentiation of bacteria before [Barbuddhe et al., 2008; Dieckmann et al., 2008; Seibold et al., 2010, Siegrist et al., 2007]. The remarkable similarity of strains within a species, which is also reflected in their MALDI-TOF MS profiles, requires higher discriminatory power of the method to differentiate strains than what is necessary for species-level identification [Sandrin et al., 2013]. As sub-species differentiation of organisms focuses on discriminating features of strains instead of focusing on the similarities of strains [Welker and Moore, 2011] which account for the majority of high intensity peaks within a spectrum, strain-level profiling may be more sensitive to small changes in spectra and thus standardized growth media are likely to play an important role for strain-level applications [Sandrin et al., 2013]. The results presented above demonstrate that reasonable standardization of growth conditions such as medium and growth time as well as a standardized extraction protocol allowed to differentiate strains of *L. brevis* based on their MALDI-TOF MS profiles, acquired from different biological replicates and prepared and measured on different days, in 90 percent of all cases.

As standard pattern recognition algorithms implemented in commercially available software packages as the Biotyper 2.0 (Bruker Daltonics, Germany) software focus on a limited number of preferential high intensity peaks reflecting intraspecies similarity for identification, different approaches are needed to obtain a higher taxonomic resolution [Seibold et al., 2010]. Thus, an in-house software application was applied for strain-differentiation, which allowed to give more weight to less intensive signals, enabling strain level differentiation of *L. brevis*.

MALDI-TOF MS showed to be a promising tool for sub-species classification of *L. brevis* strains. It's discriminatory power proved to be sufficient for the differentiation of *L. brevis* strains and could thus be applied to distinguish between contaminants of *L. brevis* which spoil beer and those which do not. The simple sample preparation and ease of handling inherent to MALDI-TOF MS makes it a valuable alternative to previously applied subtyping methods.

4.3.2 Subtyping of *Pectinatus sp.* isolates

Nineteen brewery isolates of *Pectinatus sp.* were analyzed by MALDI-TOF MS amongst which 17 isolates belonged to the species *P. frisingensis* and one isolate each belonged to the species *P. cerevisiiphilus* and *P. haikarae*. Based on the comparison of single test spectra to obtained reference spectra of the same isolate, 136 spectra were assigned to the correct reference spectrum accounting for 60 % of all spectra. The number of correct classifications of single spectra was thus significantly lower for *Pectinatus sp.* isolates than for *L. brevis* strains (90 %). This could be due to the inclusion of highly similar isolates of *P. frisingensis,* which shared a common origin.

Results also indicated that the differentiation of isolates showing a high degree of similarity in their spectra can be enhanced by keeping the variation in replicate spectra as low as possible. In general MALDI-TOF MS spectra are rather stable within a species. This is due to the fact that the majority of peaks found in spectra derive from ribosomal or structural proteins [Welker and Moore, 2011]. Previously in this work it was shown that the application of varying growth parameters, such as nutrient media or growth time, does not impair species-level identification of bacterial samples. Sub-species level differentiation, however, relies on the determination of small differences in spectra between separate strains. Thus, standardization of growth parameters such as nutrient media is crucial for strain-level profiling [Sandrin et al., 2013; Welker and Moore, 2011] to minimize evitable variability within spectra. Even if growth and measurement parameters are standardized as done in this study, a certain residual variation remains among spectra, which is far too small to impair species level identification but may influence the ability to differentiate below species level. Inevitable analytical and biological variation within spectra could hinder reproducible detection of strain-specific differences. To minimize the effect of variations, all test spectra of one strain were merged and only reproducibly found peaks were included in the resulting protein profiles. Comparing the obtained sum spectra against reference spectra, sixteen out of nineteen isolates were classified correctly. Two of the residual misclassifications occurred among isolates TMW 2.1497, 2.1499 and 2.1500 which originated from the same brewery and could therefore arise from the same source of contamination. Isolate TMW 2.1509 however definitely originated from a different brewery than isolates TMW 2.1488 and 2.1489.

Possible contamination routes for the exchange of spoilage bacteria between different breweries include the return of empty bottles or kegs or the transport of the finished product from one brewery to another for filling.

