Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry as a novel tool for detection, identification and typing of spoilage microbiota in the brewing industry

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Dissertation submitted in fulfilment of the requirements for the degree of Doctor (Ph.D.) in Sciences, Biochemistry and Biotechnology

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In Sweet Memory of Beloved Diane De Sutter,

Our Guardian Angel

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LIST OF ABBREVIATIONS

a.i.	absolute intensity
AAB	acetic acid bacteria
AAM	growth medium for acetic acid bacteria
ABC	ATP binding cassette
ABD	advanced beer spoiler detection
ATP	adenosine triphosphate
BCC	BIOTEC culture collection
BCCM	Belgian co-ordinated culture collection of microorganisms
BLAST	basic local alignment search tool
bp	basepairs
BS	beer sample
BSB	beer spoilage bacteria
bsr	beer spoilage related
CCUG	culture collection, university of Göteborg
CEM	cell extraction method
CFU	colony forming units
CLEI	chemiluminescence enzyme immunoassay
CWT	continuous wavelet transform
Da	dalton
DDH	DNA-DNA hybridization
DGGE	denaturing gradient gel electrophoresis
DHB	2,5-dihydroxybenzoate
DMS	growth medium for acetic acid bacteria
DNA	deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DYPA	general growth medium for yeast
EBC	European brewery convention
ELISA	enzyme-linked immune sorbent assay
FAFLP	fluorescent amplified fragment length polymorphism
FAME	fatty acid methyl ester
FISH	fluorescence *in situ* hybridization
GC	gas chromatography
GY	growth medium for acetic acid bacteria
GYAE	growth medium for acetic acid bacteria
IBU	international bitter unit
ICM	intact cell method
IFO	institute of fermentation
ITS	internal transcribed spacer
IVD	*in vitro* diagnostic
JCM	Japan collection of microorganisms
kDa	kilodalton
kV	kilovolt
KVE	kolonievormende eenheden
LAB	lactic acid bacteria
LMG	laboratory of microbiology Ghent University
m/z	molecular weight-to-charge
MAbs	monoclonal antibodies
MALDI TOF/TOF	matrix-assisted laser desorption/ionisation time-of-flight/time-of-flight
MALDI-TOF MS	matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

MALDI-TOF	matrix-assisted laser desorption/ionisation time-of-flight
MDR	multidrug resistance
MDS	multidimensional scaling
MLSA	multilocus sequence analysis
MRS	growth medium for lactic acid bacteria
MSP	main spectral projection
MSV	minimal similarity value
NBB	growth medium for beer spoilage bacteria
NBRC	biological resource centre, national institute of technology and evaluation
Nd:YAG	neodymium-doped:yttrium aluminum garnet
nm	nanometre
nt	nucleotides
OD_{590}	optical density at 590 nm
ORF	open reading frame
PCR	polymerase chain reaction
PMF	proton motive force
ppm	parts per million
r.int.	relative intensity
RAE	growth medium for acetic acid bacteria
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RND	resistance-nodulation cell divisions
rRNA	ribosomal RNA
q-PCR	quantitative PCR
RUO	research use only
S/N	signal-to-noise
SARAMIS™	spectra archiving and microbial identification system
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SHA	sandwich hybridization assay
sp. nov.	species nova
SSP	summary spectral profile
subsp.	subspecies
TOF	time-of-flight
UPGMA	unweighted pair group method with arithmetic mean
v/v	volume per volume
VIT®	Vermicon® identification technology®
w/v	weight per volume
YPM	growth medium for acetic acid bacteria
α-CHCA	α-cyano-4-hydroxycinnamic acid

LIST OF GENES

atpA	encoding ATP synthase
dnaK	encoding the heat-shock protein DnaK
groEL	encoding a 60 kDa chaperonin protein
pheS	encoding phenylalanyl-tRNA synthase alpha subunit
rpoA	encoding α-subunit of the RNA polymerase
rpoB	encoding β-subunit of the RNA polymerase

OUTLINE OF THE THESIS

In **PART I** the background of the thesis and the main objectives of the present study are described.

PART II consists of a comprehensive literature study on bacterial beer spoilage and the present applications of Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS). **CHAPTER 1** gives an elaborate description of the frequently encountered beer spoilage bacteria and their presence in the brewing process and in the brewery environment. The microbiological stability of beer products and the phenomenon of hop resistance are discussed as well. **CHAPTER 2** summarises the currently available culture-dependent and culture-independent techniques for the detection and/or identification of beer spoilage bacteria. Finally, **CHAPTER 3** presents the main principles of MALDI-TOF MS and provides an overview of its applications in microbiology.

PART III encompasses the experimental work performed during the study. **CHAPTER 4** describes the effect of the growth medium on the generated mass spectra and its consequences for species and strain level differentiation. **CHAPTER 5** presents the development of the MALDI-TOF MS identification database and the subsequent evaluation and validation of the taxonomical classification of the reference strains included. Moreover, the performance of MALDI-TOF MS as an accurate, rapid and high-throughput identification tool was determined using isolates from different spoiled beer and brewery samples. These studies also resulted in the reclassification of *Pediococcus lolii* strains DSM 19927^T and JCM 15055^T as *Pediococcus acidilactici* (**CHAPTER 5.2**) and in the description of a novel acetic acid bacterium, *Gluconobacter cerevisiae* sp. nov., isolated from a spoiled brewer's yeast starter culture (**CHAPTER 5.3**). **CHAPTER 6** describes the direct detection and identification of bacteria in enrichment cultures of spoiled beer and brewery samples. Finally, **CHAPTER 7** evaluated MALDI-TOF MS as a tool to differentiate between brewer's yeasts and unwanted brewing yeast strains contaminating the brewing process.

In **PART IV** the results of this study are re-evaluated and presented in a general conclusion and future perspectives section.

PART V comprises a summary of the results presented in this thesis.

PART I | BACKGROUND & OBJECTIVES

BACKGROUND

Bacterial spoilage of beer and of intermediates of the brewing process is a common threat in the brewing industry as these bacteria typically cause visible turbidity, acidity and off-flavours. Spoilage incidents instigate not only vast economic damages in terms of product recalls, but also irreversible loss of consumer confidence, leading to problems for corporate brands. Moreover, the increasing popularity of non-pasteurised, low- and non-alcoholic beers imparted that beer spoilage is of serious concern to the brewing industry worldwide. Multiple culture-dependent and culture-independent methods have been employed for the detection and sometimes species level identification of spoilage bacteria. These include growth on selective media, the use of species-specific oligonucleotide probes, PCR-based (fingerprinting) methods and the use of monoclonal antibodies. However, these methods are time-consuming and therefore lack high-throughput capacity and do not facilitate real-time interventions. Consequently, microbiological quality control requires lengthy storage of beer, which increases brewing costs and demands for more storage capacity. Furthermore, beer spoilage bacteria are taxonomically diverse while most of these methods specifically target only a narrow range of spoilage organisms. Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) has recently been introduced as a rapid, high-throughput and low-cost tool for species level identification in medical, environmental and food-related studies. MALDI-TOF MS is a soft ionisation technique allowing desorption and ionisation of biomolecules, such as proteins and peptides, in a non-destructive manner and couples high sensitivity with accuracy. The generated ions provide a peptide fingerprint that can be used to characterise and identify bacteria at species and infraspecific levels.

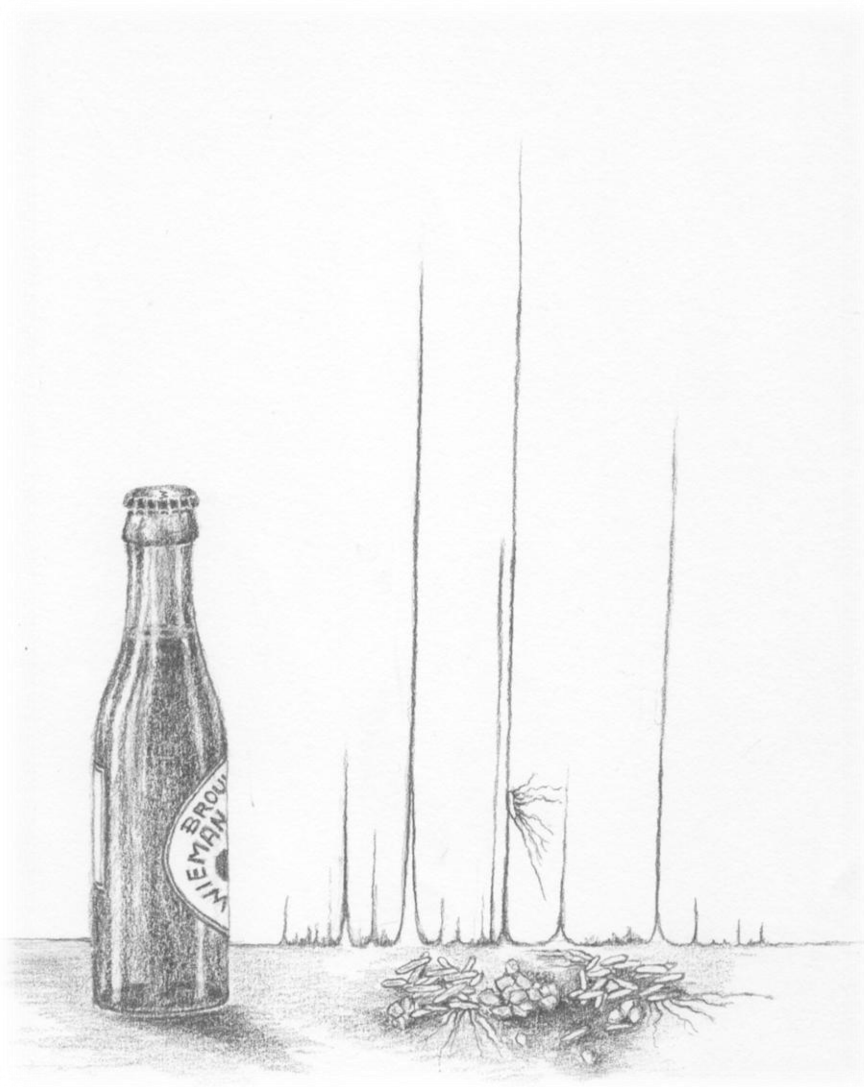
OBJECTIVES

The main objective of the present study was to investigate (***i***) the potential of Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) as a rapid, low-cost and high-throughput identification tool for spoilage bacteria isolated from beer and brewery samples. Therefore an in-house MALDI-TOF MS identification database was designed containing well-characterised reference strains originating from spoiled beer and brewery samples but also from other niches where the same species occur. Moreover, all reference strains were grown onto multiple selective and non-selective growth media to include and anticipate growth medium induced variations.

This first objective was subdivided into three specific aims.

- The investigation of the influence of the growth medium on the mass spectra generated from the reference strains; and its consequence for species and infraspecific level identification.
- The validation of the taxonomical classification of the reference strains present in the MALDI-TOF MS identification database, allowing for an accurate species level identification of unknowns.
- The application of MALDI-TOF MS for the species level identification of potential spoilage bacteria isolated from different beer and brewery samples.

Furthermore, (***ii***) the potential of MALDI-TOF MS was investigated for the detection and identification of spoilage bacteria in enrichment cultures obtained from beer and brewery samples. Finally, (***iii***) MALDI-TOF MS was also explored as a tool to differentiate among brewing and non-brewing yeast strains.



Part II | Literature Study

1 The brewing process and beer spoilage bacteria

1.1 Microbiological threats during the brewing process

The brewing process consists of multiple consecutive steps in which raw substrates are converted into a final beer product (**FIG 1.1**). The main substrate used for the production of beer is malted barley, but also other cereal grains, such as wheat, rye, oats, millet or sorghum can be used [1, 2]. The three-step-malting process starting from the raw cereal grains is necessary to modify the structure of the grains and to produce and/or activate the mashing enzymes [1-4]. In the first step, cereal grains are excessively steeped in water, aerated and drained during which the germination of the grains is stimulated [1, 3]. This natural, yet controlled germination process permits the accumulation of mashing enzymes, sugars and other soluble materials inside the grains [1, 5]. During the steeping process, various indigenous cereal microorganisms (*i.e.*, fungi, yeasts and bacteria) are able to proliferate and to produce certain off-flavours which can persist, even into the final beer product [4, 6-8] (**FIG 1.1**). Some fungal species (*e.g.*, *Fusarium* spp., *Alternaria* spp.) are able to produce secondary metabolites such as mycotoxins and these are neither removed nor degraded during the brewing process and may cause serious health problems [7, 9]. The inoculation of lactic acid bacteria (LAB) in steeping water will not only reduce microbiological contaminations, but will also improve the quality of the malt resulting in a better mash and wort filterability, a lower viscosity and less turbidity [9, 10]. Once grain germination and the concomitant intracellular modifications are completed, the grains are kilned [1, 3] during which the moisture content of the grain is decreased so that preservation and microbiological stability are enhanced [1, 5].

In the brewhouse, the malt is consecutively grinded (*i.e.*, the grist), mixed with water, mashed and filtered to remove spent grains from sweet unhopped wort [2, 3] (**FIG 1.1**). During mashing, the temperature is gradually increased and held constant for a pre-determined time to allow enzyme-catalyzed hydrolytic conversion of starch into fermentable sugars and to partially breakdown proteins [1, 3, 5, 11]. At the end of mashing, the sweet wort is separated from solids (the spent grains) via the lautering process [1, 2, 11] (**FIG 1.1**). Biological acidification of wort and mash is frequently applied in the brewing industry, especially in German breweries that operate according to the Bavarian purity law (*i.e.*, only malted barley, hops, yeasts, and water may be used for the production of beer) [3, 8, 9, 12]. Thereby, the inclusion of biologically acidified malt to the grist and/or the addition of biologically acidified unhopped wort at the start of mashing or during wort boiling are the only technological possibilities to correct the acidity of mash and wort [9, 10]. The pH reduction

will not only reduce microbiological contamination, but is also beneficial for the activity of mash enzymes, increasing the bioavailability of zinc ions, decreasing the viscosity, enhancing filterability and reducing haze and turbidity formation [1, 4, 9, 10]. The particular LAB strain(s) used for biological acidifications are often isolated from malt and should be carefully selected and under full control [1, 8, 10] (**FIG 1.1**). Usually moderately thermophilic, homofermentative, non-beer spoiling and hop-sensitive lactobacilli are selected [8].

Sweet wort represents an optimal substrate for a large group of microorganisms due to its high nutritional content, and is therefore boiled with hop [8, 12, 13] (**FIG 1.1**). During this step, the hop α-acids are extracted and subsequently isomerised into the more soluble iso-α-acids. The latter anti-bacterial compounds will also provide bitterness to the final beer [1, 14]. The same boiling step also concentrates -at least partly- the wort, removes volatile compounds and reduces the potential bacterial load [1, 3]. Moreover, the precipitation of proteins (also called the 'hot break'), the inactivation of mash enzymes, the removal of unpleasant grainy characteristics and the augmentation of wort colour and flavour complexes which all occur during this step, improve the quality of the final product [1, 3]. To avoid downstream microbiological contaminations, it should be noted, that from this critical point on, all process equipment (*i.e.*, vessels, piping structures, surfaces, air, control equipment) should comply with high hygienic standards [11, 15, 16].

The spent hops and precipitated protein-polyphenol complexes (*i.e.*, hot trub) are separated from the wort, which is cooled and aerated prior to pitching it with the brewer's yeast culture [1, 3, 17] (**FIG 1.1**). The wort should be cooled to 6 to 22°C depending on the type of fermentation foreseen, *i.e.*, traditionally 6 to 12°C for lager and 15 to 22°C for ales type beers [5]. These temperatures and the presence of nutrients, growth factors and dissolved oxygen (6 to 8 ppm) will favour the growth of multiple bacteria [8]. Although, cooled and aerated wort is sterile prior to pitching, the risk of microbiological contamination increases when wort is not immediately pitched [12]. Depending on the type of beer produced, yeast cultures with different fermentation characteristics can be pitched, for example bottom fermenting yeasts for lager beers and top fermenting ones for ale beers [1, 3], but in both cases the sugars are mainly fermented into ethanol and carbon dioxide [1, 2]. Intriguingly, the pitching yeast culture itself is one of the major sources of microbiological contamination during the brewing process, as these cultures are often recycled from previous fermentations [11, 16] (**FIG 1.1**). Hence, pitching yeast cultures contaminated with spoilage bacteria or wild-type yeast may induce spoilage in later batches of beer [6, 18, 19]. Therefore, stringent hygienic standards should be applied at all times to prevent microbiological contamination of the pitching yeast cultures [20]. After the main fermentation, the immature or green beer is matured (*i.e.*, the lagering) at low temperatures for a period of time [3] (**FIG 1.1**). During this extended maturation process the so

called cold break occurs, during which yeast cells and other precipitates settle down to the bottom of the lagering vessel [1, 2]. Subsequently, most beers are chilled and filtered [5, 11]. After a final microbiological stabilization step (*e.g.*, pasteurization, filtering), the beer is suited to be filled in bottles, cans or kegs [1, 11] (**FIG 1.1**). In the production of non-pasteurised beers, more stringent hygienic standards must be applied to prevent bacterial spoilage [21]. The use of filtration to remove contaminants in non-pasteurised beers is not always successful, as certain beer-adapted bacteria could pass through the filter membranes and consequently potentially spoil the final product [22, 23].

The filling and packaging systems have also been improved over the years resulting in lower levels of oxygen in the packaged product [10, 24]. Hence, strict aerobic microorganisms are hampered in growth; yet strict anaerobes advance in such conditions [16]. Again, hygienic standards should be outstanding in the filling and packaging hall of the brewery to avoid contamination [24, 25]; especially as the sequential growth of different types of bacteria in the form of biofilms can lead to persistent contaminations in the filling hall [8, 18, 26, 27] (**FIG 1.1**). Additional care must be taken as airborne bacteria can enter the final product prior to sealing resulting in detrimental effects to the product if no in-package pasteurization is applied [1, 8].

In general, two types of microbiological contaminations exist: primary and secondary contaminations according to the presence of the microorganisms within the brewing process [1, 12]. Primary contaminations include bacterial growth in wort, in the pitching yeast, during fermentation, in bright beer tanks and in rest beer [8, 26]. Bacterial growth on equipment, instruments, gas pipes, system dead ends, worn floor surfaces, wort aeration and cooling equipment are indications of insufficient cleaning, but are also included as primary contaminations [26]. Secondary contaminations occur in the filling and packaging hall and are introduced to the beer during bottling, canning or kegging [8, 28] (**FIG 1.1**).

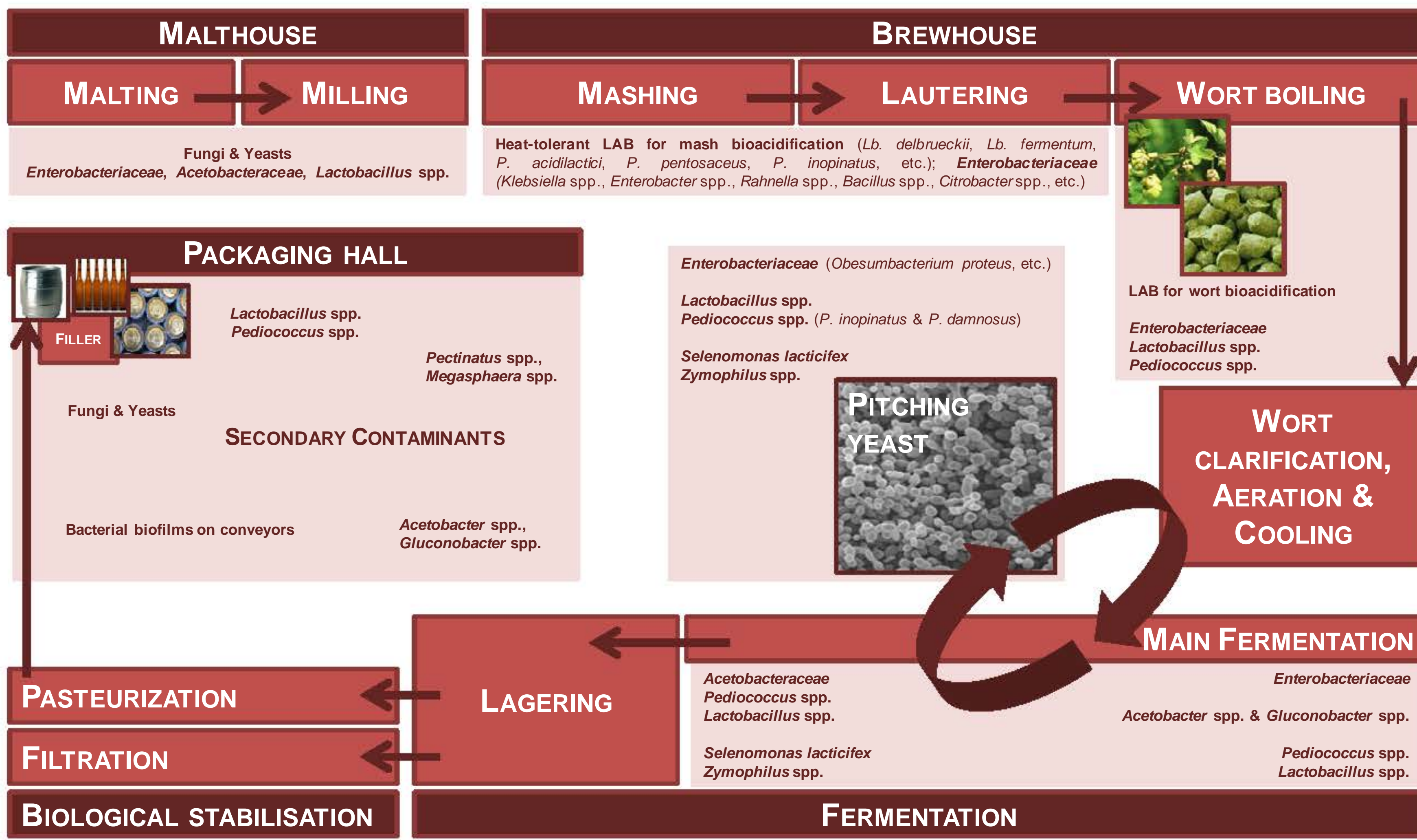


FIG 1.1. Overview of the brewing process displaying the stages during which the main beer spoilage bacteria are introduced and prevail.