MALDI-TOF MS showed a good ability to group brewing isolates of *Pectinatus sp.* according to their origin, mostly indicating that different isolates from the same brewery have a shared origin. The presented results even suggest that there is a certain potential to track contaminations of *Pectinatus sp.* based on their MALDI-TOF MS profiles. Still, to use MALDI-TOF MS as a tracking tool for recurring contaminants by comparing similarities of obtained spectra, a specific database containing extensive spectral data of the brewery's microbiome is required. Effective and reliable MALDI-TOF MS data may then be beneficial to track bacterial contaminants to their source of origin or to monitor the effect of actions taken to eliminate them.

4.4 Identification of isolates from the brewing and beverage industry

671 isolates collected from brewing and beverage environments were analyzed by MALDI-TOF MS as well as a PCR kit or 16S rDNA sequence analysis. Generally MALDI-TOF MS results showed good compliance with results obtained at the BLQ by means of a PCR kit or 16S rDNA sequence analysis (88 % of all samples). Around seven percent of the samples couldn't be identified to species level by either MALDI-TOF MS or 16S rDNA sequence analysis, however the large majority of these samples showed at least concordant identifications by both methods on the genus level. Another five percent of the samples showed differing results. Part of these non-compliances was caused by isolates belonging to the genus *Acetobacter*, which again were concordantly identified on the genus level. Especially the two species *A. fabarum* and *A. lovaniensis* couldn't be distinguished unambiguously. While MALDI-TOF MS favored *A. fabarum*, 16S rDNA sequencing favored *A. lovaniensis*. However, obtained sequences showed high similarities (≥ 99 %) to deposited sequences from more than one *Acetobacter* species. This may come as little surprise as within the species *A. pasteurianus*, which harbors both *A. fabarum* and *A. lovaniensis*, 16S rRNA gene sequences were found to display high degrees of similarity (Huang, 2013). Publicly available 16S rRNA gene sequences of *A. fabarum* [Cleenwerck et al., 2008] and *A. lovaniensis* [Lisdiyanti et al., 2000] showed 99 %

identity upon BLAST query. The residual non-compliances involved more than 20 species and were likely caused by mixed cultures, processed in two different labs, where different organisms were purified. This assumption is supported by the fact, that part of these samples were re-analyzed in both laboratories with results being perfectly concordant. Contaminations in the industry, e.g. in the filling area, often arise from sequential growth of different microorganisms such as acetic acid bacteria, yeast, lactic acid bacteria and strict anaerobes [Back, 1994b], forming a functional consortium of microorganisms in which bacteria of different species may display mutualistic interactions. As a result, process samples from brewing environments frequently contain two or more strains of different species, which at times, were found to be hard to separate by re-streaking.

As part of the bacteria could only be identified in groups by the PCR kit and MALDI-TOF MS generally delivers single species identifications some aspects of these results shall be discussed below.

88 lactic acid bacteria were identified as *L. casei/paracasei* using the commercial PCR kit. MALDI-TOF MS classified only one sample as *L. casei* while the residual 87 were assigned to the species *L. paracasei* based on their mass fingerprints. In general, taxonomy of organisms included in the *L. casei* group, i. e. *L. casei*, *L. paracasei* subsp. *paracasei*, *L. paracasei* subsp*. tolerans* and *L. rhamnosus*, is rather complicated and exacerbated by controversial nomenclature [Sato et al., 2012]. Proposals have been published for reclassification and rejection of the name *L. paracasei* [Dellaglio et al., 1991; Dicks et al., 1996]. Still, *L. casei* and *L. paracasei*, including the sub-species *paracasei* and *tolerans*, are valid taxonomical terms to date based on the proposal of Collins et al. [1989] and the rejection of requests for reclassification [Tindall et al., 2008]. As their 16S rRNA gene sequences were found to show high similarities [Huang and Lee., 2011], 16S rDNA sequencing, which is commonly used for the identification of bacteria [Petti, 2007] and is considered to be the "gold-standard" [Clarridge, 2004], is not applicable for the distinction between members of the *L. casei* group. Alternative methods such as ribotyping or multi locus sequencing are cumbersome and would have gone beyond the scope of this study. Therefore MALDI-TOF MS' ability to distinguish member of the *L. casei* group wasn't followed up. Little is known about differences concerning the beer spoilage ability of the two species *L. casei* and *L. paracasei*. Due to their rather low resistance to hop

bitter compounds, they are both generally regarded as threats for low hopped beers or beers showing elevated pH values [Suzuki, 2011, Back, 2005]. With regard to their detection as contaminations in beer, both species tend to be handled as one [Weber et al., 2008]. Distinction between the two species could, however, be beneficial for breweries if it allows the differentiation of recurring contaminants.

MALDI-TOF MS proved to be capable to distinguish between the closely related species *L. harbinensis* and *L. perolens* if appropriate reference spectra were provided. The applied Biotyper database contains two entries for *L. perolens*, DSM 12745 and the type strain DSM 12744. One of them, DSM 12745 shows spectra, which are more similar to the type strain of *L. harbinensis* (DSM 16991) than to its own species' type strain (DSM 12744). Only after the removal of the misleading reference entry DSM 12745, successful and accurate differentiation, as supported by 16S rDNA sequence analysis, between the two species was enabled. These results are in accordance with the findings of Miyamoto et al. [2005], who suggested reclassification of the strain DSM 12745 as *L. harbinensis* based on DNA-DNA hybridization analysis and underline the importance of an accurate reference database of correctly classified reference organisms to enable successful and unambiguous identification of microorganisms. It was found strongly advisable to remove reference *L. perolens* DSM 12745 from the Biotyper database and rely on a custom-tailored database like the one used in this study, when attempting to separate between the species *L. harbinensis* and *L. perolens*.

Compared to the PCR kit, higher taxonomic resolution was achieved for the differentiation between *L. buchneri* and *L. parabuchneri*. As mentioned above, higher taxonomic resolution may provide and advantage if contaminations can be traced through the production process.

In general, MALDI-TOF MS as an alternative identification tool for brewing contaminants performed equal or better compared to presently popular PCR based methods. Amongst others, it delivered accurate results for *L. brevis*, *L. lindneri* and *Pc. damnosus*, considered to belong to the most relevant beer spoilers [Suzuki, 2011] as well as for the much feared anaerobes *Megasphaera cerevisiae* and *Pectinatus frisingensis*. In addition, MALDI-TOF MS demonstrated the ability to differentiate between the closely related species *L. perolens* and *L. harbinensis*. *L.*

buchneri and *L. parabuchneri* were easily distinguished, too, outperforming the PCR kit.

Recently it has been shown that MALDI-TOF MS is able to distinguish microorganisms below species level [Dieckmann et al., 2008; Seibold et al., 2010] or between closely related organisms as the ones contained in the *L. casei* group [Sato et al., 2012]. For routine identification, the application of direct transfer methods together with poorly standardized growth conditions may represent a limiting factor for the discrimination of closely related organisms. Growth conditions have been suggested to play a major role if minor differences in spectra are responsible for the discrimination of closely related organisms [Sandrin et al., 2013]. However, rigorous standardization of growth conditions and more sophisticated sample preparation would add significantly to the required labor input, posing a major drawback in routine identification for quality control purposes.

Identification of brewery contaminants by MALDI-TOF MS requires pure cultures or at least single colonies on a plate. Cultures should be as fresh as possible, which is especially important for certain isolates of the species *L. brevis* and *L. backii* where aged cultures occasionally delivered no spectra or spectra of low quality in this study. In the presented work isolates were streaked on plates to verify that single organisms were analyzed. Successful identification of whole cells by MALDI-TOF MS was also shown for bacteria grown in broth [Vargha et al., 2006] and may present an alternative if cultures are pure and contain a sufficient amount of cells. Around 10^6 to 10^7 cells were reported as the lower limit enabling acquisition of spectra [Vargha et al., 2006; Shaw et al., 2004], which is in accordance with results obtained for *L. brevis* in this work.

In general, MALDI-TOF MS proved to be a valuable, rapid and easy to handle alternative for the identification of bacterial beer and beverage contaminants. A significant advantage compared to other techniques is for sure its broad band applicability, as there's no need to pre-specify targets [Demirev and Fenselau, 2008]. Mass spectra of unknown samples can easily be compared to a reference database containing more than 4000 spectra during a single run. This could help to speed up the identification of organisms, which are encountered in breweries but not included in PCR kits. While PCR based methods require laborious primer design and specificity testing, MALDI-TOF MS allows to react quickly and effectively to new

threats as new reference spectra can be generated easily. Compared to commonly employed methods such as PCR kits it provides a higher taxonomic resolution for certain organisms. However, currently available systems as the MALDI Biotyper require pure cultures and a sufficient amount of cell material for unambiguous identification. Additionally, high quality reference databases which may be tailored to the respective application are needed. Despite these limitations, MALDI-TOF MS is a valuable alternative for the identification of bacterial contaminations encountered in the brewing and beverage industry, especially if high sample throughput is essential and a broad range of undefined organisms is analyzed.