1.2 Microbiological stability of beer

In general, beer is a beverage with a good microbiological stability because it contains almost no oxygen (< 0.1 ppm) or nutrients to sustain bacterial growth, because these nutritive substances (sugars and amino acids) are mostly depleted by the brewer's pitching yeast during fermentation [14, 29]. In addition, the low pH (3.8 to 4.7), high carbon dioxide content (usually higher than 0.5% w/v), the presence of ethanol (0.5 to 14% v/v) and antibacterial hop compounds (approx. 17 to 55 ppm of iso-α-acids) ensure a microbiological stability [3, 14, 18, 29-33]. The inhibitory effects of molecular SO_2, the content of free amino nitrogen (FAN), the total soluble nitrogen content, and of individual amino acids and phenolic compounds on bacterial growth have been investigated and acknowledged as well [26, 30, 34-36]. And finally, the use of a final filtration step and/or pasteurization step in combination with the storage of beer at low temperatures decreases bacterial beer spoilage [8, 13, 14, 26].

Nevertheless, the brewing process itself is prone to bacterial growth because of the nutrient-rich environment of wort (presence of fermentable sugars and amino acids) and additional growth factors (vitamins, etc.) produced by the pitching yeast during fermentation [8, 12, 13]. The production time from wort boiling to the final beer packaging can take up to several weeks and gives the bacteria present in the brewery sufficient time to proliferate and subsequently spoil the beer [26]. In some cases beer spoilage bacteria survive the pasteurization processes rendering them viable but non-culturable; yet these cells might cause spoilage in a later stage [37]. Beers with elevated pH, low ethanol and carbon dioxide content and added sugars, the so-called "weak beers", are more susceptible to bacterial spoilage [8]. The increased popularity of non-pasteurised, low-alcoholic and non-alcoholic beers imparted that bacterial beer spoilage is of serious concern to the brewing industry worldwide [38, 39] as these bacteria typically cause visible turbidity, acidity and off-flavours [11, 14, 30, 40]. Such bacterial incidents instigate not only vast economic damage in terms of product recalls, but also to an irreversible loss of consumer confidence, leading to the destruction of corporate brands [21, 32, 41].

1.3 Hop resistance

Hop bells and related products, derived from the plant *Humulus lupulus* L., are added during wort boiling conferring bitterness, but also anti-bacterial properties to the beer [1, 14, 40, 42]. For centuries, it was believed that the addition of hops protected the beer from bacterial contamination. However, hop compounds only inhibit growth of most Gram-stain positive bacteria [43], but certain lactic acid bacteria (LAB) are tolerant towards hop compounds and thus grow in hopped beer [44]. A broad diversity in beer spoilage ability has been recognised among strains within the same species, for which some strains are hop-resistant while others are hop-sensitive [45-47]. Hop-resistant lactobacilli and pediococci do not differ in their phenotypic characteristics (*i.e.*, carbohydrate utilization pattern, manganese requirement, sensitivity to superoxide radicals, resistance to antibacterial agents, expression of cellular proteins or in products of metabolism) compared to the hop-sensitive strains [30, 35]. Yet, differences were found in the transmembrane pH gradient and in the intracellular ATP pool [48].

Several studies attempted to elucidate the mechanisms of hop-resistance in LAB [14, 44, 45, 49-54]. Most studies focussed on the anti-bacterial effect of iso-α-acids since these hop compounds are mainly present in beer [14]. The iso-α-acids are weak acids whose undissociated forms are responsible for the inhibition of bacterial growth. Moreover, their anti-bacterial activity increases at lower pH values [42, 46]. Buggey *et al*. [55] demonstrated that the more hydrophobic and reduced iso-α-acids have a greater anti-bacterial activity than their iso-α-acids analogues.

Iso-α-acids function as ionophores by dissipating the pH gradient across the cytoplasmic membrane and reducing the proton motive force (PMF) [40, 42, 49-51, 53] (**FIG 1.2**). Consequently, the hop-induced decrease in intracellular pH leads to the inhibition of PMF-dependent nutrient uptake, nutrient transport, essential enzyme reactions and synthesis of proteins, DNA and RNA, which all combined, finally lead to the cell death of hop-sensitive strains [14, 35, 50, 51]. The ability to resist these hop compounds appears to be multi-factorial and these mechanisms are not necessarily used at the same time [50, 56] (**FIG 1.2**). It is assumed that the resistance activation depends on several selective pressures (*e.g.*, nutrient availability, presence of ethanol and pH) and on the particular beer spoiling strain investigated [31, 46].

Studies revealed that several genetic markers correlated with hop resistance; and that each of these markers were extraordinarily homologous among different strains of different species suggesting that these markers were acquired via horizontal gene transfer [35, 45, 49, 57-59]. Consequently, such species-independent genetic markers allowed the detection of beer spoilage lactobacilli and pediococci [33, 57, 60].

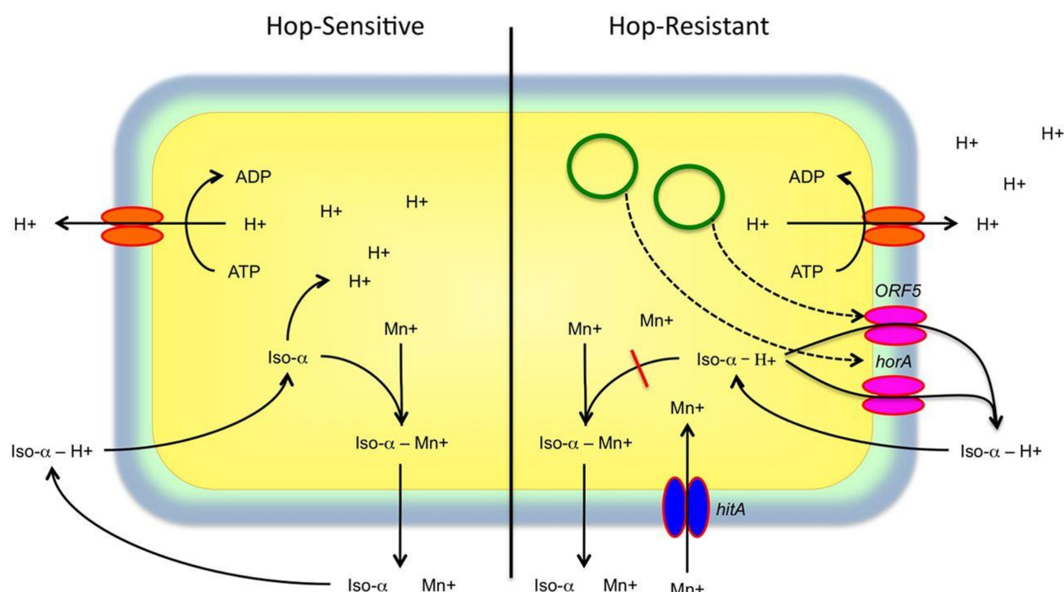


FIG 1.2. Overview of the main mechanisms of hop toxicity (left side cell) and hop resistance (right side cell) in Gram-stain positive bacteria. Green loops indicate plasmids carrying the hop resistance genes horA and ORF5. The hop-inducible HitA pump accumulates intracellular Mn+ in response to oxidative stress, decreasing efflux of Mn+ (red line). (retrieved from [6]).

The species-independent marker gene *horA* was originally identified on pRH45I, a plasmid of 15.1 kb present in the hop resistant *Lactobacillus brevis* strain ABBC45 and whose copy number multiplied with increasing hop resistance [40]. The HorA acts as an ATP-binding cassette (ABC) multidrug resistance (MDR) transporter that excretes hop compounds into the outer medium upon ATP hydrolysis and consequently maintaining the PMF and the internal pH at viable levels [44, 45] (**FIG 1.2**). Some *horA*-negative beer spoilage LAB have been reported, suggesting that these strains possessed other, *horA*-independent hop-resistance mechanisms [33, 59, 60]. Besides *horA*, other species-independent genetic markers were retrieved and correlated with hop resistance (*e.g.*, *bsrA*, *bsrB*, *ORF5* and *hitA*) [31, 33, 59, 61] (**FIG 1.2**). Another possible marker gene is *horC* which presumably encodes a transporter of the resistance-nodulation-cell divisions (RND) superfamily and confers hop resistance by potentially acting as a PMF-dependent MDR transporter [33, 49, 62] with *horB* as putative regulator of *horC* [19]. Differentiation between beer spoiling and non-spoiling pediococci for example, was accomplished via *bsrA* and *bsrB*, which are two beer spoilage-related ABC MDR transporter genes [31].

Previous studies mainly focused on the role of PMF dissipation, but the role of divalent manganese in the mechanism of hop-resistance should not be neglected [51] (**FIG 1.2**). Hop compounds also inhibit the bacterial metabolism by decreasing the intracellular manganese concentrations [51]. It is

thought that hop bitter acids exchange protons for cellular divalent cations, such as Mn^{2+} [49] (**FIG 1.2**). The hop-inducible PMF-dependent divalent cation transporter HitA accumulates intracellular divalent ions in response to oxidative stress [35, 50]. Alteration of the teichoic acids in the cell wall, which provide a constant reservoir of bound divalent cations [50, 56], was observed by Yasui *et al*. [63] as well. Furthermore, overexpressed proton-translocating ATPase increases the pumping of protons released from the hop compounds [53] and maintains the intracellular pH [50] (**FIG 1.2**). Hop stress in hop-resistant *Lb. brevis* strains also induces the expression of a broad range of proteins involved in redox homeostasis, DNA- and protein repair, and facilitates a shift towards energy balance and metabolic regulation to cope with the low pH condition and oxidative stress [6, 51].

It is clear that the hop resistance mechanisms act at various molecular levels including changes in metabolism, membrane physiology and cell wall composition [50]. Additionally, it should be noted that hop resistance is not a stable characteristic and multiple studies revealed that a decrease in hop resistance could occur after the subculturing of hop-resistant strains in the absence of hop compounds [33, 46, 49, 57].

1.4 Thriving beer spoilage bacteria – a current overview

A beer spoilage bacterium can be defined as any organism which is not deliberately introduced and is able to survive and proliferate during the brewing process (*i.e.*, wort, fermenting wort, beer after filtration or in packaged beer) [64]. Bacterial contamination could render the final beer product unfit for consumption due to visible and perceptible defects accompanied with spoilage. In general, bacterial growth and the spoilage ability is strain-dependent, but also depends on the beer type [30]. For example, beer types characterised by an elevated pH, low ethanol and hop content, the so-called "weak beers" are more prone to bacterial spoilage compared to strongly hopped beers [28]. Based on their spoilage ability, the beer spoilage bacteria are classified into five categories, namely obligate, potential and indirect beer spoilage bacteria, the less harmful indicator and latent bacteria [1, 12] (**TABLE 1.1**). Traditionally, these bacteria are further divided into two groups based upon their reaction to Gram-staining (**FIG 1.3**). In the next section, the main characteristics and their presence throughout the entire brewing process of the taxonomic heterogeneous group of beer spoilage bacteria are elaborately discussed (**FIG 1.1**, **FIG 1.4**).

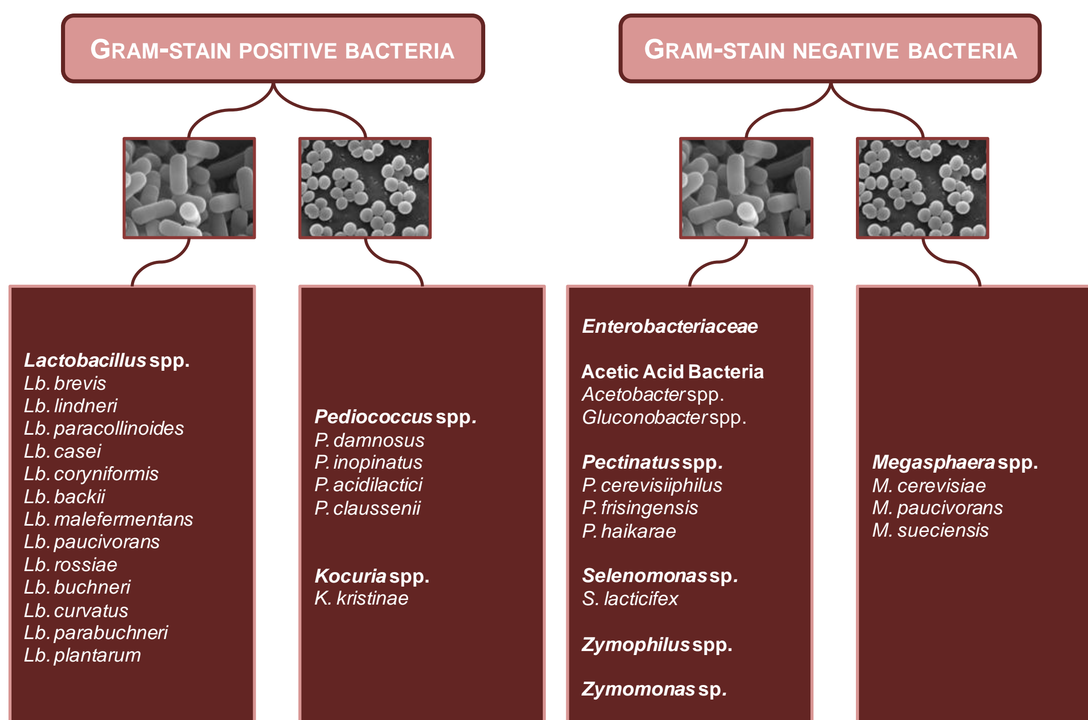


FIG 1.3. Classification of the most important beer spoilage bacteria based on their Gram-stain result and cell morphology.

TABLE 1.1. The classification of the most important beer spoilage bacteria into five categories based on their beer spoilage ability [12, 26, 29, 64-67].

Obligate beer spoilage bacteria	
Growth without extended adaptation time in strong beer types (*i.e.*, pH < 4.3, hop content of > 30 IBU, low oxygen and nutritional content and high alcohol) resulting in profound off-flavours, precipitates and turbidity. Occur throughout the entire brewing process.	*Lb. brevis, Lb. lindneri, Lb. buchneri, Lb. coryniformis, Lb. curvatus, Lb. casei, Lb. paracollinoides, Lb. plantarum, Lb. rossiae, Lb. backii, Lb. casei, P. damnosus, Pe. cerevisiiphilus, Pe. frisingensis, M. cerevisiae, S. lacticifex*
Potential beer spoilage bacteria	
Growth occurs after extended adaptation time in beer characterised by high pH and oxygen content, low IBU and alcohol content, low degree of fermentation (*i.e.*, high residual fermentable extract). Their adaptation to the beer environment and consequent proliferation in beer will transfer them to the obligate beer spoilage bacteria.	*Lb. acetotolerans, Lb. amylolyticus*, *Lb. collinoides*, *Lb. harbinensis, Lb. malefermentans, Lb. parabuchneri, Lb. paracasei, Lb. paucivorans, K. kristinae, P. inopinatus, P. claussenii, P. acidilactici*, *Z. raffinosivorans, Zm. mobilis*
Indirect beer spoilage bacteria	
Do not grow in the final beer, but may proliferate at certain stages of the brewing process (e.g., in pitching yeast cultures, early fermentation stage, etc.) resulting in off-flavours eventually carried to the final product. Beer intermediates characterised with a low CO_2 content high pH and residual fermentable extract; especially primed beer types.	*O. proteus*, *R. aquatilis,* other *Enterobacteriaceae* (*i.e*., *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., *Pantoea* spp., *Serratia* spp., *Escherichia* spp., etc.), *Clostridium* spp., *Z. paucivorans*
Indicator bacteria	
Do not grow in beer, appear as a consequence of insufficient cleaning or errors in the brewing process (*e.g.*, leakage, biofilm formation in piping system). Presence is often accompanied with obligate and potential beer spoilage bacteria.	*A. pasteurianus, A. cerevisiae, G. oxydans, Pantoea* spp., *Klebsiella* spp.
Latent bacteria	
Sporadically encountered (for example contaminated process water or construction work) and may survive certain stages in the brewing process. If they are more frequently encountered, they should be categorised as indicator bacteria.	Spore forming bacteria (*i.e*., *Clostridium* spp. and *Bacillus* spp.), *Enterobacteriaceae*

IBU = International Bitterness Units; *Lactobacillus* (*Lb.*), *Pediococcus* (*P.*), *Pectinatus* (*Pe.*), *Megasphaera* (*M.*), *Selenomonas* (*S.*), *Kocuria* (*K.*), *Zymophilus* (*Z.*), *Zymomonas* (*Zm.*), *Obesumbacterium* (*O.*), *Rahnella* (*R.*), *Acetobacter* (*A.*), *Gluconobacter* (*G.*)

1.4.1 *Gram-stain positive beer spoilage bacteria*

The most important Gram-stain positive beer spoilage bacteria are the lactic acid bacteria (LAB). Beer spoilage by these microorganisms leads to enhanced turbidity and ropiness due to increased production of hazes and extracellular polysaccharides [3, 68]. Moreover, spoilage by LAB causes unpleasant changes to the final beer product such as increasing sourness (production of lactic and acetic acid) and the formation of atypical off-flavour compounds, such as diacetyl (2,3-butanedione) and acetoin (3-hydroxbutanone) [14, 69]. The production of diacetyl is a serious problem as its threshold in beer is low (~ 0.15 ppm) and has, compared to lactic acid (threshold of 300 ppm in beer), a bigger impact on the lighter flavoured beers in which this characteristic aroma and taste is highly undesirable [14, 70]. Most LAB species are intrinsically resistant to ethanol, especially at ethanol levels found in finished beers (0.5 to 14% v/v ethanol). Furthermore, these species are mostly acidophilic and prefer environments with higher CO_2 and lower O_2 levels [18]. Some LAB strains resist temperatures above 70°C for quite a long time and are thus able to survive the mashing process [12], and other stages of the brewing process [8, 11] (**FIG 1.1**). The none-spore-forming, non-motile and catalase-negative genera *Lactobacillus* and *Pediococcus* are recognised as the most hazardous beer spoilage bacteria, since these bacteria are responsible for approximately 60 to 70% of microbiological incidents [24, 28, 49].

The obligate heterofermentative *Lactobacillus brevis* is the most encountered obligate beer spoiling LAB through the entire brewing process and is even found in air and water supplies of the brewery [12, 28, 71, 72] (**TABLE 1.1**). *Lb. brevis* grows optimally at 30°C and pH 4-6 [29] and causes super-attenuation due to its ability to ferment starch and dextrines [64, 70]. This species is physiologically versatile and well-studied within the brewing microbiology. It served for instance as a model organism to study the mechanisms of hop resistance [14, 44, 45, 50, 51, 53]. Cell morphologies among *Lb. brevis* strains can differ significantly, but the tendency to form long rods (up to 50 μm) seems to be a shared characteristic [12].

Lactobacillus lindneri is the second major obligate beer spoiling LAB [28, 73, 74]. It grows in high hop concentrations (25-45 EBC bitter units), at lower temperatures (19-23°C) [14, 73, 74], survives thermal treatment (up to 17 pasteurisation units), low pH (around 3.8), high ethanol concentrations (up to 7-9% v/v), and is even highly tolerant against acid disinfection agents [12, 70, 73, 74]. *Lb. lindneri* is hard to culture in defined media, but grows more rapidly in lager beer, in which increasing cell lengths (up to 20 μm) are observed [22, 70, 73, 75, 76]. Yet, *Lb. lindneri* strains are known to pass through final filtration [28]. Strains of this species are mostly primary contaminants; and have also been isolated from different areas in the brewing environment (*i.e.*, yeast propagators, fermentation

rooms, storage cellars, bright beer tanks, filtration area, filling lines) [73, 74]. In contrast with other LAB, all *Lb. lindneri* strains are capable of spoiling beer [14]. However, Suzuki *et al*. [33] generated the first hop-sensitive, non-spoiling variant of *Lb. lindneri* via serial subculturing in MRS broth.