4.5 Effect of plasmid curing on MALDI-TOF MS spectra of *Lactobacillus brevis* TMW 1.313

Genomic analysis showed that *L. brevis* strains can harbor up to nine distinct plasmids [Fukao et al., 2013]. Marker genes, which are associated with *L. brevis'* potential to grow in beer such as horA, horC or ORF5 were reported to be located on plasmids [Sami et al., 1997b; Samiet al., 1998; Suzuki et al., 2004; Suzuki et al., 2004b]. It was also demonstrated that the loss of a certain plasmid, referred to as pRH45, impaired the ability of a mutant of *L. brevis* ABBC45 to grow in beer [Sami et al., 1998].

Previously in this work it was described that no specific and reproducible biomarker peaks, which would allow delineation between strains with a high or low potential to spoil beer, could be found among the spectra of the 17 different *L. brevis* strains from our culture collection. Plasmid curing experiments with *L. brevis* TMW 1.313 were therefore carried out to check, whether a non-beer spoiling variant of the strongly beer spoiling strain would exhibit distinct and reproducible differences in its mass peptide finger print obtained by MALDI-TOF MS when compared to the wild type.

Plasmid curing of *L. brevis* TMW 1.313 was carried out by repeated sub-culturing at elevated temperatures and the effects on growth behavior in beer and on MALDI-TOF MS spectra were observed. Loss of plasmids or at least partial loss of plasmid sequences was confirmed by specific PCR primers designed to amplify a region of the pRH45II and pLB925 A04 plasmids, found within the genome of *L. brevis* TMW 1.313. Next to its disability to grow in beer, variant *L. brevis* TMW 1.313 PC, which

was obtained by plasmid curing, showed changes in colony morphology when compared to TMW 1.313. Similar changes from rough to smooth colonies were observed in a study with a probiotic *L. brevis* strain, which lost plasmid-located genes potentially associated with stress response through plasmid curing [Fukao et al., 2013] and were thus interpreted as additional evidence confirming the loss of plasmids.

MALDI-TOF MS was successfully shown to be able to detect plasmid insertions in *Escherichia coli* through detection of β-lactamase, which confers resistance to penicillin type antibiotics and is commonly included in cloning vectors [Russell et al., 2007]. Recently it was shown that MALDI-TOF MS has the potential to differentiate between strains carrying different mobile genetic elements such as SCC_{mec}, which encodes for the *mecA* gene and confers resistance to methicillin and β-lactam antibiotics [Malachowa and DeLeo, 2010; Shah et al., 2011]. In a study by Shah et al. [2011], SELDI-TOF MS, a variant of MALDI-TOF MS, was applied for the differentiation between methicillin-resistant *Staphylococcus aureus* and methicillin-sensitive *Staphylococcus aureus* based on seven key ions.

However, to the best of our knowledge, there have been no reports so far on the effects of plasmid curing of strains, which naturally harbor plasmids, on their MALDI-TOF MS profile.

Comparison of MALDI-TOF MS spectra of the wild-type strain *L. brevis* TMW 1.313 and the non-spoiling variant *L. brevis* TMW 1.313 PC showed two reproducibly found peaks for *L. brevis* TMW 1313 which were absent in spectra of *L. brevis* TMW 1.313 PC. Both signals were not found in any of the other examined *L. brevis* strains from our culture collection, indicating that these signals were strain-specific and were thus not further considered as general markers for spoilage potential. Comparison to spectra of *L. brevis* strains, which were grown in beer prior to MALDI-TOF MS analysis, however, showed peaks at identical m/z for strains TMW 1.315 and 1.317. These findings may support the hypothesis, that the presence of the signals is associated with growth behavior in beer in some way. To elucidate the potential function of the proteins behind the two peaks, a database search was run against the proteome of *L. brevis* as calculated from its genome sequences based on the predicted protein sizes. However, none of the two peaks could be associated with a

well-characterized protein. Thus, the identity and function of the proteins causing the two signals remains unclear.

4.6 Comparative analysis of the beer spoilage potential of *Lactobacillus brevis* brewery isolates by MALDI-TOF MS and gene marker analysis

100 brewery isolates of *L. brevis* were examined to determine their ability to grow in beer using a resazurin assay. Subsequently, they were checked for the presence of potential marker gene sequences, which were previously deduced from the whole genome sequences of two strongly beer spoiling strains (TMW 1.313 and 1.465) and two strains with a low potential to spoil beer (TMW 1.1326 and 1.6) in AiF project 16292. Additionally MALDI-TOF MS spectra for all isolates were acquired to compare their protein mass fingerprints. The isolates were assigned to four groups based on the resazurin assay: non-beer spoilers, weak beer spoilers, beer spoilers and strong beer spoilers. Separation of the groups was confirmed by ANOSIM. Both results from MALDI-TOF MS as well as results obtained from Multiplex PCR were compared to the grouping based on the physiological test. Gene marker data, as visualized by HiT-MDS, did not correlate well with the previous grouping as the same configuration of presence and absence of gene sequences in isolates could be found for isolates belonging to different spoilage groups. Prediction of growth behavior of lactobacilli in beer through the use of marker gene sequences such as horA, horC and ORF5 was reported to be problematic before by Haakensen et al. [2008]. These genes, are located on plasmids and may thus be lost if strains proliferate in nutrient broth over multiple passages [Haakensen et al., 2008]. With the exception of horA, only chromosomal gene marker sequences previously established were used in this study. Thus it is unlikely that the loss of plasmids, or the presence of plasmids in only part of the population of an isolate, may have had a major impact on the results. Still, if bacterial cells derived from the same isolate, can differ in their plasmid profile, it is also possible that mobile genetic elements may have an influence on chromosomal gene configuration. Isolates were purified based on repeated sub-culturing of single colonies on mMRS and visual examination was used to check their phenotypic uniformity. As cultures were never diluted to a concentration, where only single cells would have been obtained, it can neither be precluded that isolates may have consisted of a mixture of strains of the same species with different genotypes but identical or highly similar colony morphology. Furthermore, the tested marker genes

were retrieved from the genome sequences of four strains of *L. brevis* only, and may thus not reflect the heterogeneity within the entire species *L. brevis.*

Additionally isolates were compared based on their MALDI-TOF MS spectra. Therefore twelve test spectra were merged to a sum spectrum. The different groups obtained from the resazurin test overlapped, when isolates were distributed based on their MALDI-TOF MS data and distribution was visualized by HiT-MDS. Apparently, spread within the two groups containing strong beer spoilers (SB) and beer spoilers (B) was smaller than within the group of non-beer spoilers (NB). This finding is in accordance with the results presented earlier in this work on MALDI-TOF MS spectra of *L. brevis* strains from our culture collection.

To emphasize on the differences between different beer spoiling groups, DAPC was used for clustering of MALDI-TOF MS spectra acquired from the 100 *L. brevis* isolates. The resulting Figure 11 shows that DAPC indeed delivers a better separation of the four groups, though still outliers are observed which are located closer to the centre of a neighboring group than to the centre of their own group.

In a second approach, MALDI-TOF MS' ability to trace bacterial contaminations was tested. Test spectra of the 100 *L. brevis* isolates were acquired and compared to reference spectra of the same isolates previously deposited in a database. While only 42 % of isolates were correctly matched to the corresponding reference spectrum, 74 % were assigned to the correct brewery of origin and 71 % matched either their own reference spectrum or at least a reference spectrum of an isolate belonging to the same spoilage group. The relatively low number of correct identifications (42 %) may be surprising, especially as it was previously described in this work that around 90 % of the analyzed *L. brevis* strains from our culture collection could be differentiated based on their protein mass fingerprints. Those strains, however, were originating from different environments and with a single exception, all of them showed minor differences in RAPD-PCR patterns. In contrast, brewery isolates frequently shared the brewery of origin or were even isolated at the same time in the same brewery on different spots. It is thus likely, that at least part of the isolates were recurring contaminations, which may be linked to a common source of origin. The fact that around 70 % of the isolates were linked to the correct brewery could be seen as an indication in support of this hypothesis. There are, however, also ways for beer-spoiling bacteria to spread among different breweries such as the transport of

matured beer from one plant to another for filling or the return of empties. Therefore it can't be precluded that highly similar types of spoilage bacteria are found in different breweries, which could contribute to the misclassifications. Additionally certain limiting factors, which have already been discussed above, concerning the results from marker gene analysis, also apply for MALDI-TOF MS analysis, namely limitations with regard to sample purity and potential genetic heterogeneity which may as well influence protein mass patterns. Still, MALDI-TOF MS showed a better ability to differentiate between different spoilage groups than Multiplex PCR of marker genes. This may be due to the fact that generally more than 100 peak signals are detected within mass spectra, contributing to the protein mass fingerprint. In contrast the number of marker genes which can be included in a multiplex PCR assay is rather limited.