Three facultative heterofermentative, weak beer spoiling isolates were collected from a Japanese brewery by Funahashi *et al.* [67]. At that time, none of these isolates (LA-6 as representative strain) showed any significant homology to the other established *Lactobacillus* spp. [67]. Back *et al*. [77] found however a high similarity with isolates previously collected from spoiled lemonades and consequently described them as the novel species, *Lactobacillus perolens*. In 2005, research by Miyamoto *et al*. [78] revealed that the former isolates should be assigned to *Lactobacillus harbinensis*. Additional experiments could, unfortunately, not be performed as none of the isolates were initially deposited in international culture collections.

In the study of Funahashi *et al*. [67], a second strain set comprising three obligate heterofermentative isolates (LA-2 as representative strain) was recovered. They seemed closely related to *Lactobacillus collinoides*, which is generally not regarded as a beer spoilage bacterium [54]. However, the isolates collected by Funahasi *et al*. [67] possessed a strong beer spoilage capacity. In 2004, these isolates were taxonomically revised and assigned to the novel described species *Lactobacillus paracollinoides* [76]. *Lb. paracollinoides* (formerly known as "*Lb. pastorianus*" in the brewing industry [70]) strains exhibit a strong beer spoilage ability [33] and are hard-to-culture upon primary isolation from brewery environments due to their profound adaptation to beer [37, 75, 79].

Other obligate beer spoiling lactobacilli, *Lactobacillus casei* and *Lactobacillus coryniformis*, are facultative heterofermentative LAB which occur especially in weakly hopped beers (*e.g.*, wheat beers) [12] and sweet wort [80]. These secondary contaminants spoil beer by haze and sediment formation, and by the production of copious amounts of diacetyl. In 2006, a novel homofermentative, obligate beer spoilage species "*Lactobacillus backi*", closely related to *Lb. coryniformis*, was described based on three isolates retrieved from different types of spoiled beer (*i.e.*, lager, pilsner and wheat beer) [81]. These strains are capable to grow at 15°C and are highly resistant to hop compounds (up to 32 EBC bitter units) [81]. "*Lb. backi*" remained taxonomically invalid until it was included in the Approved List of Bacterial Names as *Lactobacillus backii* in 2013, when the species was validated by Tohno *et al*. [82]. Over the last couple of years, this spoilage bacterium has been increasingly reported from the brewing environment [28, 80, 83]. Furthermore, it was more recently recovered from beer bottles found in a 170 year old shipwreck discovered in the Baltic Sea off the Åland Isles, together with *Lactobacillus malefermentans* [84]. The latter has also been isolated from a top fermented beer [85].

Several other *Lactobacillus* spp. were recently correlated with beer spoilage. In 2010, the novel species *Lactobacillus paucivorans* was collected from a beer storage tank, but classified as a potential beer spoiler only because of its inability to grow in high iso-α-acids concentration (> 5 ppm) [66]. The acetic acid tolerant *Lactobacillus acetotolerans* was isolated from Chinese beer, characterised by low hop and alcohol content, but also from a German non-alcoholic beer and from conventional wheat beers [71]. *Lactobacillus rossiae* was recovered from a German yeast culture containing wheat beer and has been isolated from biofilms in the filling hall [83].

Strains of other LAB species such as *Lactobacillus amylolyticus*, *Lactobacillus buchneri*, *Lactobacillus curvatus*, *Lactobacillus fermentum*, *Lactobacillus fructivorans*, *Lactobacillus parabuchneri*, *Lactobacillus paracasei*, *Lactobacillus paraplantarum* and *Lactobacillus rhamnosus* have also been reported to have comparatively weak to strong beer spoilage ability [14, 21, 29, 64, 80, 86-92] (**TABLE 1.1**).

The thermo-tolerant, malt-associated *Lactobacillus delbrueckii* can survive flash and bottle-pasteurization temperatures and results in a weak growth and disturbing haze formation in hopped beers [12, 29]. *Lb. delbrueckii* has been encountered as a contaminant of sweet wort [29], but was also applied in the biological acidification of malt, mash or wort [4, 9]. The potential as an obligate beer spoilage bacterium is for *Lactobacillus plantarum* less common than the species described above. Yet, in weakly hopped beers (*e.g.*, wheat beers) *Lb. plantarum* can cause distinct off-flavours (*i.e.*, diacetyl) [12, 80], but nevertheless, *Lb. plantarum* has been applied as a starter culture in steeping water during malting to enhance malt quality [4, 9, 93].

The homofermentative beer spoilage species belonging to *Pediococcus* are micro-aerophilic and have an optimal growth temperature of 22 to 25°C. *Pediococcus* spp. are, as compared to *Lactobacillus* spp., more common contaminants at the end of the fermentation or during the storage of the final beers and are particularly present in beers fermented at lower temperatures [13]. They are capable of proliferating very well in beer and spoil the beer by the concomitant production of lactic acid, ropiness and copious amounts of diacetyl [64, 94].

Pediococcus damnosus is the most encountered obligate beer spoiling *Pediococcus* species [8, 12], prevailing in late fermentation stages, in pitching yeast cultures and final beer products [12, 14, 94] (**TABLE 1.1**). Haze formation is less common, as the cells tend to settle down rather quickly and consequently form sediment deposits at the bottom [28]. Strains of this species were recently recovered together with *Lactobacillus* spp. from beer bottles found in a 170 year old shipwreck discovered in the Baltic Sea off the Åland Isles [84].

The potential beer spoiling *Pediococcus inopinatus* grows in beer characterised by a high pH and low ethanol and hop contents [58] (**TABLE 1.1**). Strains of this species frequently contaminate the pitching yeast, and are rarely encountered in other stages of fermentation [29, 95]. Contaminations by either *P. damnosus* or *P. inopinatus* extend the time needed for fermentation and the produced beers contain high diacetyl concentrations [8]. Ropiness in beers has been attributed to pediococci but also depends on the presence of particular fermentable sugars [96]. Slime production has been associated with *P. damnosus*, but also with *Pediococcus acidilactici* and *Pediococcus claussenii* [92, 97].

Pediococcus pentosaceus and *P. acidilactici* have never been reported to cause any defects in finished beer [14], but have been isolated from malts and are used in the biological acidification of mash and/or wort [4, 9, 64, 71, 90]. Compared to *P. pentosaceus* (able to ferment maltose at temperatures up to 44°C), *P. acidilactici* grows at temperatures above 50°C, but is unable to ferment maltose resulting in a weaker growth in wort [98, 99]. *P. claussenii* has also been isolated from the brewing environment [100]. Pittet *et al*. [92] sequenced the complete genome of a slime-producing *P. claussenii* persisting in the brewing environment probably due to its capability in biofilm formation.

Next to the LAB, the potential beer spoiling *Kocuria kristinae* (formerly known as *Micrococcus kristinae*) is also a Gram-stain positive beer spoilage bacterium, growing in beer characterised by low ethanol and hop content and a pH above 4.5 [8] (**TABLE 1.1**). Its spoilage capacity is characterised by a slight haze formation and the production of an atypical fruity estery off-flavour [12].

Other Gram-stain positive non-LAB beer spoilage species are *Clostridium* spp. and *Bacillus* spp. (**TABLE 1.1**). Their heat resistant spores can enter the brewery and subsequently survive the mashing and boiling steps in the brewing process [101]. Generally, these bacteria are hop-sensitive and are unable to grow in low pH environments, but growth can occur in improperly treated wort and results in unwanted proteolytic activity and a range of detrimental metabolites (*i.e.*, butyric acid, caproic acid, propionic acid and valeric acid and sulphur compounds) [65]. Certain *Bacillus* spp. were isolated from two types of home-brewed beers and were capable of growing in commercially available beer [60]. Further research revealed that these isolates carried *horA*, a marker gene correlated with hop resistance [14]. Spoilage by *Bacillus* spp. may lead to an excessive acidification and N-nitrosamine production, as these strains are able to reduce nitrate to nitrite [6]. Subsequently, the nitrite reacts with the amines and amides present in the wort to form N-nitrosamines [13, 95, 102] which are of serious concern when their legal levels (20 µg/L) are exceeded [8, 26, 103]. *Bacillus coagulens* has been reported to produce copious amounts of lactic acid in sweet wort kept at 55 to 70°C for more than two hours [8]. *Clostridium* spp. have been isolated from wort as well and also from other brewery intermediates [26].

1.4.2 *Gram-stain negative beer spoilage bacteria*

1.4.2.1 *Enterobacteriaceae*

Enterobacteriaceae are indirect beer spoilage bacteria prevailing mostly in pitching yeast, cold wort and early stages of fermentation [6, 102] (**TABLE 1.1**, **FIG 1.1**). Although they do not thrive well in the adverse conditions during fermentations (*i.e.*, low pH and high alcohol concentration), they persist in dormant state and revive in the recycled pitching yeast and subsequently cause spoilage in the next fermentation batch [6, 80]. Therefore, microbiological quality screening of the pitching yeast cultures is of utmost importance and acid washing of the contaminated yeast culture prior to pitching is traditionally advised to remove the spoiling bacteria [13, 20, 95]. *Enterobacteriaceae* have not only been isolated from pitching yeast, but also from the brewing environment such as the plate of mash and lauter vessels [26]. Both examples bare an inadequate cleaning management [13, 80].

Growth and spoilage by *Enterobacteriaceae* are facilitated by the presence of high oxygen levels and relatively high pH (5.0 to 5.5; growth stops at pH < 4.3) [29]. They decrease the rate of fermentation by directly competing with the brewer's yeast for nutrients, resulting in a lower fermentation degree [104]. Certain metabolites produced by these bacteria will negatively affect the final flavour and aroma profile of the beer, resulting in a parsnip-like or fruity flavour [103], and sulphuric (dimethyl sulphide and dimethyl disulphide) or phenolic (guaiacol) off-flavours [13, 29]. These bacteria do not produce any turbidity or ropiness, but they can produce diacetyl, acetaldehyde, fusel alcohols and acetic acid [12, 29]. Unacceptable levels of nitrite are accumulated under anaerobic conditions, as these facultative anaerobes are able to reduce nitrate to nitrite [29]. Subsequently, N-nitrosamines can be formed [13, 95, 102] which are of serious concern when their legal levels are exceeded [8, 26, 103]. The presence of biogenic amines in beer is also linked to contaminations by *Enterobacteriaceae* during the early stage fermentations as a result of the decarboxylation of free amino acids [8]. Moreover, certain *Enterobacteriaceae* are known to harbour strains that are pathogenic for humans [105].

Obesumbacterium proteus is a noted contaminant of pitching yeast slurries at a level of 1 cell per 100 yeast cells [13, 20, 103, 104] (**FIG 1.1**). During early fermentation *O. proteus* grows rapidly causing a drop in the fermentation rate which leads to an inferior product of high specific gravity and pH [102, 103]. *O. proteus* tolerates ethanol concentrations up to 6% v/v and is responsible for increased levels of organo-sulphur compounds, various alcohols and diacetyl which are thought to contribute to the parsnip-like smell of *O. proteus* contaminated beer [103]. *Rahnella aquatilis* also grows well in wort and produces an excessive amount of vicinal diketones (*i.e.*, diacetyl) in beer [8]. Other genera belonging to the *Enterobacteriaceae* have been frequently encountered in the brewery as well, *e.g.*,

Citrobacter, *Enterobacter*, *Pantoea*, *Serratia*, *Klebsiella* and *Escherichia* [6, 13, 65, 80, 106]. However, hygienic standards and equipment have dramatically changed over the past 40 years, and *Enterobacteriaceae* are now less prevalent [6, 80].

1.4.2.2 *Acetic acid bacteria*

Acetic acid bacteria (AAB) have been isolated from the brewing environment, as these bacteria are resistant to hop compounds and to high levels of ethanol (up to 13% v/v) [8, 10, 43]. These bacteria are strict aerobic and normally do not persists in wort or beer once anaerobic conditions arise during fermentation and after packaging [6, 107] (**FIG 1.1**). Nevertheless, they may contaminate packaged beer when air is present in the headspace of bottles as a result of faulty packaging [8]. Growth of beer spoiling AAB under micro-aerophilic conditions has been reported [89, 108]. Moreover, AAB are frequently encountered in and around beer dispensing systems [10, 26].

Beer spoilage by AAB is characterised by the production of acetic acid (converted from ethanol), off-flavours (*e.g.*, oxidation of glycerol to dihydroxyacetone), turbidity and ropiness [107]. Optimal growth is achieved at 25-30°C and at pH 5-6, but some strains can develop at pH values as low as 3.6-3.8 [101]. Slime forming AAB are considered to be primary colonisers in brewery plant's biofilms, contributing to the favourable conditions for other beer spoilage bacteria as they reduce oxygen levels and provide an acidic environment [1, 25, 27].

Both *Acetobacter* spp. and *Gluconobacter* spp. are AAB which are ubiquitously present in the brewing environment (*i.e.*, in the mash, wort, brewing liquors and adjuncts, in the pitching yeast and in packaged beer) [8, 10]. *Acetobacter* spp. thrive in alcohol rich niches whereas *Gluconobacter* spp. prefer sugars as carbon source [8]. *Acetobacter* spp. oxidize ethanol to acetic acid and subsequently acetic acid to CO_2 and H_2O, while *Gluconobacter* spp. are not able to perform this latter oxidation step [107, 109-111]. The most frequently encountered AAB in beer and the brewery environment are *Acetobacter aceti*, *Acetobacter cerevisiae*, *Acetobacter pasteurianus* and *Gluconobacter oxydans* [6, 64]. However, improved brewing technology reduced the oxygen levels in packaged beer, resulting in a decreased threat of these aerobic beer spoilage bacteria for the brewing industry. Instead, the Gram-stain negative strict anaerobic bacteria have emerged [8].

1.4.2.3 *Strict anaerobic beer spoilage bacteria*

Together with the increased production of non-pasteurised beer and with the improved bottling technology, which results in a reduced oxygen content in packaged beers, the strict anaerobic beer spoilage bacteria have surfaced over the past few years [14, 38, 64, 112-114]. Compared to other frequently encountered beer spoilage bacteria (*e.g.*, *Pediococcus* and *Lactobacillus*), contamination

with these strict anaerobes is much more serious as they produce offensive odours and excessive turbidity in packaged beers [1, 24]. The following strict anaerobic genera have been associated with beer and brewery environment, *i.e.*, *Megasphaera*, *Pectinatus, Selenomonas* and *Zymophilus*.

Both *Megasphaera* spp. and *Pectinatus* spp. are common spoilers of finished beers characterised by pH 4.1-4.3 and ethanol concentration lower than 5.2% v/v [28, 114]. They grow between 15 to 40°C, with a growth optimum of 30°C and are typical secondary contaminants that prevail in the filling and packaging hall [28, 39]. Both species are tolerant to hop compounds and can grow in beer with bitterness ranging between 33 to 38 EBC bitter units [24].

Pectinatus spp. have been correlated to 20-30% of the bacterial spoilage incidents, and spoil beer by producing considerable amounts of H_2S (rotten egg smell), acetoin, methyl mercaptan, acetic acid and propionic acid [8, 28, 29, 112, 115]. Hence, beer contaminated with *Pectinatus* spp. does not only show massive sedimentation and turbidity, but also unpleasant flaws in odours and taste [28, 114]. The true biological origin of this species is not known, but they have been isolated from wort, from lubrication oil mixed with beer and water, in drainage and water pipe systems, in the air and on the floor of the filling hall, in steeping water of malt before milling, etc. Altogether it is suggested that water is most likely the source of contamination and that some of these species are rather oxygen tolerant [24, 112, 116]. Helander *et al.* [117] found high similarities between the lipopolysaccharide structure of *Pectinatus* and that of plant-associated bacteria, suggesting that *Pectinatus* entered the brewing environment via the various plant materials utilised (*e.g.*, cereals, hops, rice). *Pectinatus cerevisiiphilus* grows faster than *Pectinatus frisingensis* strains, yet the latter is more resistant to ethanol [29, 118, 119]. *Pectinatus haikarae* which has so far only been associated with low-alcohol beer has also been isolated from brewery bottling plants [120]. The latter species has a preference for lower temperatures; and produces, unlike *Pe. cerevisiiphilus*, catalase which may enhance survival in oxygenic niches in the brewery [16].

Megasphaera spp. spoil beer less frequently compared to *Pectinatus* spp. [12] and spoilage leads to turbidity and production of H_2S, CO_2, H_2 and smaller amounts of acetoin [8]. *Megasphaera* spp. will also produce acetic acid, butyric acid, propionic acid, caproic acid and valeric acid [112, 120-122]. *Megasphaera cerevisiae* is less acid and alcohol-tolerant compared to *Pectinatus* spp. as it is already restricted in growth with pH < 4.5 and ethanol concentration above 2.8% v/v [112]. Other *Megasphaera* spp., such as *Megasphaera paucivorans and Megasphaera sueciensis* are also correlated with beer spoilage as they were originally isolated from spoiled Italian and Swedish beer, respectively [120].

Spoilage induced by *Zymophilus* spp. results in turbidity and production of acetic acid and propionic acid [13, 112]. The potential beer spoilage bacterium *Zymophilus raffinosivorans* spoils only beer characterised by high pH values (> 5) and low ethanol concentrations (< 5% v/v) and has also been isolated from pitching yeast [8, 64] (**TABLE 1.1**). *Zymophilus paucivorans*, on the other hand, is not regarded as a beer spoiling species due to its inability to grow below pH 6 [112]. *Selenomonas lacticifex* is also considered to be a potential beer spoilage bacterium, as it has been recovered from brewer's pitching yeast but so far never from beer directly [29] (**FIG 1.1**, **TABLE 1.1**). However, artificial "forcing tests" prove that *S. lacticifex* purified from contaminated pitching yeast culture was able to grow in beer [8] causing turbidity and producing acetic acid, lactic acid and propionic acid [112].

1.4.2.4 *Zymomonas*

The facultative anaerobic species *Zymomonas mobilis* subsp. *mobilis* has been recovered frequently from ales and primed beers [6]. The increased occurrence of this species in ale breweries is thought to arise from the use of invert sugars or glucose syrups as priming agent in beer [8]. The potential beer spoiling *Zm. mobilis* subsp. *mobilis* is unable to ferment maltose and maltotriose, but ferments glucose, fructose and sucrose [10, 123] (**TABLE 1.1**). The species spoils beer by increasing turbidity and producing high levels of acetaldehyde, hydrogen sulphide and trace amounts of dimethyl sulphide, acetoin, glycerol, acetic acid and lactic acid [3, 13, 26, 123]. This species is not only acid tolerant [13], but survives ethanol concentrations up to 13% v/v (produced in high gravity brewing) and is proven to be metabolically active even up to 15% v/v ethanol [121, 124]. This high ethanol tolerance emphasises the importance of the species in the brewing environment. In the UK, contaminations with *Zymomonas* sp. have been associated with an inadequate cleaning of kegs and casks in ale breweries [13]. *Zm. mobilis* subsp. *mobilis* has never been recovered from lager breweries, probably due to lower fermentation temperatures (8-12°C) and their stringent carbohydrate requirements [64].