5 Summary

The growth of microbial contaminants in industrially produced beverages can cause turbidity, haze and off-flavors resulting in quality loss often rendering the product undrinkable. Accurate, fast and easy to perform identification and differentiation methods for spoilage organisms are thus prerequisites to ensure effective quality control. So far, MALDI-TOF MS was shown to be a valuable tool for the identification of bacteria in clinical applications and is increasingly used for the analysis of food associated bacteria.

Data on the application of MALDI-TOF MS for microorganisms associated with beer and beverage spoilage is however limited. Therefore this work focused on key issues relating to the applicability of MALDI-TOF MS for the identification and differentiation of contaminants which can be found in industrial process and beverage samples.

The most appropriate sample preparation method for the analysis of beer and beverage spoiling bacteria was determined by analyzing three strains as model organisms which belonged to the species *L. brevis, Ln. mesenteroides* and *Pc. claussenii*. Taking into account the quality and reproducibility of the obtained spectra, a simple extraction procedure using ethanol, formic acid and acetonitrile proved to be well suitable for reliable and accurate identification. Direct preparation of whole cells onto the MALDI-TOF MS target failed to constantly deliver spectra for *L. brevis* and thus required repeated analysis. Neither mechanical nor enzymatic pre-treatment of the cells was found to improve spectral quality for any of the tested organisms.

Cultivation of bacteria under different growth conditions may be a concern in terms of portability of results. Though spectra from microorganisms grown under varying conditions such as different nutrient media, presence or absence of oxygen and growth time showed specific reproducibly found signals, these variations were only minor and did not impair identification on the species level. However, if higher taxonomic resolution is desired, rigorous standardization may be beneficial if not required to minimize variations.

In order to verify MALDI-TOF MS potential for the identification of beer and beverage spoilage bacteria, 671 isolates obtained from industrial brewing and beverage environments were analyzed by MALDI-TOF MS. A commercially available PCR kit as well as 16S rDNA sequencing were used as a reference method. Around 88 % of

these samples were identified concordantly by MALDI-TOF MS and the reference method. Next to showing a higher taxonomic resolution compared to the PCR kit, MALDI-TOF MS also allowed to analyze a broader range of organisms as acquired spectra could be compared to a reference database containing several thousand reference spectra.

Besides offering fast routine identification, MALDI-TOF MS could also be of vital interest as a subtyping tool. Being able to distinguish between isolates from different origins or predicting spoilage behavior of unknown isolates is an asset for effective microbiological monitoring not only in the beverage and brewing industry. Two organisms which play a major role in spoilage incidents in the brewing industry due to their prevalence and their detrimental effects on product quality were chosen to further investigate MALDI-TOF MS' taxonomic resolution: *L. brevis* being the most frequently encountered spoilage organism in beer and isolates of the genus *Pectinatus*, which are much feared for their particular unpleasant metabolites. Multiple spectra of 17 strains belonging to the species *L. brevis* were recorded and compared to reference spectra obtained from the same strains. Based on this database approach, more than 90 % of all single spectra were assigned to the correct corresponding reference spectrum. Examination of misclassifications indicated that strains showing highly similar MALDI-TOF MS spectra also showed similar growth behavior in beer as all but one of the 19 misclassifications occurred among strains grouped into the same spoilage type by a physiologic growth assay.

In contrast to the species *L. brevis*, which comprises strains with a high beer spoilage potential as well as strains which are even unable to grow in low hopped beers, isolates of *P. frisingensis, P. haikarae* and *P.cerevisiiphilus* generally exhibit a certain beer spoilage potential and are thus regarded as a serious threat in the brewing industry. Among the 19 isolates of *Pectinatus* analyzed, 17 were found to belong to the species *P. frisingensis.* Analysis of these isolates was carried out as done before with *L. brevis*. On the basis of single spectra, only about 60% of all acquired spectra could be assigned to the correct reference spectrum. Generation of sum spectra from single spectra was able to increase the number of correct assignments to 14 out of 17 isolates by eliminating minor, non-reproducible variations among spectra. However, part of these isolates shared a common brewery of origin and could thus even originate from the same source of contamination within a plant.

The results obtained in this work show that MALDI-TOF MS represents a valuable alternative for the identification of beverage and beer spoiling bacteria in routine quality control. In addition a certain potential for the subtyping of beer spoiling organisms could be demonstrated. Though subtyping requires optimized growth and preparation procedures as well as dedicated software tools, the beneficial additional information on contaminants provided may justify the increased labor input.