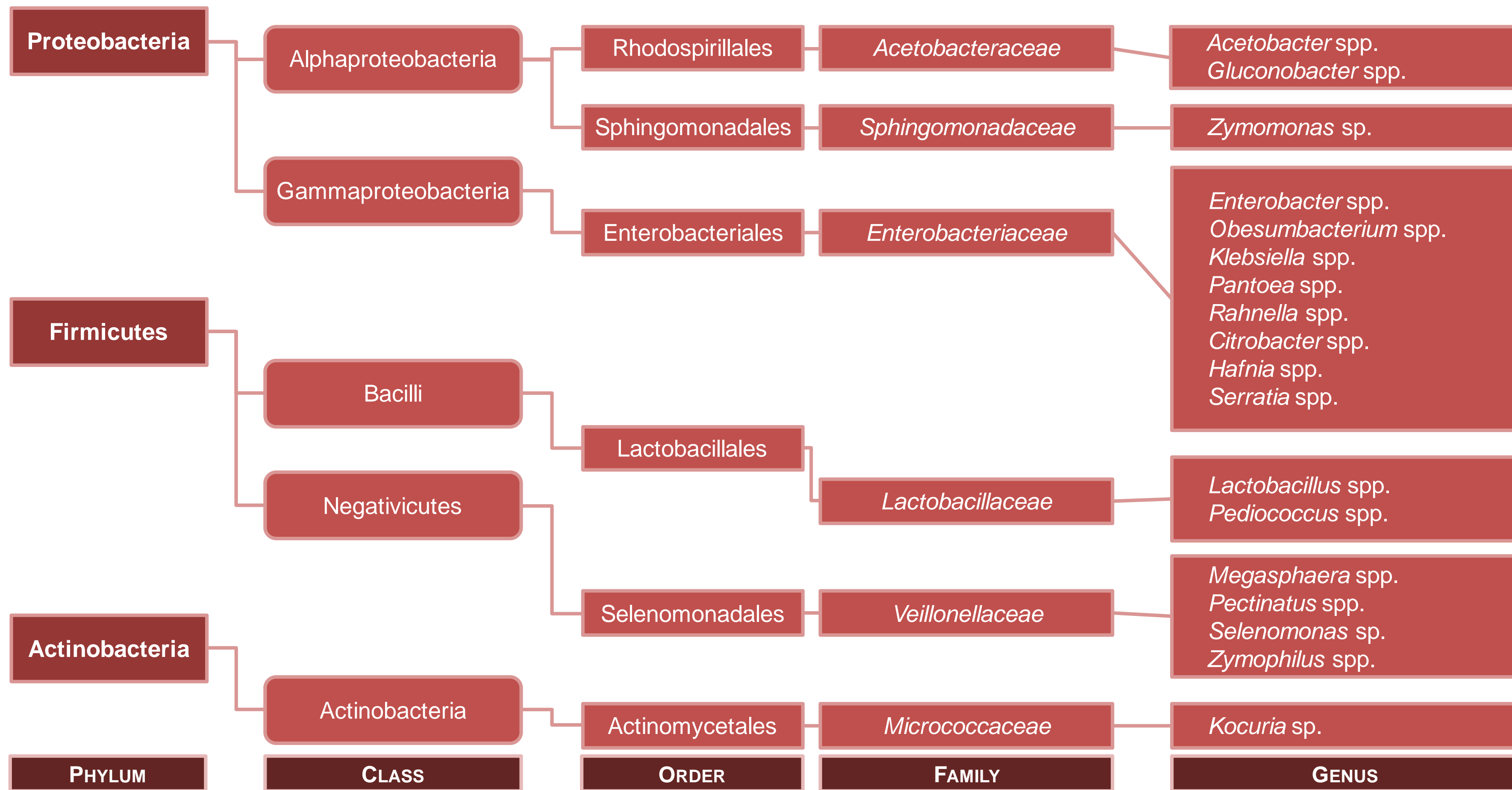


FIG 1.4. The taxonomic classification of the main beer spoilage bacteria.

2 Current approaches for the detection and/or identification of beer spoilage bacteria

Beer has been generally considered as a beverage with a relatively high microbiological stability (**CHAPTER 1**). Nevertheless, bacteria can prevail and spoil not only brewery intermediates (*e.g.*, wort, brewery additives, pitching yeast) but also packaged beer products. Therefore, quality control is necessary. This control step has however some drawbacks, such as the lengthy storage of the final products, resulting in increasing brewing costs and demands for more storage capacity [118]. Another disadvantage is that beer products are sometimes already released to the market before microbiological test results become available [8]. So should bacterial spoilage be detected after release, the products have to be recalled from the market resulting in serious financial and commercial damage for the respective brand [26, 49].

Beer spoilage bacteria adversely affect the brewing process and final beer quality [8]. Hence rapid, simple, sensitive and reliable detection methods are needed to control and minimise bacterial spoilage in the brewery [58]. Besides early detection, species level identifications are necessary to assist the determination of spoilage capability, to track the source of contamination and finally, to prevent future spoilage [1, 45, 125, 126]. Currently, beer spoilage bacteria are primarily detected using selective isolation media, an approach that is time-consuming and neither sufficiently sensitive nor specific enough [23, 28]. Furthermore, subsequent characterization and identification of spoilage bacteria is mostly carried out via phenotypic tests, but these tests are again time-consuming and lack specificity [12]. Therefore, a range of different culture-dependent and culture-independent detection and/or identification techniques have been developed and applied in the brewing industry (**TABLE 2.1**). These include ATP bioluminescence, the use of monoclonal antibodies, the application of species-specific oligonucleotide probes, ribotyping, PCR-based detection and fingerprinting methods and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole-cell proteins. All these different methods are elaborately discussed in this chapter, but their main characteristics and major benefits and drawbacks are listed in **TABLE 2.1**.

2.1 Detection of beer spoilage bacteria via growth on selective and non-selective media

The most common detection method is the traditional incubation of beer and brewery samples onto selective and non-selective culture media [26, 64, 79, 127-129]. Different types of culture media have been developed, yet not a single medium can be used to detect all potential beer spoilage bacteria at the same time [64, 65, 121, 127-134]. This detection approach is rather time-consuming as it usually takes more than a week to observe visible colonies on agar plates or turbidity in enrichment broths [14, 28, 135]. Fastidious beer spoiling bacteria are often reluctant to grow at laboratory conditions due to their extensive lag phase, caused by low cell concentration, sub-lethal damages and/or extensive growth requirements [79, 128, 129]. Moreover, some bacterial cells are too excessively adapted to their stressful environment (*i.e.*, starvation, environmental compounds, and extreme temperatures), rendering them sometimes viable but nonculturable [26, 79]. With this time-consuming approach, most beer products are already released for sale before the microbiological test results become available.

New detection and/or identification methods have been developed and are discussed below. However, these techniques need an enrichment step (approximately 24-72 hours) in order to overcome the cell detection limit [129]. Hence, the search for the optimal liquid cultivation medium that allows a more efficient enrichment and thereby a decrease in time of analysis is ongoing [16, 128, 129].

Strains isolated from contaminated beer or brewery samples are often reinoculated and subsequently incubated in different types of beer in order to determine their beer spoilage capability [14]. Growth during these so-called 'forcing tests' can take up to one month or even longer [56]. Alternatively, hop-gradient agar plates with additional ethanol mimicking the beer environment, allow a more comprehensive and accurate culture-dependent determination of the beer spoilage capability of the obtained isolates [136].

2.2 Phenotypic features for identification of isolated spoilage bacteria

Characterization and identification of spoilage bacteria can be carried out via phenotypic tests (*e.g.*, Gram-staining, cell morphology, carbohydrate assimilation or fermentation pattern analysis). Yet, such tests are again time-consuming, lack specificity and the interpretation is often difficult and inconclusive [12, 74, 87, 94, 95, 122, 137]. In practice, most of the beer spoilage bacteria grow very weakly and slowly, making the test results based on growth response unreliable [74]. Moreover,

plasmid loss due to environmental changes may cause alteration to the phenotypes, as many plasmids code for carbohydrate utilization pathways [95].

Another method to identify beer spoilage bacteria is the extraction and analysis of species-specific metabolic end-products, including volatile and non-volatile organic acids, from a liquid enrichment culture [138]. As such, Suihko and Haikara [122] detected strict anaerobic strains based on their production of short-chain fatty acids (*e.g.*, propionic acid, butyric acid, valeric acid) using gas chromatography (GC) analysis. The production of diagnostic fatty acid methyl ester (FAME)-profiles have also permitted the identification of certain beer spoiling LAB species [139]. This technique requires 10^7 to 10^{10} CFU/mL sample in order to extract enough fatty acid for analysis, making this technique more suitable for bacterial identification instead of early detection [139].

2.3 ATP bioluminescence

The detection of ATP, the direct energy source of living cells, is a common tool for hygienic monitoring (*i.e.*, hygienic surface swabbing of process machinery, pipelines, water analysis) and in a lesser extent for product quality control in the brewing industry [140, 141]. The emission of light via the luciferin/luciferase assay is directly correlated to the amount of ATP present in the sample [140, 142]. ATP is released upon cell death which makes its detection in a sample indicative for the presence of viable cells at the time of analysis [13, 142]. However, false-positive ATP signals derived from beer matrix, culture medium and/or other background compounds interfere with the analyses [24, 36, 143, 144]. Nevertheless, the measurement of ATP bioluminescence has been commercialised and is frequently applied in the brewing industry worldwide for the detection of all brewery contaminants [36, 65, 140, 143]. For example, the detection of bacterial ATP-yielding abilities in beer can be used as a rapid pre-screening method to detect potential beer spoilage bacteria isolated from the brewing environment [91].

2.4 Immuno-based approaches

The use of monoclonal antibodies (MAbs) has been investigated as an alternative detection and/or identification tool for beer spoilage bacteria [68, 145]. Interaction between group- or species-specific MAbs and targeted antigens is rapidly detected using a membrane-based fluoroimmunoassay, a chemiluminescence enzyme immunoassay (CLEI) or an enzyme-linked immune sorbent assay (ELISA) [143, 145-150]. For example, Whiting *et al.* [148] reported a drastic decrease in detection

limit compared to conventional methods when a membrane-based fluoroimmunoassay was applied for the detection of *Pediococcus* spp. in pitching yeast slurries. March *et al*. [145], on the other hand, reported that CLEI (using MAb LA-4) successfully detected 18 out of 19 LAB isolates (obtained from six different and distant Spanish breweries) after an enrichment step of 48 h. The application of specific MAbs was also successful in the determination of the spoilage capacity of isolated contaminants, as their targeted antigens were proven to be related to beer spoilage capability [143, 147, 149].

These immunological approaches struggle with false-positive background signals due to non-specific binding of MAbs and cross-reactions with closely related species [150, 151]. This method is therefore ill-suited for high-throughput analysis and is mostly limited to a single or small group of species. Moreover the development of group- and/or species-specific MAbs is rather complicated, very expensive and time-consuming [151].

2.5 Genotypic detection and/or identification methods

2.5.1 *Oligonucleotide probe-based approaches*

The use of group- and species-specific oligonucleotide probes based on a genomic target site allows detection and/or identification of beer spoilage bacteria [24, 73, 118, 152-154]. Beimfohr and Snaidr [118] designed over 440 different oligonucleotide probes for the detection of beer spoiling *Lactobacillus* spp., *Pediococcus* spp., *Megasphaera* spp. and *Pectinatus* spp. using fluorescence *in situ* hybridization (FISH) analysis. The main advantage of this technique is that the probes interact directly with the target nucleotide sequences inside the cell without the need of a prior extraction and PCR step [49, 151, 155]. Different nucleotide sites can be targeted, but RNA sequences are preferred due to their ubiquitous distribution and abundant presence in viable cells [118, 151, 156]. This early-stage-detection significantly decreases the time of analysis even when a primary enrichment step is used [75, 151]. The FISH analysis approach was also used in microbial diversity studies of biofilms formed on conveyors in a brewery's filling and packaging hall [25]. Asano *et al*. [75] coupled the microcolony (small colonies that consist of at least eight closely associated cells) method with FISH allowing the detection of 'true' beer spoilage bacteria. Bacterial cells are entrapped on membrane filters and subsequently incubated onto advanced beer spoiler detection (ABD) medium prior to FISH analysis. As such, specific detection of beer spoiling LAB has been obtained [75, 79]. This approach also leads to a more accurate depiction of CFU present compared to conventional culturing methods [37, 157].

The RNA-based sandwich hybridization assay (SHA) permits detection of a large group of beer spoilage *Lactobacillus* spp. and *Pediococcus* spp. present in yeast slurries using only four group-specific probes [152]. The VIT® (Vermicon identification technology)-Bier plus *Lactobacillus brevis*-kit (Vermicon®) allows the detection of all known beer spoiling LAB and simultaneously allows the identification of *Lactobacillus brevis* and *Pediococcus damnosus*. The VIT®-Bier *Megasphaera/Pectinatus* kit (Vermicon®) on the other hand detects both *Megasphaera cerevisiae* and *Pectinatus* species. Lastly, the commercialised HybriScan^{TM} detection kit (Fluka Analytical, [158]) is capable to detect 25 beer spoilage species starting from an enriched sample. This kit is based on colorimetric SHA, using only two different group-specific probes targeting the 16S rRNA gene [129, 158]. This approach is however ill-suited for species-level differentiation and only a limited group of beer spoilage bacteria is targeted [158]. Finally it should be noted that all methods mentioned above require specific imaging systems to confirm and/or to visualise hybridization results [118, 156].

2.5.2 *PCR-based detection and/or identification methods*

Numerous PCR-based methods were introduced for the detection and/or identification of beer spoilage bacteria [33, 75, 125, 159]. Group- or species-specific primer pairs were developed to amplify certain targets of interest, mostly the 16S rRNA gene or the 16S – 23S rRNA gene internal transcribed spacer (ITS) region [54, 56, 63, 125, 160, 161]. However, the distinction between beer spoiling and non-spoiling strains is impossible using these types of targets [33]. The detection of genes involved in hop resistance (*e.g.*, *horA*, *horC*, *hitA*, *ORF5*) has proven to be successful in the determination of the beer spoiling capability of LAB strains [33, 35, 45, 49, 59, 60]. Such species-independent detection allows the discovery of new and true beer spoilage species that emerge occasionally in the brewing industry [33, 45, 60]. It is noted that these plasmid-bound genes may get lost after serial subculturing in hop-deprived enrichment medium, eventually leading to misinterpretations of the obtained results [61]. Moreover, it remains difficult to discriminate based on a single marker gene [35, 162]. Multiplex PCR enables simultaneous detection of different marker genes within a single PCR reaction using multiple primer pairs, and thereby also detecting multiple beer spoilage species [24, 125, 163, 164].

Quantitative PCR (q-PCR) analysis will not only amplify, but also quantify the DNA target in ‘real-time’ [57]. Juvonen *et al*. [113] designed the first group-specific primer pair which targeted the 16S rRNA gene of all established strict anaerobic beer spoiling species in a single q-PCR reaction. Moreover, the determination of melting point curves (T_m) allows discrimination between the obligate (*i.e.*, *Megasphaera* spp. and *Pectinatus* spp.) and potential (*i.e.*, *Zymophilus* spp. and

Selenomonas lacticifex) beer spoilage bacteria [113]. The use of different probe markers enables q-multiplex PCR, allowing group- or species-level differentiation among beer spoilage bacteria [57, 113, 159]. Multiple commercially available detection and/or identification kits adopted the q-PCR principle. For example, the PIKA Weihenstephan™ (Life Technologies™) screening kit detects a broad range of spoiling bacteria in a single test. Another illustration is the foodproof® Beer Screening Kit (BIOTECON Diagnotics) which detects and mostly identifies about 30 species belonging to the genera *Lactobacillus*, *Pectinatus*, *Megasphaera* and *Pediococcus* within a 24-72 h experiment that includes a pre-enrichment step [71, 83]. Similarly, the GeneDisc® Cycler (Pall Corporation) detects and identifies about 20 relevant beer spoilage bacteria belonging to the genera *Lactobacillus*, *Pediococcus*, *Megasphaera* and *Pectinatus* using the q-PCR approach [126]. This system uses the patented GeneDisc®-plates pre-loaded with specific primers and probes, and the kit is supplied together with the ready-to-use PCR master mixture for an easy and simple sample preparation [126]. However, a pre-enrichment step of about 24 to 72 hours is necessary for samples with a low bacterial load. [71, 126].

The PCR-based methods are extremely sensitive. Unfortunately, the elimination of several PCR inhibitors present in the beer matrix (*e.g.*, polyphenols, proteins, cell debris) is necessary during sample preparation (*e.g.*, membrane filtration, centrifugation, washing steps) to prevent false-negative results [63, 129, 161]. Also, the presence of yeast cells increases the cell detection limit [102] so that the adequate removal of yeast cells is advised [152, 165]. Prior to PCR-based analyses, DNA must be extracted from the bacterial cells present in sample material [160]. During DNA extraction no distinction will be made between viable and dead cells or naked DNA (for example derived from LAB used during bioacidification), resulting in inevitable false-positive results [23, 160]. Even after an enrichment step, the high load of naked DNA and dead cells present may still lead to false-positive results [23]. Therefore, the detection of only viable cells was investigated using RNA-targets in reverse transcriptase PCR, but the results were not satisfying (*i.e.*, labour-intensive, high detection limit, false-positive results) [23]. Particularly in the brewery quality control, the use of PCR-based analysis is limited due to its high costs, complexity, pre- and post-PCR operations, the risk of false-negative and false-positive results, the need for enrichment, etc. [165, 166]. Moreover, when novel beer spoilage bacteria emerge, former detection kits have to be updated (*i.e.*, novel primer pairs and probes have to be designed) in order to include these novel species as well [163].

2.5.3 *Fingerprinting techniques*

Ribotyping is a DNA fingerprinting technique analysing the relative numbers and positions of the rRNA genes within the bacterial genomic DNA [74]. Restriction endonuclease digestion of extracted genomic DNA is performed, followed by Southern hybridization with probes targeting the rRNA genes [97]. Different restriction enzymes (*e.g.*, *Eco*RI, *Hin*dIII, *Bam*HI) generate different sets of DNA fragments resulting in various fingerprints [102]. Besides species level identification, this method enables strain level differentiation, given the restriction enzymes used [102, 122, 167]. Ribotyping has proven its applicability in the characterization of beer spoiling *Megasphaera* spp., *Lactobacillus* spp., *Obesumbacterium* spp., *Pectinatus* spp., *Pediococcus* spp. and *Zymophilus* spp. [74, 94, 97, 102, 122, 137, 167, 168]. A major advantage of such strain level differentiations is that they permit source tracking of the respective bacterial contaminants throughout the brewing process and in the brewery environment [97, 102, 137].

Ribotyping is laborious and time-consuming, yet an automated analytical system developed by Qualicon (DuPont) facilitated a wider adoption of the technology in brewing microbiology [74]. The Qualicon RiboPrinter™ performs all actions automatically starting from DNA extraction to final Southern blot data-analysis [97]. The initial investment and running costs are high compared to alternative identification methods and it does not allow high-throughput analysis (8 samples in 8 h) [137]. The identification of unknowns can be hampered due to an incomplete profile database [74, 97, 122, 137].

Other DNA-fingerprinting methods, such as restriction fragment length polymorphism (RFLP) [113] and random amplified polymorphic DNA (RAPD) PCR [32, 35, 169] have been successfully applied as brewery's quality control methodologies and serve as an identification and typing tool allowing differentiation among beer spoiling and non-spoiling strains. For example, Hayashi *et al*. [35] were able to classify multiple *Lactobacillus brevis* strains according to their beer spoiling capability using RAPD PCR fingerprinting. In total 440 random primers were tested and one primer finally enabled distinctions among beer spoiling and non-spoiling strains. The one specific amplified fragment included the *hitA* gene, which is associated with the hop resistance mechanism [35]. Similarly, Fujii *et al*. [56] reported a novel locus that is highly specific for the differentiation of beer spoiling strains belonging to *Lactobacillus brevis*, *Lactobacillus collinoides*, *Lactobacillus coryniformis* and *Pediococcus damnosus* based on RAPD PCR fingerprinting analysis. The specific locus is thought to be related to the production of the beer spoiling strain specific teichoic acids [56].

Protein profiling using SDS-PAGE on total protein extracts has also been successfully used to identify multiple beer spoilage isolates [26, 74, 94, 170]. Generated SDS-PAGE profiles represent

strain-specific gene expression patterns which can be compared with a reference database comprising SDS-PAGE profiles to identify at species level or even at infraspecific level [26, 170]. Although final profile analyses are straightforward, the sample preparation protocol is rather extensive and should be performed very standardised (*e.g.*, preparation of polyacrylamide gels, protein extraction of intact cells, electrophoretic separation, staining).

2.6 The ongoing search for improved, alternative methods

Most of the above-mentioned methods specifically target one or a narrow range of beer spoiling species, while others are time-consuming, lack high-throughput capacity and do not facilitate real-time interventions. Moreover, aspecific interaction may occur between designed primer pairs, probes or MAbs resulting in false-negative outcome. Numerous rapid and specific detection methods have been investigated; however, none of these methods enables the identification of the heterogeneous group of beer spoilage bacteria simultaneously, except for ribotyping and SDS-PAGE analysis. At present, rapid, more sensitive and simple detection and/or identification tools with more throughput potential and preferably, tackling a broader range of beer spoilage bacteria are still being developed. Culture-independent methods, such as metagenome and amplicon sequencing analyses, have emerged and enabled the in depth analysis of the microbial diversity, possibly including low abundant members and viable but non-culturable bacteria present in complex (food) samples [106, 171, 172]. Even though metagenome and amplicon sequencing analyses are becoming less expensive, the turnaround time to obtain raw sequences and further processing of the data remains low and often requires highly skilled staff [171, 173]. At present, this technique is not yet suited for the day-to-day microbiological quality control in brewing industry.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has already been introduced as a high-throughput tool for species level identification in medical, environmental and food-related studies [109, 174-181]. The presented study aimed to validate this technique as a suitable identification tool for beer spoilage bacteria present in spoiled beer samples and brewery intermediates. MALDI-TOF MS and how it serves bacterial identifications will be discussed in more detail in the following chapter.

TABLE 2.1. Overview of characteristics, major benefits and drawbacks of currently established methods used for the detection and/or identification of beer spoilage bacteria (BSB).

	Method	D/I/T	Target	Sensitivity	Time of analysis	Benefits	Drawbacks	References
GROWTH	Culturing on selective and non-selective growth media[¥]	D	All BSB	1-5 culturable cells/sample	days - weeks	Sensitive (when appropriate culture condition are applied); detection of culturable cells; easy-to-perform	Time-consuming (days up to weeks); often unreliable results; viable but nonculturable cells are not detected	[49, 64, 120, 126-132, 134]
ATP	Detection of bacterial ATP using the enzyme coupled luciferin/luciferase assay	D	All BSB	50 cells/sample	<1 h (48 h*)	Rapid tool for hygienic monitoring	Interference with chemicals (false-negative results); expensive read-out system; false-positive results; variable sensitivity; poor reproducibility	[13, 91, 140, 142]
IMMUNO	Immuno-based assays using MAbs	D / I	Species- or group-specific	3-40 cells/100 mL sample	<1 h (48 h*)	Rapid and sensitive; quantification possible	Expensive and tedious design of MAbs; cross-reactions & background interferences; expensive read-out system; no distinction between viable and dead cells	[36, 143, 145, 146, 149, 150] [179, 180]
PROBE	Hybridization of oligonucleotide probes onto specific target sequences[¥]	D / I	Species- or group-specific	10^2-10^5 CFU/mL sample; 1-5 CFU / membrane (microcolony approach)	3 h (48 h*)	Without DNA extraction; quantification possible; detection of viable cells	Investment costs; enrichment step preferred (microcolonies); design of probes; different probemarkers for multiplex detection	[24, 73, 75, 117, 151-153, 157]
PCR	End-point amplification of target DNA sequences	D / I	Species- or group-specific	10^3-10^8 cells/100 mL sample	3-6 h (48 h*)	Easy-to-use; detection of spoilage capabilities	Enrichment and/or pre-filtration step needed; DNA-extraction; PCR-inhibition from beer matrix (false-negative results); unable to distinguish between viable or dead cells; presence of naked DNA (false-positive results); post-PCR processing (only for end-time PCR); primer development; high investment costs	[124, 160, 161]
	Real-time amplification and quantification of target DNA sequences[¥]	D / I	Species- or group-specific	10^4-10^5 cells/100 mL sample	2-3 h; (24-72 h*)	Less post-PCR manipulations; real-time follow-up; better cost/benefit ratio		[83, 112, 125, 159]
FINGERPRINTING	Ribotyping, restriction enzyme-pattern analysis of genomic DNA using Southern blot analysis[¥]	I / T	All BSB	Pure cultures	8 h	Automation possible; easy-to-perform; standardised; objective identification; typing possible	High investment and running costs; time-consuming; pure cultures are required; cumbersome sample preparations; identification database-dependent; selection of restriction enzymes (ribotyping); no high-throughput analyses	[67, 88, 94, 97, 121, 137, 167, 168]
	SDS-PAGE protein-profiling	I / T	All BSB	Pure cultures	1-2 days	Objective identification; gene expression-based protein patterns		[26, 94]

[¥]commercialised;

*time of pre-enrichment of sample;

Abbreviations: D, Detection; I, Identification; T, Strain level differentiation possible; MAbs: Monoclonal antibodies

3 The application of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry in microbiology

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a soft mass spectrometric technique which allows the ionization of biomolecules, such as proteins and peptides, in a non-destructive manner [90, 184]. MALDI-TOF MS couples sensitivity with accuracy and is thus well-suited for the detection of high and low molecular weight proteins that comprise a significant portion of the microbial cell [185]. Most microbial proteins detected by MALDI-TOF MS are highly abundant in the cytosol, have a high basicity and are of medium hydrophobicity [186]. Moreover, this technique has a number of advantages over other mass spectral methods especially if used for the analysis of intact microbial cells, such as its ability to detect proteins in a broad *m/z* (molecular weight to charge ratio) range and its remarkable tolerance for impurities (*e.g.*, salts) which eliminates time-consuming sample purification steps [187, 188].

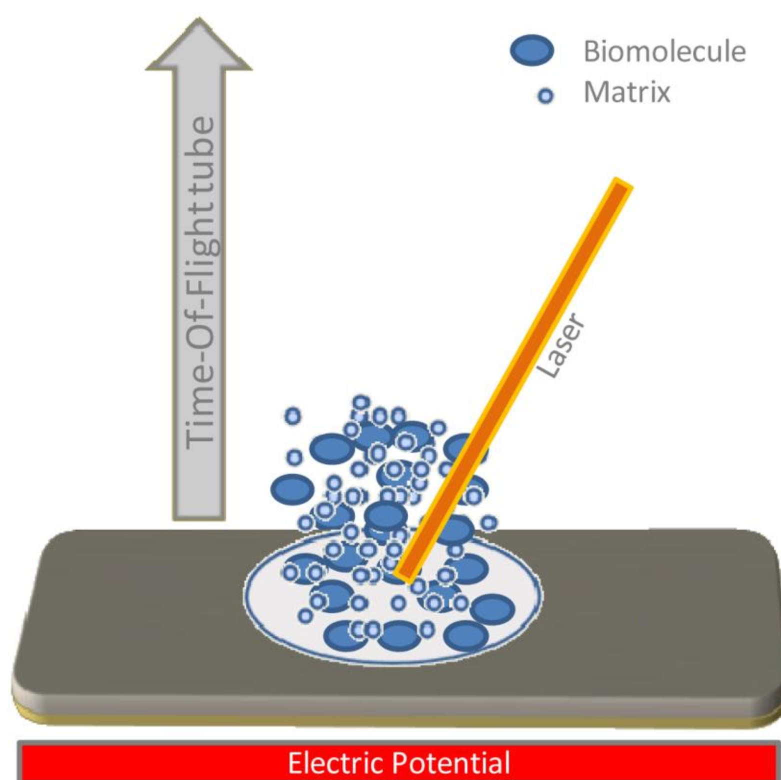


FIG 3.1. The laser irradiates the sample spot enabling desorption/ionisation of the biomolecules. During this process, a plume containing gas phase ions from the biomolecules and the matrix compounds is formed above the target plate. The ions generated are subsequently accelerated by a high electrical potential and separated in the mass analyser.

Microbial intact cells or crude extractions thereof are deposited onto a metal target plate and subsequently overlaid or pre-mixed with an appropriate organic matrix solution (**FIG 3.1**) [90, 184, 189]. After loading the target plate in the vacuum of the mass spectrometer's ion source, a laser irradiates the sample spot with nanosecond pulses enabling its high energetic desorption and subsequent ionization of the biomolecules [90, 189]. The matrix molecules adsorb the laser energy resulting in the transfer of solid phase biomolecules into gas phase ions with minimal fragmentation (**FIG 3.1**) [90, 176]. To date, the exact mechanisms of this desorption/ionization process are not well understood [176, 190]. The generated ions are subsequently accelerated by a high electric potential and separated in the mass analyser (*i.e.*, the TOF tube) according to their *m/z* ratio (**FIG 3.2**) [90, 187]. The ions, which are mostly single charged, accelerated in the same electric field have the same kinetic energy, but have different velocities depending on their molecular weight which enables their separation in the field-free, vacuum TOF tube (**FIG 3.2**) [90, 191, 192]. The generated MALDI-TOF mass spectrum represents the intensity (*i.e.*, the quantity of the ionised molecules) in function of the *m/z* value, and are typically unique for a certain microorganism, allowing for an accurate identification of unknown strains (**FIG 3.3**) [175, 176, 193].

Different data analysis approaches have been employed to analyse MALDI-TOF mass spectra generated for the species level differentiation of microorganisms, such as the library-based and proteomic-based analysis methods. The library-based approach is most commonly used and correlates the generated mass spectra (*i.e.*, full-spectrum or extracted peak tables) from unknowns with a collection of mass spectra derived from well-characterised reference strains using different pattern matching algorithms [194-198]. When comparing entire mass spectra one must consider that all peaks are used during analysis even if these are not directly linked to the studied microorganism (*e.g.*, medium associated peaks, peaks linked to instrumental variations or to sample treatment) [189, 192, 196], and that this method depends on a solid and reproducible identification database comprising mass spectra generated from well-characterised reference strains of interest [191]. Robustness of the library-based identification approach increases when only a limited number of genus-, species- or strain-specific biomarker peaks are selected for further analysis [192, 199].

In the proteomics-based approach, extracted *m/z* peak tables can also be compared to *in silico* generated protein spectra predicted from available genome data of microorganisms present in publicly available protein databases [184, 191, 197, 200, 201]. This approach relies strongly on the availability of the genome data, the mass accuracy of the mass spectrometer, the proteome database completeness and fidelity (*e.g.*, post-translational modifications) [189, 191, 202].

The next chapter mainly focuses on the application of MALDI-TOF MS for the identification of bacteria. However, multiple research groups investigated and proved its applicability for the identification of fungi, viruses, protozoa, algae etc. as well [191, 203-208].

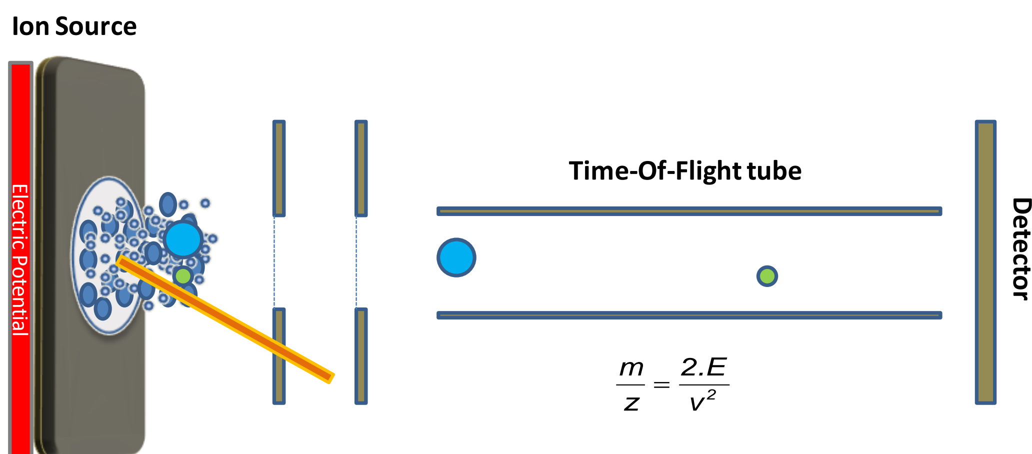


FIG 3.2. After the matrix-assisted laser desorption/ionisation process, ions are accelerated via an electric potential and subsequently separated in the time-of-flight (TOF) tube. The ions accelerated in the same electric field have the same kinetic energy, but differ in velocities depending on their respective molecular weights. Lower molecular weight ions (green dot) will travel faster than the high molecular weight ions (blue dot) through the field-free, vacuum TOF-tube and hence hit the detector first. *m*, molecular weight; *z*, charge; *E*, energy and *v*, velocity.

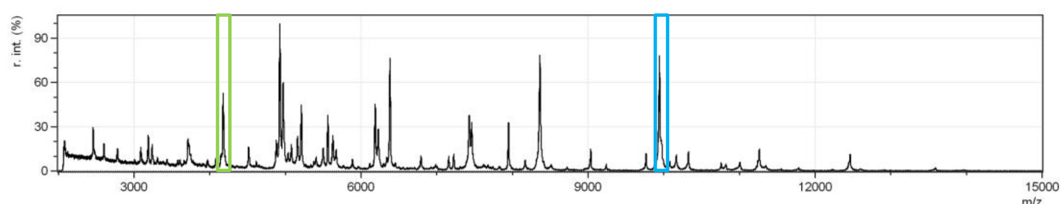


FIG 3.3. MALDI-TOF mass spectrum generated from *Gluconobacter oxydans* strain LMG 1406. The mass spectrum represents the individual intensities of all detected ions in function of their *m/z* value in the 2- to 15-kDa range. The green and blue rectangles represent the peak signal from the low and high molecular weight ion highlighted in **FIG 3.2**, respectively. r. int., relative intensity.

3.1 From culture to mass spectrum

Reproducibility is the major factor to consider when characterizing intact microorganisms using MALDI-TOF MS, especially when using the library-based identification approach [175, 202, 209]. Experimental factors (*e.g.*, growth condition, cell concentration, sample treatment, data acquisition) contribute to mass spectral variation and hence alter mass spectral reproducibility [191, 194, 210-215].

Studies revealed that the mass spectra generated from bacteria consist of signals derived mostly from ribosomal and other abundant proteins [90, 186, 202, 216-221]. For proteins that are of ribosomal origin [220], the effect of growth medium on the mass spectra and thus on the identification result is expected to be minimal [202, 222]. However, growth medium can influence the expression pattern of other proteins [191, 202, 223] and hence alter the mass spectrum [217, 224]. Additionally, medium compounds can interfere with the ionization of the bacterial biomolecules [191, 212, 217], especially when bacterial cells have the tendency to adhere onto the culture medium surface when grown onto solid medium [217, 218]. Nevertheless, several studies found that variations related to the growth medium had no impact on species level identification of unknowns [177, 217, 225-230]. Not only growth medium, but the age of the cell culture also induces variations to the mass spectra, as cells adapt to changes in the growth medium (*i.e.*, nutrient depletion and build-up of waste products) [220, 231-234], but again not to an extent that species level identification was affected [229, 234].

Two principal sample treatment protocols [the intact cell method (ICM) and the cell extraction method (CEM)] have been employed to generate MALDI-TOF mass spectra from bacteria [201]. During ICM, intact cells are directly smeared onto the MALDI-TOF MS target plate and coated with the selected matrix [191, 201, 235]. This method is less expensive, easier and faster compared to the CEM [210]. However, the term 'intact cell' is not strictly accurate, as the integrity of the cell wall can be disrupted by the matrix solution prior to MALDI-TOF MS analysis [202]. Some research groups even mixed intact cells with 0.1% trifluoroacetic acid solution [201, 236] or employed an on target extraction using for example 70% formic acid [201, 228, 232] to enhance cell lysis prior to analysis. Instead of picking cells from solid medium, cells can also be harvested from liquid culture and subsequent deposited (with or without additional washing steps) onto the target plate [209, 215, 237, 238]. The CEM approach on the other hand assists the disruption of the microbial cell wall and removes compounds (*e.g.*, salts, cell debris, medium derivates) that may interfere with co-crystallisation of sample and matrix [197, 201, 239]. This results in a more homogeneous disposition on the target plate and hence improves the reproducibility of the desorption/ionization process

[240]. Goldstein *et al*. [215] favoured the CEM for profiling different *Staphylococcus aureus* strains, as this method yielded mass spectra with increased data richness (*i.e.*, more peaks and broader mass ranges) and reproducibility.

A pre-treatment using lysozyme prior to ICM or CEM enhanced the reproducibility and quality of the generated mass spectra without influencing data analysis [191, 241, 242]. Other chemical or physical adaptations enhancing extraction efficiency were reported as well, such as the use of corona plasma discharge, ethanol, heat treatment, freeze-thawing, micro-beads, surfactants and sonication [201, 203, 211, 224, 243-247].

Other factors affecting mass spectral quality and reproducibility are the used matrix compound [*e.g.*, sinapinic acid, α-cyano-4-hydroxycinnamic acid (α-CHCA), ferulic acid, 2,5-dihydroxybenzoate (DHB)] and matrix solvent composition [191, 201, 210, 239]. Different types of matrix compounds enable the analysis of different types of biomolecules. For instance, sinapinic and ferulic acid which favour high molecular weight ions and α-CHCA which favours low molecular weight ions are mostly used for the detection of proteins, while DHB is more suitable in detecting glycopeptides and lipids [191, 197]. The matrix solvent selection is equally important and affects spot deposition, protein solubility, extraction efficiency, crystallisation, shot-to-shot reproducibility, peak intensity and data richness [191, 201, 210, 214]. Finally, it was demonstrated that the manner of deposition of the sample/matrix (*e.g.*, dried-droplet method, sandwich method) can induce variation to the generated mass spectra as well [197, 201, 213].

Instrument hardware and settings [210], the selected mass range [184, 202], the ion mode (*i.e.*, positive or negative) [219, 243], the use of automated or manual spot targeting [191, 202, 248], etc. should be carefully selected and optimised to generate reproducible and high quality mass spectra. The reproducibility can also differ within individual laboratories (intra) and especially across multiple laboratories (inter) and depends on the robustness of the procedure used to generate a mass spectrum starting from a cell culture [191, 201].

3.2 Commercially available MALDI-TOF MS identification databases

When using the library-based approach, multiple well-characterised reference strains should be included to sufficiently cover the intra species diversity and to obtain accurate and reliable identifications [184, 202, 228, 249-252]. Some commercially available and user-friendly MALDI-TOF MS identification systems supported with appropriate data analysis software package and a built-in mass spectral database are discussed below [202, 239].

The Bruker Daltonics MALDI BioTyper is the most commonly used MALDI-TOF MS identification system [198]. The accompanying identification database consists of numerous main spectral projections (MSPs) of the most relevant clinical microorganisms, and such MSPs can be easily created and added to personal in-house identification databases [185, 242]. Unknowns are identified based on three scores: (i) counting matching signals of their generated mass spectra with in-house MSPs and (ii) vice versa and (iii) correlating consistency of signal intensities of these matched signals [202, 222, 229, 253]. The three obtained scores are then multiplied and normalised to a value of 1000 and finally log-transformed [202, 240]. Identification log scores above 2 and between 1.7 and 2 are considered to be reliable at the species and genus levels, respectively [192, 236, 251, 254].

The VITEK MS RUO (Research Use Only, bioMérieux) [formerly the Axima Assurance system with SARAMIS™ (Spectra ARchiving And Microbial Identification System), Shimadzu], the VITEK MS IVD (*in vitro* diagnostic, bioMérieux), and Andromas (Andromas SAS) are other commercially available MALDI-TOF MS identification systems [176]. Such systems also allow an user-friendly approach to process raw data (*i.e.*, perform smoothing, normalization and baseline subtraction) and then compare generated mass spectra with a built-in identification database that again mainly comprises mass spectra of clinically relevant microorganisms [191, 202, 221, 255]. For instance, the VITEK MS IVD database consists of reference mass spectra generated from ten different strains (*i.e.*, different isolation source, year and origin) per species grown at different cultivation conditions (*i.e.*, incubation time, growth medium, growth medium supplier) and several mass spectrometers [255].

Comparisons between two or more commercially available systems for the identification of clinical microorganisms revealed that differences in identifications were mainly based on the discriminatory potential of closely related species, the completeness of the database and the incorporated intra-species diversity [204, 222, 236, 252, 256-258].

3.3 Current applications of MALDI-TOF MS in microbiology

Numerous applications of MALDI-TOF MS are related to public health and food safety. Rapid and accurate identification and classification of microorganisms, especially food-borne and human pathogens, is crucial for timely infection control, initiating appropriate treatment and defining associated risks that affect the patient and health-care personnel [184, 215, 251]. In the clinical diagnostic laboratories, the biochemical identification systems that dominated for decades are partially replaced with rapid, cost-effective and high-throughput MALDI-TOF MS identification systems [202, 251].

Multiple studies reported the successful application of MALDI-TOF MS as identification tool for clinically relevant species [174, 249, 250], such as *Arcobacter* spp.[218], *Bacteroides* spp. [259], *Brucella* spp. [231], *Burkholderia cepaciae* complex [236, 260], *Campylobacter* spp. [218], *Helicobacter* spp. [218], *Legionella* spp. [235], *Listeria* spp. [221], *Mycobacterium* spp. [212, 261], *Salmonella* spp. [217], *Stenotrophomonas* spp. [253], *Streptococcus* spp. [193, 209, 238], *Pantoea* spp. [230], and *Vibrio* spp. [237]. MALDI-TOF MS has also been employed in the battle against bioterrorism [191, 229]. For example, discrimination of *Bacillus anthracis* spores from spores produced by closely related *Bacillus* spp. was facilitated using small acid-soluble proteins as biomarker peaks [211, 262].

In general, MALDI-TOF MS has been extensively investigated in the field of clinical microbiology. However, fewer investigations have been conducted in the field of environmental [184, 263-266] or food microbiology [187, 192, 267, 268] where most studies so far concentrated on potential human- or plant-pathogens. Angelakis *et al.* [269] reported MALDI-TOF MS as a rapid and accurate tool for species level identification and authenticity check of probiotic strains isolated from probiotic food products. Other research groups also examined the potential of MALDI-TOF MS to differentiate among lactic acid bacteria present in the fermentation industry [179, 181, 242, 270, 271]. MALDI-TOF MS also enabled differentiation between acetic acid bacteria belonging to the genera *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* involved in the industrial production of vinegar [109]. Kern *et al.* [272] also demonstrated the potential of MALDI-TOF MS as a species level identification tool for spoilage isolates obtained from the beverage industry.

3.4 Beyond species level identification

3.4.1 *Infraspecific level discrimination*

Multiple studies have demonstrated the successful application of MALDI-TOF MS for the species level discrimination of bacteria. However, infraspecific level discrimination is even more important in certain applications such as epidemiological studies, source-tracking, antibiotic resistance investigations, etc. Closely related strains yield similar mass spectra, yet in some cases subtle reproducible discrepancies enabled discrimination among mass spectra of different strains belonging to the same species [198, 248, 265, 266]. The mass spectral reproducibility and quality are equally important to enable accurate and reliable infraspecific level discrimination [215]. Therefore, standardised growth conditions (*e.g.*, culture medium, incubation time) and optimised data acquisition for the generation of mass spectra are advised [210, 214, 229, 237, 266].