6 Zusammenfassung

Mikrobiologischer Verderb führt in Getränken zu Trübungen und sensorischen Beeinträchtigungen und somit zu einer Qualitätsminderung, die bis hin zur Ungenießbarkeit des Getränkes gehen kann. Um eine effiziente Qualitätskontrolle zu gewährleisten werden präzise, schnelle und einfach anwendbare Methoden zur Identifizierung und Differenzierung dieser Verderbniserreger benötigt. MALDI-TOF MS wurde bereits umfangreich in der medizinischen Mikrobiologie eingesetzt und hat sich für diese Anwendung als wertvolles Diagnose-Tool etabliert. In letzter Zeit wird diese Methode auch verstärkt zur Analyse von Keimen verwendet, die in der Lebensmittelmikrobiologie von Bedeutung sind.

Zur Anwendung von MALDI-TOF MS für die Untersuchung von Bakterien, die in Brauereien oder generell in der Getränkeindustrie von Bedeutung sind, lagen allerdings noch keine ausreichenden Daten vor. Diese Arbeit beschäftigte sich deswegen mit Schlüsselfragen der Anwendbarkeit von MALDI-TOF MS zur Identifizierung von Kontaminanten im Getränkebereich.

Um die optimale Probenvorbereitung für die Analyse von bier- und auch getränkeverderbenden Bakterien zu finden, wurde je ein Stamm der Spezies *L. brevis, Ln. mesenteroides* und *Pc. claussenii* als Modellorganismen verwendet. Dabei hat sich unter Berücksichtigung der Reproduzierbarkeit und Qualität der Spektren gezeigt, dass ein einfaches Extraktionsprotokoll mit Ethanol, Ameisensäure und Acetonitril zu den besten Ergebnissen hinsichtlich der Zuverlässigkeit der Identifizierungen führt. Das direkte Aufbringen unbeschädigter Zellen auf den MALDI-TOF MS Probenträger erwies sich als weniger geeignet, da in mehreren Fällen für die Spezies *L. brevis* im Automatik-Modus keine Spektren aufgezeichnet werden konnten. In diesen Fällen mussten die Messungen im manuellen Modus wiederholt werden. Weder eine mechanische noch eine enzymatische Vorbehandlung der Proben vor Anwendung des Extraktions-Protokolles konnte die Qualität der Spektren für die getesteten Spezies verbessern.

Um die Übertragbarkeit der Identifizierungsergebnisse zu gewährleisten, musste der Einfluss verschiedener Wachstumsbedingungen der Organismen auf die anschließend aufgenommenen MALDI-TOF MS Spektren geprüft werden. Obwohl die Spektren der Bakterien, die auf verschiedenen Nährmedien gewachsen waren,

aerob oder anaerob angezogen wurden oder auch in unterschiedlichen Wachstumsphasen analysiert wurden, spezifische reproduzierbare Signale beinhalteten, waren diese Schwankungen vergleichbar gering und hatten keine negative Auswirkung auf die Identifizierung der korrekten Spezies. Für eine bessere taxonomische Auflösung hingegen, die über das Spezies-Niveau hinaus geht, dürfte eine möglichst präzise Standardisierung der Wachstumsbedingungen von Vorteil, wenn nicht sogar zwingend erforderlich sein um die Variabilität der Spektren so gering wie möglich zu halten.

Um die Einsetzbarkeit von MALDI-TOF MS zur Identifizierung von bier- und getränkeverderbenden Bakterien zu verifizieren, wurden 671 Isolate aus der Brau- und Getränkeindustrie gesammelt und analysiert. Als Referenzmethode wurde einerseits ein PCR Kit herangezogen, der die meisten in der Brauindustrie relevanten Bakterien abdeckt. Für alle mittels Kit nicht erfassten Organismen wurde eine 16S rDNA Sequenzierung durchgeführt. 88 % aller Proben ergaben übereinstimmende Identifizierungen mittels MALDI-TOF MS und auch der Referenzmethode. Im Gegensatz zum PCR Kit zeigte MALDI-TOF MS dabei eine bessere taxonomische Auflösung und erlaubte darüber hinaus auch die Identifizierung einer breiteren Auswahl an Organismen, da die Identifizierung auf dem Vergleich der Spektren zu einer Datenbank beruht, in der mehrere tausend Referenzspektren hinterlegt sein können.