MALDI-TOF MS has been successfully applied in infraspecific level discrimination of biological warfare agents, food-borne pathogens, clinical infections (*e.g.*, antibiotic resistance, serotyping, pathogenicity), source tracking, manufacturing of probiotic starter cultures in the fermentation and pharmaceutical industry [177, 198, 229, 237, 242, 245, 265, 266, 273, 274].

Siegrist *et al*. [266] addressed the problem of faecal contaminations of surface waters and employed MALDI-TOF MS to categorise strains of the indicator organism *Escherichia coli* according to their source (*i.e.*, avian, bovine, canine or human). Similarly, Giebel *et al*. [265] reported the potential of MALDI-TOF MS as source tracking tool for environmental *Enterococcus* strains in recreational waters. Discrimination among nitrogen-fixation *Frankia* strains based on their specific host infection groups was reported by Hahn *et al*. [275]. Seibold *et al*. [229] classified *Francisella tularensis* strains, the causative agent of tularaemia, with respect to their membership in one of the four subspecies showing significant differences in virulence regarding animal and human infections. Sato *et al*. [245] enabled discrimination between two out of three subspecies of *Bifidobacterium longum* based on ribosomal protein profiling using a proteomics-based approach, and thereby supported the proposal of reclassification of the species into two subspecies (*i.e.*, subsp. *longum* and subsp. *infantis*) instead of three (including subsp. *suis*). In contrast, Zeller-Péronnet *et al*. [242] were not able to discriminate among the subspecies of *Leuconostoc mesenteroides*, and thus could not reproduce the study of De Bruyne *et al.* [177]. Furthermore, both studies differed in the used sample treatment protocol, data acquisition and data analysis approach, which clearly affected infraspecific level discrimination [198, 276].

3.4.2 *Direct MALDI-TOF MS detection and identification of bacteria from specimen*

Research groups have explored the potential of MALDI-TOF MS for the direct detection and identification of bacteria present in biological specimens without the need for time-consuming cultivation procedures to isolate the bacteria of interest [194, 204, 239, 277-286].

Different sample treatment protocols have been optimised in order to separate the bacterial cells of interest from interfering background compounds such as blood cells, commensal bacteria, degradation products, salts, proteins, lipids, etc. [184, 239, 263]. These protocols include washing steps, differential centrifugation, gel-based separation, filtration and affinity-based methods (*e.g.*, immuno magnetic beads) [197, 201, 205, 219, 239, 263, 278-281, 285-291]. In clinical laboratories, the commercially available Sepsityper kit (Bruker Daltonics) contains reagents required for the preprocessing of blood specimens that tested positive for bloodstream infections after enrichment in liquid medium [239, 277, 283, 284, 290, 292]. The Sepsityper kit includes a lysis solution which

disrupts only blood cells rendering bacterial cells intact, and a washing solution that eliminates interfering background compounds [277, 281, 282, 292]. Taken together, such separation methods enabled the generation of reproducible, good quality mass spectra that are well-suited for MALDI-TOF MS identification. Furthermore, such separation methods can effectively increase the enrichment factor and hence detection sensitivity [201, 263]. However, multiple research groups were unable to circumvent an initial enrichment step as initial bacterial load was too low (lower than 10^6 CFU/mL in positive blood cultures), resulting in mass spectra where bacterial peaks were indistinguishable from background peaks [277, 279]. Wang *et al.* [286] reported that at least 10^5 CFU/mL were required to allow accurate and reliable species level identification directly from positive urine samples [278]. Li *et al*. [263] combined membrane filtration and vancomycin-conjugated magnetite nanoparticles to selectively separate and concentrate Gram-stain positive bacteria in tap water and reservoir water, thereby achieving a detection limit of 5×10^2 CFU/mL (2 L sample was required). Increasing sample volume could assist in a more accurate identification [263, 284], but this is not always achievable, especially for clinical specimens [293].

3.4.3 *MALDI-TOF MS analysis of specimen-containing mixed populations*

To date, the analysis of bacterial mixtures by MALDI-TOF MS remains challenging, and multiple studies have investigated and evaluated its potential to accurately identify the individual compounds [184, 239, 243, 263, 294, 295]. The differentiation among members of a mixture depends on the sample treatment, data acquisition, data analysis algorithms, etc. [176, 240, 280, 294-296]. In general, biomolecules derived from the most abundant microorganism present in the mixture suppress those generated from less abundant microorganisms during the desorption/ionization process. The limited dynamic range of MALDI-TOF MS makes it complex to discriminate between the individual microorganisms, especially when their relative concentration varies a lot [200, 239, 279, 282, 286, 296, 297]. As described above, the direct identification of positive clinical samples was successful using MALDI-TOF MS, at least with some minimal sample preparation. However, identifications were often hampered when co-infections were present. Such analysis result in the identification of the most abundant microorganism present only, or in no identification at all [204, 205, 239, 277, 281-283, 285, 298]. Furthermore, such positive identification did not exclude the presence of additional microorganism, and conventional slower screening methods should always be combined with MALDI-TOF MS [176, 298].

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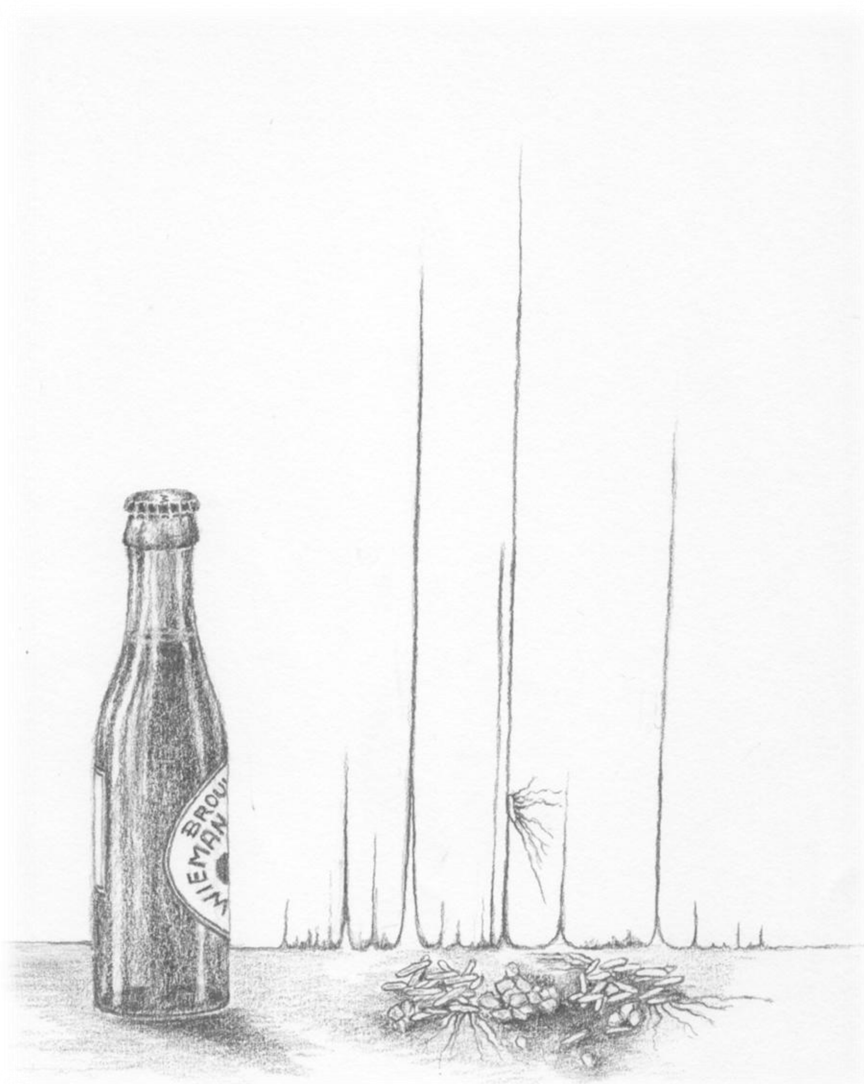

Part III | Experimental Work

PREAMBLE

Beer is a beverage with normally good microbiological stability because it contains almost no oxygen and nutrients for bacterial growth. In addition, low pH, high CO_2-content and the presence of ethanol and antibacterial hop compounds ensure microbiological stability. Nevertheless, beer spoilage due to bacteria is a common problem in the brewing industry and causes important economic losses worldwide. The taxonomically diverse group of beer spoilage bacteria are currently detected and/or identified using a range of culture-dependent or culture-independent methods. It is clear that these methods are time-consuming and often lack high-throughput capacity and do not facilitate real-time interventions. Furthermore, these methods mostly target only a narrow range of beer spoilage bacteria. Therefore, MALDI-TOF MS was investigated as an alternative, rapid, low-cost and high-throughput identification tool for the most prevalent beer spoilage acetic acid bacteria (AAB) and lactic acid bacteria (LAB) by constructing an identification database and evaluating its applicability for the identification of novel spoilage bacteria, and subsequently validated by state-of-the-art taxonomic standards. Therefore, different studies were set up, of which the results will be presented in the following chapters.

CHAPTER 4 describes the effect of the growth medium on the generated mass spectra and its consequences for species and strain level differentiation. **CHAPTER 5** presents the development of the MALDI-TOF MS identification database and the subsequent evaluation and validation of the taxonomical classification of the reference strains included. Moreover, the performance of MALDI-TOF MS as an accurate, rapid and high-throughput identification tool was established via isolates obtained from different spoiled beer and brewery samples. These studies also resulted in the reclassification of *Pediococcus lolii* strains DSM 19927^T and JCM 15055^T as *Pediococcus acidilactici* (**CHAPTER 5.2**) and in the description of a novel acetic acid bacterium, *Gluconobacter cerevisiae* sp. nov., isolated from a spoiled brewer's pitching yeast culture (**CHAPTER 5.3**). **CHAPTER 6** describes the direct detection and identification of bacteria in enrichment cultures of spoiled beer and brewery samples. Finally, **CHAPTER 7** evaluated MALDI-TOF MS as a tool to differentiate between brewer's yeasts and unwanted brewing yeast strains contaminating the brewing process.

4 The effects of the growth medium on matrix-assisted laser desorption/ionisation time-of-flight mass spectra: a case study of acetic acid bacteria

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SUMMARY

The effect of the growth medium used on the matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectra generated and its consequences for species and strain level differentiation of acetic acid bacteria (AAB) were determined by using a set of 25 strains. The strains were grown on five different culture media that yielded a total of more than 600 mass spectra, including technical and biological replicates. The results demonstrate that the culture medium can have a profound effect on the mass spectra of AAB as observed in the presence and varying signal intensity of peak classes, in particular when culture media do not sustain optimal growth. The observed growth medium effects do not disturb species level differentiation but strongly affect the potential for strain level differentiation. The data proved that a well-constructed and robust MALDI-TOF MS identification database should comprise mass spectra of multiple reference strains per species grown on different culture media to facilitate species and strain level differentiation.

4.1 Introduction

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) has been applied in medical [1], environmental [2-4] and food [5-8] related studies as an excellent cost- and time-effective tool for the identification of microorganisms. Different types of identification approaches were recently reviewed [9]. These include linking of protein biomarkers derived from the mass spectra to a corresponding experimental database or comparing mass spectra with *in silico*-generated spectra predicted by whole-genome sequence analyses [10, 11]. Yet, the most frequently used approach is the comparison of mass spectra of unknowns with those of well-characterised reference strains stored in a profile database. These comparisons are conducted using either a peak- or curve-based algorithm [9, 12]. With a peak-based algorithm, the presence of specific biomarker peaks in the unknown isolate's mass spectrum is verified [6, 13]. A curve-based method considers the complete spectrum, *i.e.*, not only the presence of certain peaks but also the variation in peak signal intensity. The major drawback of these pattern recognition approaches is that they can be prone to experimental variations [14].

Previous studies revealed that MALDI-TOF mass spectra of bacteria consist of signals derived mostly from ribosomal and other abundant proteins [15-18]. For proteins that are of ribosomal origin [19], the effect of growth conditions on the mass spectra and thus on the identification result is expected to be minimal [20]. However, growth conditions can influence the expression pattern of other proteins [21] and hence alter the mass spectrum [16, 22]. Additionally, growth medium compounds can interfere with the ionisation of the bacterial biomolecules, especially when the bacterial cells have a tendency to adhere to the culture medium surface [15]. Nevertheless, several studies found that variations related to the growth medium had no impact on species level identification of unknowns [16, 23-26].

We are investigating the use of MALDI-TOF MS as an identification tool for bacterial isolates originating from spoiled beer and brewery samples and are constructing an identification database including spectra of strains originating from spoiled beer and brewery samples but also from other niches where the same species occur in order to encompass the species' phenotypic diversity wherever possible. Beer spoilage bacteria are taxonomically diverse and include several acetic acid bacteria (AAB) which can be grown on different culture media.

The present study investigated the effect of the growth medium on the generated mass spectra using a set of 25 AAB grown on five different culture media and its consequences for species and strain level differentiation.

4.2 Material and Methods

4.2.1 *Strains and growth media*

Twenty-five strains of AAB (see **TABLE S 4.1** in the supplementary material) were obtained from the BCCM/LMG bacteria collection (Ghent University, Ghent, Belgium) (http://www.belspo.be/bccm/) and cultured according to the provider's instructions and on additional selective and nonselective growth media. *Acetobacter s*trains were grown on acetic acid medium (AAM) [1.0% (w/v) D-glucose, 1.5% (w/v) bacteriological peptone, 0.8% (w/v) yeast extract, 0.3% (v/v) acetic acid, 0.5% (v/v) ethanol and 1.5% (w/v) agar, adjusted to pH 3.5] [27]; deoxycholate-mannitol-sorbitol (DMS) agar [0.1% (w/v) D-glucose, 0.1% (w/v) D-mannitol, 0.1% (w/v) sorbitol, 1.0% (w/v) bacteriological peptone, 0.3% (w/v) yeast extract, 1.5% (w/v) calcium lactate, 0.1% (w/v) potassium phosphate, 0.01% (w/v) sodium deoxycholate, 0.002% (w/v) magnesium sulphate, 0.003% (w/v) bromocresol, 1.5% (w/v) agar, pH 4.5] [28]; GY agar [5% (w/v) D-glucose, 1% (w/v) yeast extract and 1.5% (w/v) agar]; GYAE agar [GY agar supplemented with 1% (v/v) acetic acid and 2% (v/v) ethanol] and YPM agar [0.5% (w/v) yeast extract, 0.3% (w/v) bacteriological peptone, 2.5% (w/v) D-mannitol, and 1.5% (w/v) agar]. *Gluconacetobacter* and *Gluconobacter* strains were cultured on the same growth media, with the exception of GYAE agar which was replaced by Reinforced AE (RAE) agar [4% (w/v) D-glucose, 1% (w/v) yeast extract, 1% (w/v) bacteriological peptone, 0.338% (w/v) $Na_2HPO_4.2H_2O$, 0.15% (w/v) citric acid, 1% (v/v) acetic acid and 2% (v/v) ethanol, a layer of this medium containing 1% (w/v) agar is topped with a layer containing 2% (w/v) agar] [29]. AAB are strictly aerobic bacteria, and cultures were always incubated at 28°C. The incubation time on each medium was kept constant per strain but varied between 24 and 72h among strains in order to obtain sufficient growth; yet some strains grew so poorly on, at most, one growth medium that mass spectra of acceptable quality (see below) could not be obtained.

4.2.2 *MALDI-TOF MS sample preparation and data acquisition*

Resuscitated lyophilised cultures were subcultured twice on each culture medium and cell extracts were prepared from the subsequent third, fourth and the fifth generation. Hence, variation induced by resuscitation and adaptation to the growth medium was excluded and three generations, *i.e.*, three biological replicates, were included. Five mg of wet cells taken from single colonies was suspended into 300 µL Milli-Q water, after which 900 µL pure ethanol was added. After centrifugation (3 min, 20817 × g, 4°C), 50 µL 70% formic acid and 50 µL acetonitrile were added to the bacterial cell pellet. After vigorous shaking and centrifugation (3 min, 20817 × g, 4°C), 1 µL of the supernatant (= the cell extract) was spotted in duplicate onto a MALDI-TOF MS stainless steel target plate to obtain two technical replicates. Immediately after drying, the spots were overlaid with 1 µL matrix solution, which consisted of 5 mg α-cyano-4-hydroxycinnamic acid dissolved in 1 mL acetonitrile/trifluoroacetic acid/Milli-Q water solvent (50:2:48). Prior to analysis, the mass spectrometer was externally calibrated with a peptide mixture of adrenocorticotropic hormone (fragment 18-39; Sigma-Aldrich), insulin (Sigma-Aldrich), ubiquitin (Sigma-Aldrich), cytochrome c (Sigma-Aldrich) and myoglobin (Sigma-Aldrich). A 4800 Plus MALDI TOF/TOF™ Analyzer (AB Sciex, USA) was used in the linear mode and covered a mass range from 2 to 20 kDa. The mass spectrometer used a 200 Hz frequency tripled Nd:YAG laser operating at a wavelength of 355 nm. Generated ions were accelerated at 20 kV through a grid at 19.2 kV into a 1.5 m, linear, field-free drift region towards the detector. For each spot, 40 subspectra resulting from 50 laser shots from randomised positions within the spot were collected and presented as one spectrum (a total of 2000 laser shots).

The laser intensity was set between 4200 and 5700 procedure defined units. With every set of measurements comprising 384 spots, the Bacterial Test Standard (Bruker Daltonics) was included as positive control.

4.2.3 *MALDI-TOF MS data analysis*

The mass spectra were retrieved as t2d files from the 4800 Plus MALDI TOF/TOF™ Analyzer via the 4000 Series Explorer software (AB Sciex). Data Explorer 4.0-software (AB Sciex) was used to convert the t2d files into text files that were subsequently used as input files for the BioNumerics 7.1 software package (Applied Maths, Belgium). A spectrum was considered to be of acceptable quality if the absolute signal intensity of the highest peak was > 500 counts, if more than 5 peaks with a signal-to-noise (S/N) ratio of > 20 were detected in the 3-20 kDa range and if there were no repetitive signals in the 2.1-3 kDa range. The spectral data were imported by using an optimised preprocessing template in the BioNumerics 7.1 software package [30]. After the data were imported, the preprocessing involved consecutive continuous wavelet transform (CWT) noise estimation, a Savitsky-Golay filter smoothing and baseline subtraction with the rolling disk algorithm. Each peak with a S/N ratio of at least 5 and an absolute intensity of at least 6 counts was annotated. For each strain grown on each culture medium, both technical and biological replicates were combined into a single summary spectral profile (SSP). A peak-matching analysis was conducted with constant and linearly varying tolerance values of 1 *m/z* and 800 ppm, respectively [30]. The minimum peak detection rate was set at 100%, meaning that each summary peak occurred in each individual spectrum of the technical and biological replicates. The use of the 100% peak detection rate during this summarizing procedure excluded any technical and biological variation from the analysis. The signal intensity for each data point in the SSP was calculated by averaging the respective signal intensities in the technical and biological replicates.

The peak-based data analysis matches all of the peaks in the SSP to a peak class using constant and linearly varying tolerance values of 2 *m/z* and 800 ppm, respectively [30]. The dataset obtained was converted into a binary character set. The latter dataset was then used to compare the SSPs using the binary Dice coefficient. The curve-based data analysis of SSPs was conducted by using the Pearson product-moment correlation coefficient. In order to visualise the variation between the SSPs, multidimensional scaling (MDS) plots were created as described by De Bruyne *et al.* [24]. In brief, the MDS algorithm starts with the similarity matrix generated after either peak- or curve-based analysis and then assigns a location to each data point in the *n*-dimensional space using a nonlinear least squares fit, minimizing the distances between the data points [24]. The resulting data positions can be displayed by three-dimensional visualization. The degree of variation among the five SSPs of each strain was described by the minimal similarity value (MSV) which was defined as the lowest average similarity level among the five SSPs.

4.3 Results and Discussion

4.3.1 *Media that do not sustain optimal growth can influence the SSP strongly*

A total of 17 AAB could be cultured on all five media as described above (see **TABLE S 4.1** in the supplementary material). The MDS plots obtained after peak-based numerical analysis of the five

SSPs of each of these strains revealed that the diversity among the SSPs was variable, suggesting a strain-dependent effect of the growth medium on the mass spectra (**FIG 4.1**).

The MSVs among SSPs of the 11 *Acetobacter* strains ranged from 50.5% (*Acetobacter fabarum* LMG 24630) to 81.0% (*A. fabarum* LMG 1701) with an average of 66.1% ± 9.4% (**FIG 4.1**). The SSPs of strain LMG 1701 shared 31 (52%) out of 60 peak classes. In contrast, only 12 (23%) out of 53 peak classes were common to the SSPs of strain LMG 24630. Nine additional shared peak classes could be detected in the SSPs obtained from the latter strain grown on four culture media but not YPM agar. Strain LMG 24630 did not grow well on the latter medium, and longer incubation times were required to obtain sufficient cell material for MALDI-TOF MS analysis. It is unclear what caused the aberrant SSP, but these slow growing subcultures may have been sampled in a different physiological state, which may affect MALDI-TOF mass spectra [31]. A similar decrease in the number of shared peak classes and concomitantly in the MSV was observed for *Acetobacter aceti* strain LMG 1504^T when it was grown on this culture medium. A total of 52 peak classes were detected for this particular strain, 19 of which were common to all five SSPs, and again 7 additional peak classes were present in the SSPs generated after growth on four media but not on YPM agar (**FIG 4.2**). This was again associated with poor growth on YPM agar. Other strains like *A. aceti* LMG 5 (MSV of 66.9%) or *A. fabarum* LMG 1701 (MSV of 81.0%) grew well on YPM agar, which resulted in SSPs that were more comparable to the strain-specific SSPs generated from growth on the other four culture media (**FIG 4.1**).

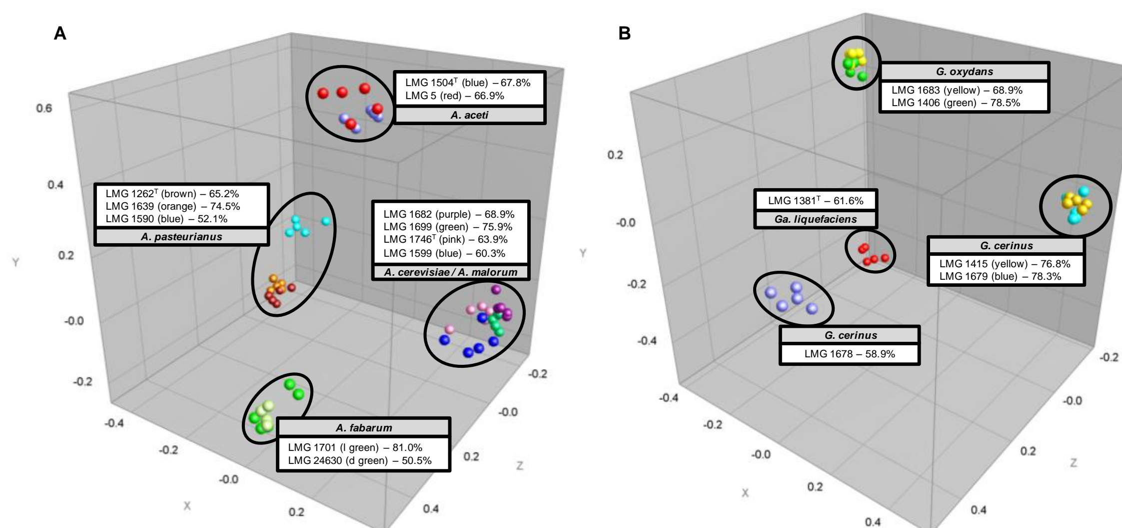

FIG 4.1. MDS plots obtained after peak-based numerical analysis of the SSPs of 11 *Acetobacter* strains (**A**) and 1 *Gluconacetobacter* (*Ga.*) and 5 *Gluconobacter* (*G.*) strains (**B**) grown on five culture media. For each strain, single-coloured dots represent the SSPs derived from the five different culture media. The boxes display the MSVs among the respective strain-specific SSPs. The higher the level of similarity between two SSPs, the more closely located the dots are to each other.

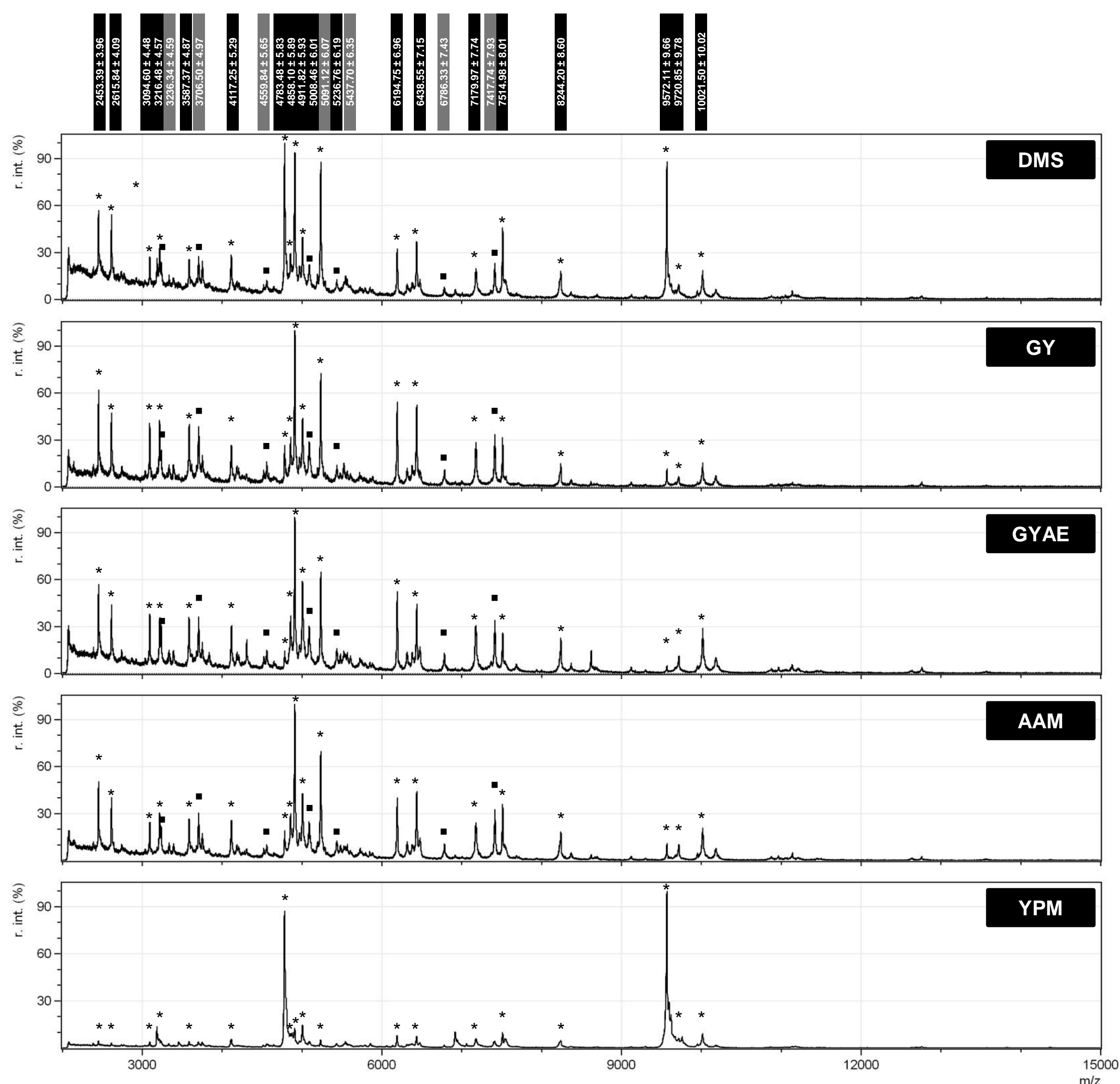

FIG 4.2. Mass spectra derived from *A. aceti* strain LMG 1504^T grown on the five culture media indicated in the black boxes on the right. All of the shared peak classes (19 out of 52) of the five SSPs are indicated by asterisks, and the corresponding m/z values are represented in the black boxes at the top. The seven additional peak classes after omission of the YPM-derived SSP are indicated by squares within the spectra, and the corresponding m/z values are shown in the grey boxes at the top. r. int., relative intensity.

The five SSPs of strain *Acetobacter pasteurianus* LMG 1590 had 10 (19%) out of 53 peak classes in common, yet the signal intensities of these common peak classes varied with the growth medium used (**FIG 4.3**). The most intense peak observed after cultivation of LMG 1590 on GY, GYAE and AAM agar was detected in the peak class at *m/z* 5268.97 ± 6.22; each of these growth media comprises D-glucose as the sole carbon source. On the other hand, peak class *m/z* 6790.66 ± 7.43 had the highest signal intensity in the SSPs generated after growth on the other two culture media, which comprised D-glucose (DMS agar), D-mannitol (YPM and DMS agar), and sorbitol (DMS agar) as carbon sources. Similar results were obtained for three other *Acetobacter* strains grown on

multiple media, suggesting a link between certain medium compounds, *i.e.*, the carbon source, and peptides detected. Remarkably, seven peak classes were unique for the DMS agar-derived SSP (**FIG 4.3**). These seven peak classes were not present in SSPs of other strains grown on DMS agar, indicating that these are strain-specific rather than DMS agar-specific peak classes. A detailed comparison of SSPs generated on each of the five growth media revealed similar observations for the other *Acetobacter* strains where no growth medium-specific peak classes were found. This contrasts with results obtained by Dieckmann *et al.* [16] who detected two medium-related peak classes in the mass spectra of isolates grown on a blood-containing culture medium. However, they smeared the bacterial cells directly onto the MALDI-TOF MS target plate, an approach that increases the potential for contamination with medium-derived peaks [32].

The MSVs among SSPs of the *Gluconacetobacter* and *Gluconobacter* strains ranged from 58.9 to 78.5% with an average of 70.5% ± 8.7% (**FIG 4.1**). The lowest MSVs were observed for strains *Gluconobacter cerinus* LMG 1678 (58.9%, 15 out of 52 peak classes were shared) and *Gluconacetobacter liquefaciens* LMG 1381^T (61.6%, 19 out of 53 peak classes were shared), and for both strains, this was related to the RAE agar-derived SSPs, as both strains grew poorly on this culture medium. Omission of the RAE agar data improved the MSVs to 80.5 and 83.4% for strains LMG 1678 and LMG 1381^T, respectively. The highest MSV (78.5%) was observed for strain *Gluconobacter oxydans* LMG 1406, which grew well on all media, including the RAE agar, and whose SSPs had 25 (48%) out of 52 peak classes in common (see **FIG S 4.1** in the supplementary material).

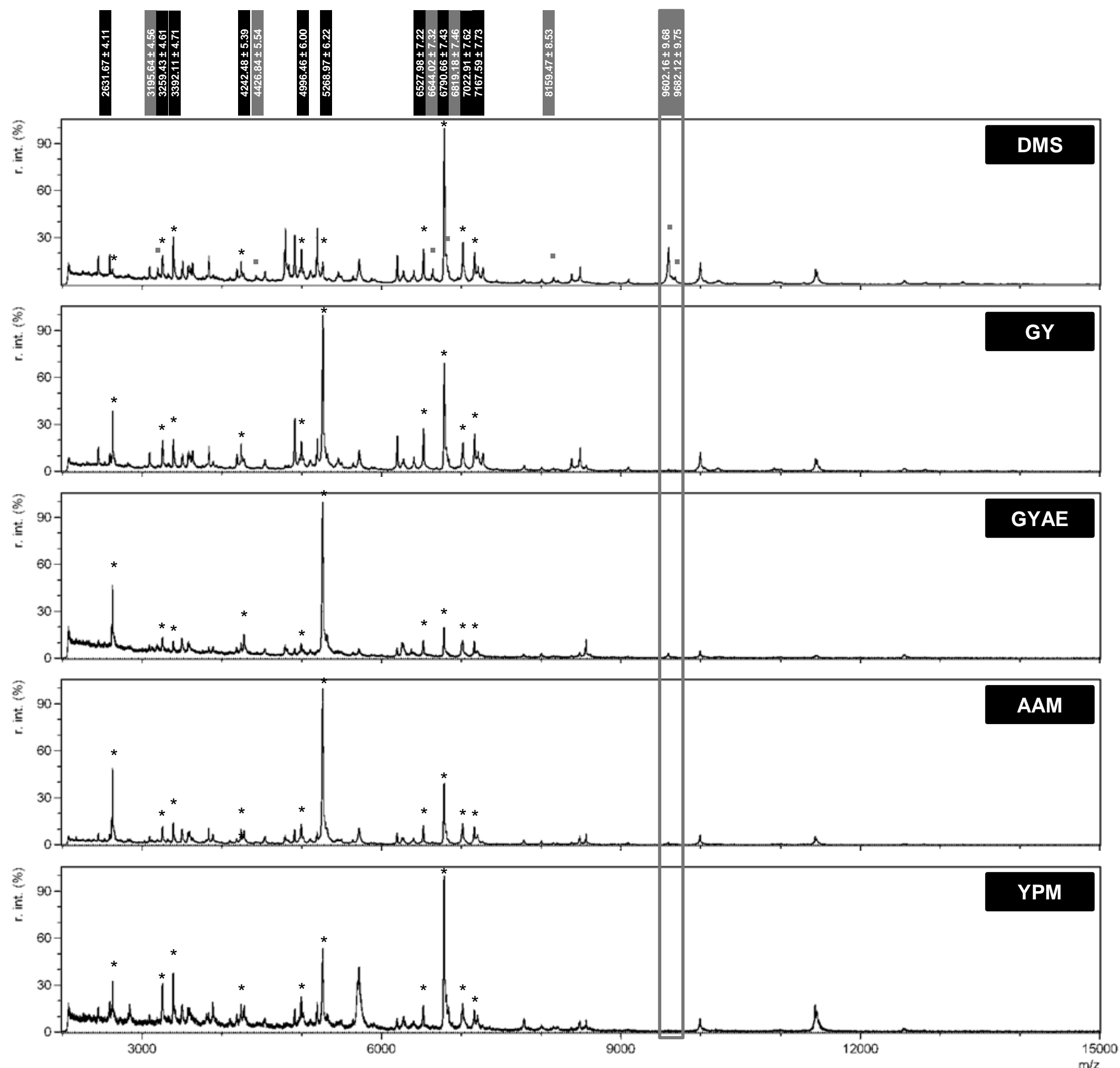

FIG 4.3. Mass spectra derived from *A. pasteurianus* strain LMG 1590 grown on five different culture media. The shared peak classes (10 out of 53) are indicated by asterisks, and their m/z values are shown in the black boxes at the top. The seven unique peak classes detected after culturing on DMS agar are indicated by grey squares, and the corresponding m/z values are shown in the grey boxes at the top. The two most abundant peak classes of these seven DMS specific peak classes are inside the rectangle. r. int., relative intensity.

4.3.2 *The culture medium used affects strain level differentiation*

The SSPs of eight *G. oxydans* strains (see **TABLE S 4.1** in the supplementary material) grown on AAM were compared using the binary Dice coefficient, as strain level differentiation is best accomplished by the presence-or-absence analysis of specific peak classes [13, 26, 33, 34]. An MSV of 54.1% was obtained and only 11 (13%) out of 85 peak classes were shared (**FIG 4.4**). Although these 11 shared peak classes had the same *m/z* value, a strain-dependent variation in peak signal intensity was observed (**FIG 4.4**). When the same eight strains were grown on YPM and GY agar only 8/88 (9%, MSV of 59.5%) and 12/79 (15%, MSV of 49.3%) peak classes, respectively, were shared (**TABLE 4.1**). An analysis of all of the SSPs showed that only 7 (7%) out of 105 peak classes were consistently present in these *G. oxydans* SSPs, irrespective of the growth medium used (**TABLE 4.1**). However, the same analysis also revealed many strain-specific peak classes in SSPs obtained after growth on each of the three culture media (**TABLE 4.2**) (to identify strain-specific peak classes, the SSPs of all of the strains obtained after growth on the same culture medium were considered). The number of strain-specific peak classes varied with the growth medium used (**TABLE 4.2**). For instance, the LMG 1674 SSPs were characterised by four and two strain-specific peak classes when that strain was grown on AAM and GY agar, respectively; however, no strain-specific peak classes were found when LMG 1674 was grown on YPM agar. Similarly, the LMG 1683 SSPs were characterised by 3, 4 and 0 strain-specific peak classes when that strain was grown on AAM, YPM, and GY agar, respectively. It should also be noted that some of these strain-specific peak classes are present in SSPs obtained after growth on multiple culture media. For example, the peak class characterised by an *m/z* value of about 5591 was present in each of the strain LMG 1676 SSPs, while for strains LMG 1683 and LMG 1398, each strain-specific peak class was observed after growth on a single growth medium only (**TABLE 4.2**).

Likewise, five *Acetobacter cerevisiae* strains (see **TABLE S 4.1** in the supplementary material) were cultured on AAM, DMS, YPM, and GY agar and their SSPs were compared by using the binary Dice coefficient. Eight (6%) out of 142 peak classes were common to all of the SSPs generated after growth on all four culture media (see **TABLE S 4.2** in the supplementary material). The same analysis also revealed strain-specific peak classes (see **TABLE S 4.3** in the supplementary material), and again the number of strain-specific peak classes varied with the growth medium used. For example, the LMG 1545 SSPs were characterised by two, eight, five and five strain-specific peak classes when that strain was grown on AAM, DMS, YPM, and GY agar, respectively. Moreover, some of these strain-specific peak classes were common to SSPs obtained after growth on multiple media. For instance, eight strain-specific peak classes (*m/z* values of about 4300, 5335, 6366, 6399, 6574, 7889, 10676, 12737) were present in each SSP of strain LMG 1699 grown on the four different culture

media. Similarly, two strain-specific peak classes characterised by *m/z* values of about 6560 and 7126 were shared in all of the LMG 1599 SSPs. In contrast, the four SSPs of strain LMG 1545 had no strain-specific peak classes that were present in each of the SSPs, but still several peak classes were present in two or three SSPs (see **TABLE S 4.3** in the supplementary material).

These results confirm that the number of strain-specific peak classes is culture medium-dependent and demonstrate that the selected culture medium affects the potential for strain level differentiation.

TABLE 4.1. Peak classes common to SSPs derived from eight *G. oxydans* strains grown on AAM, YPM and GY agar[a].

m/z value	Peak class		
	AAM[b]	YPM[c]	GY[d]
2460.83 ± 3.97	X		
3090.78 ± 4.55			X
3188.40 ± 4.55	X		X
4178.58 ± 5.34	X	X	X
4514.83 ± 5.61			X
4926.60 ± 5.94	X	X	X
4968.53 ± 5.97	X	X	X
5211.47 ± 6.17		X	
5627.75 ± 6.50	X		X
6186.62 ± 6.95	X	X	X
6382.37 ± 7.11	X	X	X
7431.76 ± 7.95	X		X
8362.86 ± 8.69	X	X	X
9942.86 ± 9.95	X	X	X

[a] Only the peak classses marked with an X are present in the respective medium, and the *m/z* values of the peak classes common to all of the SSPs are in bold (7/105 [7%]).

[b] Eleven (13%) of 85 peak classes shared

[c] Eight (9%) of 88 peak classes shared

[d] Twelve (15%) of 79 peak classes shared

TABLE 4.2. Strain-specific peak classes found in SSPs derived from eight *G. oxydans* strains cultured on three growth media[a].

	m/z value for indicated peak class		
Strain	AAM	YPM	GY
LMG 1398	3867.46 ± 5.09		
			4616.35 ± 5.69
		5318.36 ± 6.25	
		10644.00 ± 10.52	
LMG 1406	3728.15 ± 4.98		
		4616.79 ± 5.69	
	5036.67 ± 6.09		
			5406.82 ± 6.33
		6341.08 ± 7.07	
		7158.83 ± 7.73	
		7224.86 ± 7.78	
			9971.87 ± 9.98
	10822.60 ± 10.66	**10821.40 ± 10.66**	
LMG 1408[T]	**2627.62 ± 4.10**	**2627.42 ± 4.10**	**2627.51 ± 4.10**
	3910.06 ± 5.13		**3910.36 ± 5.13**
			3944.98 ± 5.16
			4356.70 ± 5.49
			5318.39 ± 6.25
			6819.21 ± 7.46
	7852.49 ± 8.28	**7852.91 ± 8.28**	
			7922.93 ± 8.34
			8719.65 ± 8.98
	10643.90 ± 10.52		
LMG 1519	**4895.25 ± 5.92**		**4894.64 ± 5.92**
		5035.04 ± 6.03	
		5395.70 ± 6.32	
		5934.74 ± 6.75	
			9795.83 ± 9.84
LMG 1581		5267.04 ± 6.21	
			6608.63 ± 7.29
			7254.69 ± 7.80
	7369.34 ± 7.90		
			7741.57 ± 8.19
	7829.83 ± 9.86		
		9827.70 ± 9.86	**9828.18 ± 9.86**
	10770.50 ± 10.62		**10770.30 ± 10.62**
LMG 1674			2911.32 ± 4.33
	5394.77 ± 6.32		
	7676.08 ± 8.14		**7675.78 ± 8.14**
	9967.32 ± 9.97		
	10797.20 ± 10.64		
LMG 1676		3681.46 ± 4.95	
			3728.02 ± 4.98
			3894.66 ± 5.12
		4082.31 ± 5.27	
	5591.13 ± 5.47	**5591.62 ± 6.47**	**5591.79 ± 6.47**
	5954.07 ± 6.76		5954.59 ± 6.76
	6239.26 ± 6.99	6239.71 ± 6.99	6239.69 ± 6.99
		6652.23 ± 7.32	
			6904.59 ± 7.52
	7255.28 ± 7.80		
		7395.64 ± 7.92	
	7796.98 ± 8.24	**7796.61 ± 8.24**	**7796.76 ± 8.24**
	7867.55 ± 8.29	**7867.55 ± 8.29**	**7867.43 ± 8.29**
	7938.71 ± 8.35		
			8170.35 ± 8.54
			9812.64 ± 9.85
		12487.6 ± 11.99	**12488.40 ± 11.99**
LMG 1683		3309.27 ± 4.65	
		3970.36 ± 5.18	
		5334.88 ± 6.27	
		6679.51 ± 7.34	
	6956.09 ± 7.56		
	7905.84 ± 8.32		
	14902.00 ± 13.92		

[a] The strain-specific peak classes common to the strain-specific SSPs of bacteria grown on two or three different culture media are in bold.