Neben der Einsatzmöglichkeit als schnelle Routinemethode zur Identifizierung der jeweiligen Spezies, bietet MALDI-TOF MS auch die Möglichkeit eine Sub-Spezies-Typisierung der Isolate vorzunehmen. So kann es im mikrobiologischen Monitoring eines Lebensmittelbetriebes von enormem Vorteil sein, wenn zwischen Isolaten mit verschiedener Herkunft oder unterschiedlichem Wachstumsverhalten unterschieden werden kann. Um die Potentiale, die MALDI-TOF MS hinsichtlich Sub-Spezies-Typisierung in der Getränkeindustrie bietet auszuloten, wurden zwei in der Brauindustrie als Verderbnisorganismen relevante Organismen herangezogen: *L. brevis* als der am häufigsten aus Bier isolierte Verderbniserreger sowie Isolate der Gattung Pectinatus, die aufgrund ihrer sensorisch besonders unangenehmen Stoffwechselprodukte in der Brauindustrie besonders gefürchtet sind. Von 17 Stämmen der Spezies *L. brevis* wurden MALDI-TOF MS Spektren aufgezeichnet und mit Referenzdatenbankeinträgen der gleichen Stämme abgeglichen. Basierend auf

dem Vergleich einzelner aufgezeichneter Spektren konnten mehr als 90 % aller Einzelspektren zum korrekten Referenzspektrum zugeordnet werden. Bei genauerer Betrachtung der falsch zugeordneten Spektren, zeigte sich, dass Stämme die sehr ähnliche MALDI-TOF MS Spektren aufwiesen meist auch über ein vergleichbares Wachstumsverhalten in Bier verfügten. 19 Spektren wurden falsch klassifiziert, wovon alle bis auf ein einziges Spektrum dem Referenzspektrum eines Stammes zugeordnet worden waren, der auf Basis eines physiologischen Wachstumstests in Bier das gleiche Wachstumspotential zeigte.

Im Gegensatz zur Spezies *L. brevis*, die sowohl Stämme mit hohem Verderbspotential beinhaltet, als auch Stämme, die nicht in Bier oder nur in sehr niedrig gehopften Bieren wachsen können, zeigen Isolate, die den Spezies *P. frisingensis, P. haikarae* und *P. cerevisiiphilus* zuzuordnen sind ein generelles Verderbspotential und werden in der Brauindustie daher als ernsthafte Bedrohung angesehen. Von den 19 Isolaten der Gattung *Pectinatus*, die analysiert wurden, gehörten 17 der Spezies *P. frisingensis* an. Die Analysen wurden dabei analog zu denen der Stämme der Spezies *L. brevis* ausgeführt. Beim Abgleich von Einzelspektren gegen Referenzdatenbankeinträge derselben Isolate, konnten allerdings nur 60% aller aufgenommener Spektren zur richtigen Referenz zugeordnet werden. Das Aufsummieren der einzelnen Testspektren eines Isolates zu einem Summenspektrum, welches dann wiederum gegen die Referenzdatenbankeinträge abgeglichen wurde, führte zu einer Verbesserung der korrekten Identifizierungsquote. Von 17 Isolaten konnten so 14 der dazugehörigen Referenz zugeordnet werden. Ein Teil dieser Isolate stammten allerdings aus derselben Brauerei und könnten somit auch derselben Kontaminationsquelle innerhalb einer Brauerei entsprungen sein.

Zusammenfassend kann gesagt werden, dass MALDI-TOF MS eine werrtvolle Alternative zur Identifizierung von getränke- und bierverderbenden Bakterien in der Routinediagnostik im Rahmen der Qualitätskontrolle bietet. Zusätzlich kann diese Methode bei geeigneter Probenvorbereitung und –aufbereitung auch zur Sub-Spezies-Typisierung heranzgezogen werden. Dies setzt zwar die Verfügbarkeit geeigneter Software sowie einen höheren Arbeitsaufwand voraus, könnte aber wertvolle zusätzliche Informationen bezüglich Verderbnispotential oder Herkunft der Kontaminanten liefern.

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8 Appendix

Prior printed publications

Kern, C.C., Usbeck, J.C., Vogel, R.F., Behr, J., 2013. Optimization of Matrix-Assisted-Laser-Desorption–Ionization-Time-Of-Flight Mass Spectrometry for the identification of bacterial contaminants in beverages. Journal of Microbiological Methods 93 (3), 185–191.

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