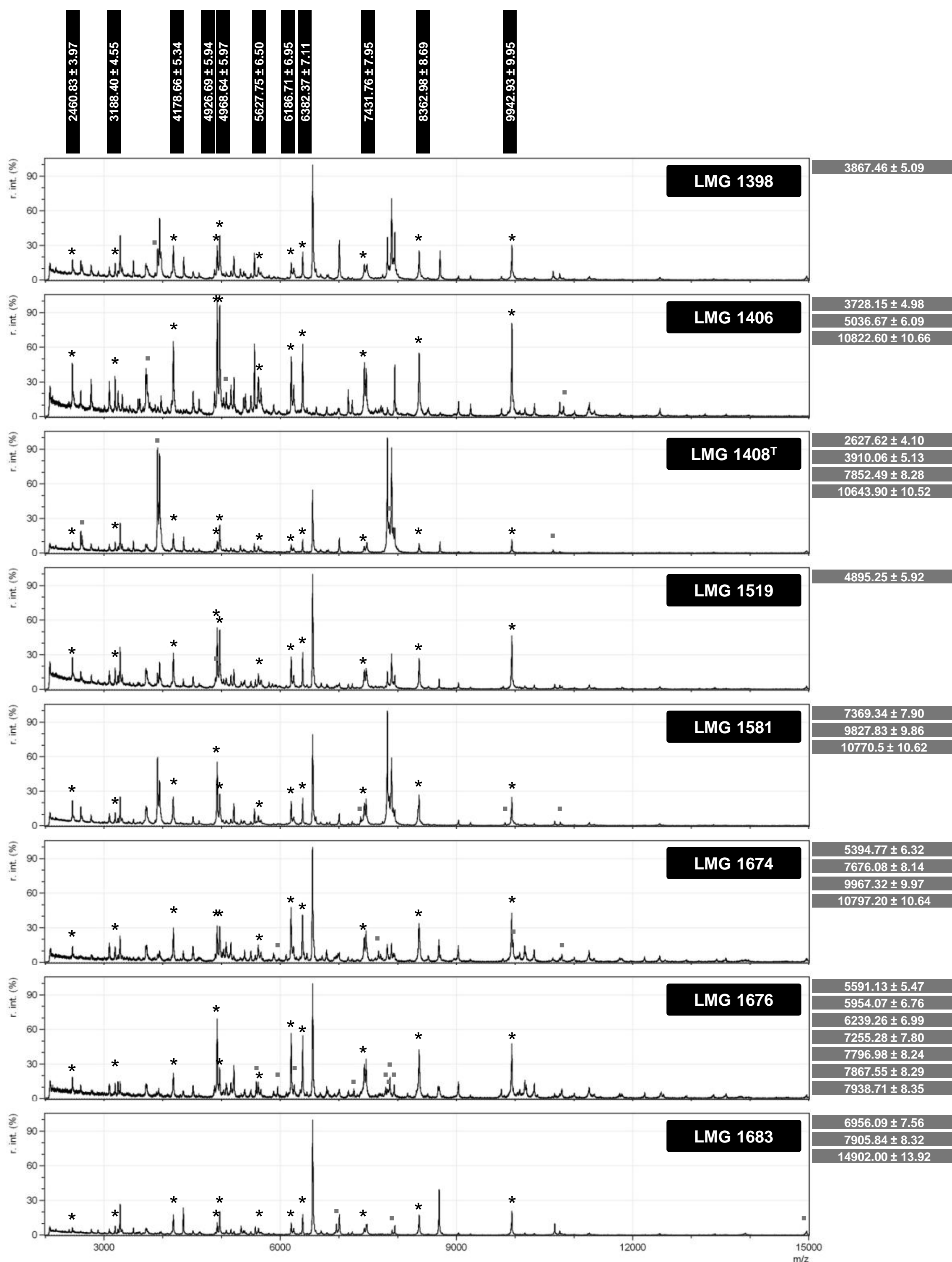

FIG 4.4. Mass spectra generated for eight *G. oxydans* strains grown on AAM agar. The shared and strain-specific peak classes are indicated by asterisks and squares, respectively. Annotations of the peak classes are shown in black or grey boxes for, respectively, the common or strain-specific peaks. r. int., relative intensity.

4.3.3 *A growth medium-dependent core set of peak classes decreases with increasing number of strains*

During the analysis of strain-specific peak classes the number of shared peak classes appeared to decrease when more strains were examined. To investigate this in more detail, the SSPs of the eight *G. oxydans* strains grown on GY, AAM, and YPM agar were re-analysed. Per growth medium, the effect of a sequential inclusion of an SSP of an additional strain was analysed in all possible combinations. Subsequently, the number of shared peak classes was determined for all of the possible combinations and plotted as a function of the number of strain SSPs sequentially added to the data set. The plots represented in **FIG 4.5** visualise this decrease in shared peak classes with increasing numbers of strain SSPs. The lowest set of shared peak classes [8 (9%) out of 88] was found when the eight strains were grown on YPM agar, rather than on AAM [11 (13%) out of 85], or GY agar [12 (15%) out of 79] (**TABLE 4.1**). Seven out of 105 peak classes were common to the SSPs of the strains grown on the three media (**TABLE 4.1**). The same study was performed for the five *A. cerevisiae* strains grown on four culture media. Although only five strains were examined, a similar decrease in shared peak classes was observed after growth on AAM, DMS, YPM, and GY agar (see **FIG S 4.2** in the supplementary material).

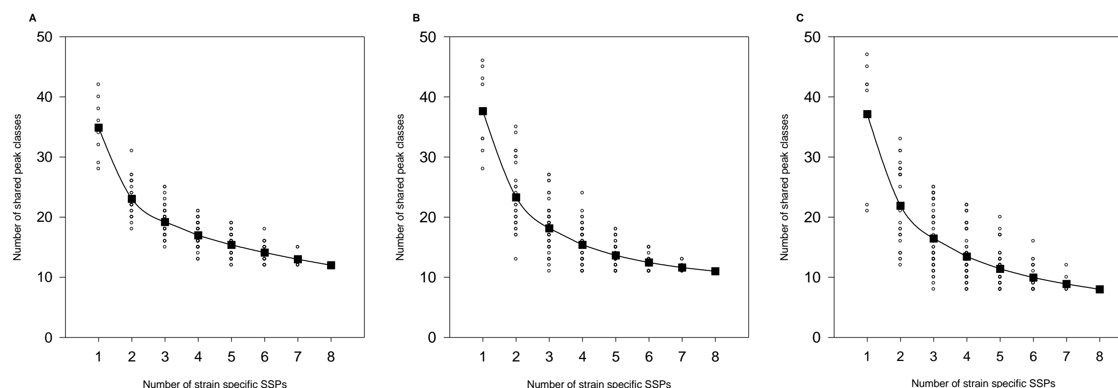

FIG 4.5. The decrease in shared peak classes among SSPs of eight *G. oxydans* strains grown on GY (**A**), AAM (**B**), and YPM agar (**C**). The number of shared peak classes is plotted as a function of the number of strain-specific SSPs sequentially added. Squares represent the average of shared peak classes per sequential addition of a strain-specific SSP.

These data suggest that there is a core set of peak classes that is consistently present when multiple strains of a species are examined and that this core is culture medium-dependent. The data confirm that multiple reference strains grown on multiple growth media should always be analysed to cover the intraspecies diversity when constructing a MALDI-TOF MS identification database [35]. It also demonstrates that the decrease in shared peak classes can be used as a measure of taxonomic intraspecies diversity.

4.3.4 *Species level differentiation is growth medium-independent and is not biased by similarity coefficient used*

The MDS plots derived from the peak-based numerical analysis as visualised in **FIG 4.1** demonstrated that most of the AAB species were clearly distinguishable, irrespective of the growth medium used. One exception, however, is *A. malorum* strain LMG 1746^T, which could not be distinguished from the *A. cerevisiae* strains (**FIG 4.1**). Similar results were obtained in the study by Andrés-Barrao *et al.* [5]. It is well known that these two species are very closely related and can only be distinguished by minimal differences revealed primarily by sequence analysis of the 16S-23S rRNA gene internal transcribed spacer region [36, 37]. In addition, two strains, *i.e.*, *A. pasteurianus* LMG 1590 and *G. cerinus* strain LMG 1678, did not group with the other strains of these respective species (**FIG 4.1**). *Gluconobacter cerinus* LMG 1678 had only 2 out of 96 peak classes in common with other *G. cerinus* strain SSPs, suggesting that it may be erroneously assigned to this species. The latter was confirmed by sequence analysis of the housekeeping genes *rpoB* and *dnaK* as described by Cleenwerck *et al.* [38], and strain LMG 1678 should therefore be considered as misclassified (A.D. Wieme, **CHAPTER 5.1**). Similarly, *A. pasteurianus* strain LMG 1590 also grouped separately and had only 5 out of 109 *A. pasteurianus* SSP peak classes in common, which confirms the established taxonomic diversity of this species [39].

In addition to the peak-based analysis using the binary Dice coefficient, the same data set was also analysed using the Pearson product-moment correlation coefficient, taking into account the complete molecular mass range (2-20 kDa) and the averaged signal intensities. In general, the MDS plots were similar to those based on the comparison of SSPs by means of the binary Dice coefficient and thus facilitate species differentiation with the same exceptions discussed above (**FIG 4.6**). The MSVs obtained through the curve-based numerical analysis of the 11 *Acetobacter* strain SSPs ranged from 44.7 to 92.0% with an average of 68.4% ± 13.6%. For the SSPs of the *Gluconacetobacter* and the *Gluconobacter* strains the MSVs ranged from 65.0 to 94.4% with an average of 81.9% ± 11.3%.

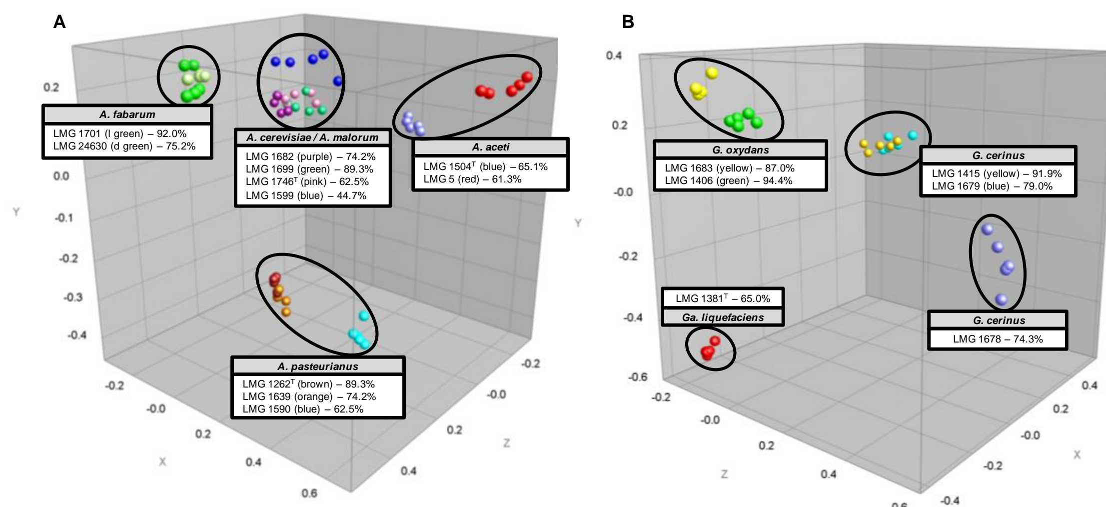

FIG 4.6. MDS plots obtained after curve-based numerical analysis of the generated SSPs of 11 *Acetobacter* strains (**A**) and 1 *Gluconacetobacter* (*Ga.*) and 5 *Gluconobacter* (*G.*) strains (**B**) grown on five different culture media. For each strain, single-coloured dots represent the SSPs derived from growth on five different culture media. The MSVs among each specific strain's SSPs are shown within the respective boxes.

In general, the curve-based analysis yielded more variability between strain-specific SSPs obtained after growth on different culture media than did peak-based analysis. However, curve-based analysis of some strains like *A. pasteurianus* LMG 1262^T, yielded MSV values among SSPs that were higher than those obtained by peak-based analysis (MSV of 89.3% compared to 65.2%), and this was related to errors or problems during the peak class assignment. For instance, in *A. pasteurianus* LMG 1262^T SSPs the peak characterised by *m/z* 7211.49 ± 7.77, which was present in the YPM-derived SSP, was not assigned to the *m/z* 7197.90 ± 7.76 peak class (**FIG 4.7**; see **FIG S 4.3** in the supplementary material). Also, a double peak that was present in the *G. oxydans* strain LMG 1406 SSPs (see **FIG S 4.1** in the supplementary material) was not consistently annotated during preprocessing because of the limited resolution achieved by MALDI-TOF MS analysis. This double peak was therefore not detected during summarization and had no influence on the peak-based numerical analysis (MSV of 78.5%), while it was accounted for in the curve-based analysis (MSV of 94.4%).

In conclusion, the present study demonstrates that the culture medium can have a profound effect on the mass spectra of AAB, as observed in the presence and varying signal intensity of peak classes. Growth media that do not sustain optimal growth can influence the SSP strongly. The growth medium effects do not disturb species level differentiation but strongly affect the potential for strain level differentiation. A decrease in the number of shared peak classes was observed with an increasing number of strains examined and may be indicative of the degree of intraspecies diversity. Altogether, our data demonstrate that a well-constructed and robust MALDI-TOF MS identification database ideally comprises mass spectra of multiple reference strains per species grown on different culture media.

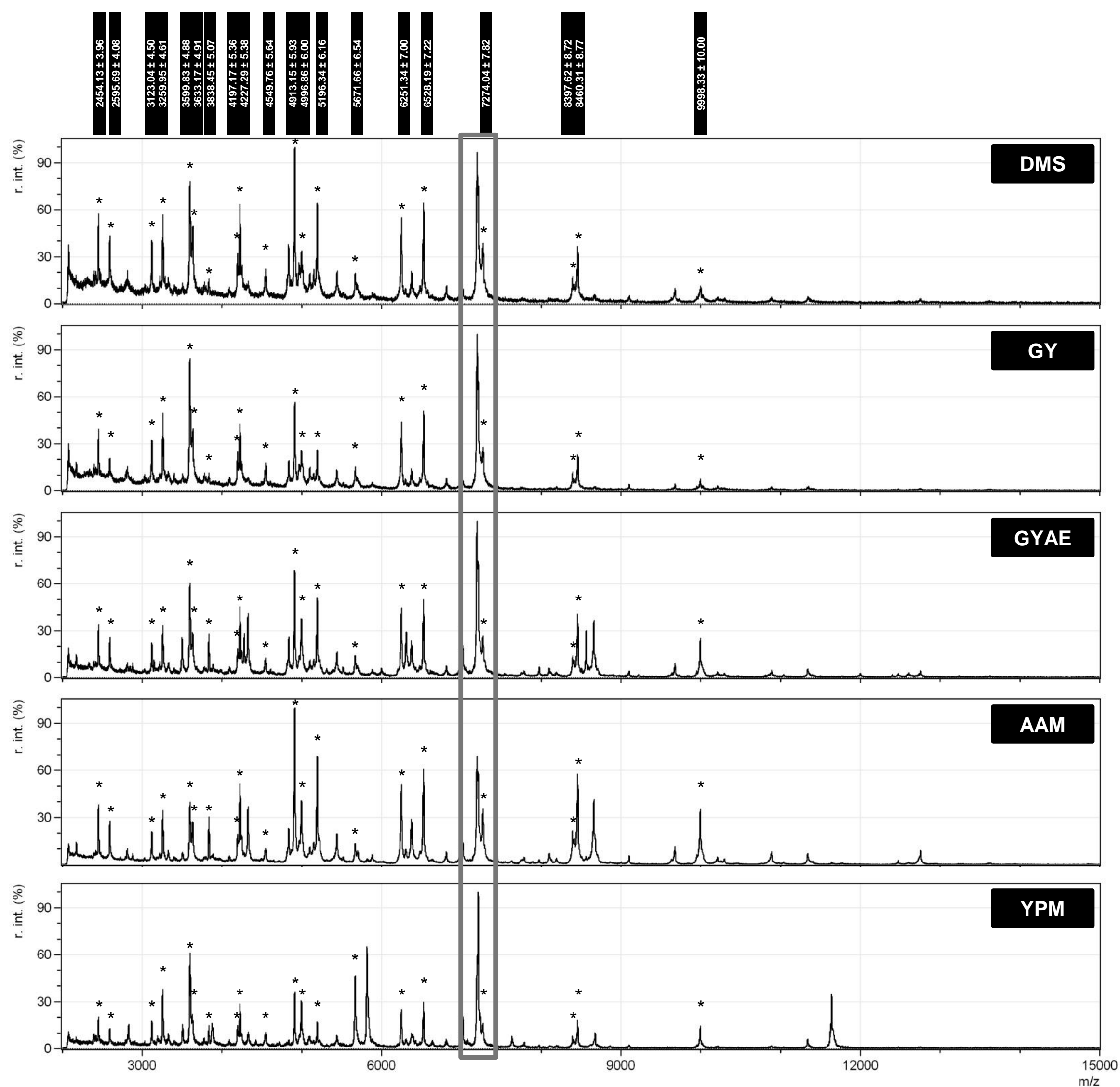

FIG 4.7. Mass spectra of *A. pasteurianus* strain LMG 1262ᵀ grown on five different culture media. The shared peak classes are indicated by asterisks and annotated in the black boxes at the top. The rectangle highlights the peak that shifted in the mass spectrum derived from the YPM SSP and that was erroneously missed by the peak detection algorithm. r. int., relative intensity.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

TABLE S 4.1. List of 25 acetic acid bacteria examined in the present study.

Species	Strain	Isolation source (geographical origin)
Acetobacter aceti	LMG 5	Beer (Belgium)
	LMG 1504[T]	Beech-w ood shavings of vinegar plant
Acetobacter cerevisiae	LMG 1545[a]	Film in a rice vinegar fermentor (Japan, Aichi)
	LMG 1599	Brew ing yeast (United Kingdom)
	LMG 1625[Ta]	Ale beer in storage (Canada, Toronto)
	LMG 1682	Beer (Ireland)
	LMG 1699	Brew ery (United Kingdom)
Acetobacter fabarum	LMG 1701	Beerw ort (South Africa, Alberton)
	LMG 24630	Kefir grains (Belgium, Gembloux)
Acetobacter malorum	LMG 1746[T]	Rotting apple (Belgium, Ghent)
Acetobacter pasteurianus	LMG 1262[T]	Beer (The Netherlands)
	LMG 1590	Not know n
	LMG 1639	Beer (The Netherlands)
Gluconacetobacter liquefaciens	LMG 1381[T]	*Diospyro* sp., dried fruit (Japan)
Gluconobacter cerinus	LMG 1415	Beer (The Netherlands)
	LMG 1678	Amstel beer (The Netherlands, Leiden)
	LMG 1679	Amstel beer (The Netherlands, Leiden)
Gluconobacter oxydans	LMG 1398[a]	Beer (United Kingdom)
	LMG 1406	Ropy beer
	LMG 1408[Ta]	Beer
	LMG 1519[a]	Ropy sample of top fermented beer (United Kingdom)
	LMG 1581[a]	Beer (The Netherlands, Delft)
	LMG 1674[a]	Beer (The Netherlands, Delft)
	LMG 1676[a]	Amstel Beer (Belgium, Leuven)
	LMG 1683	Beer (Ireland)

[a] Strains that failed to grown on one culture medium (*A. cerevisiae*) and on two or more culture media (*G. oxydans*).
LMG: BCCM/LMG Belgian Co-ordinated Collections of Microorganisms/Laboratory of Microbiology Ghent University, Ghent (Belgium); other culture collection number can be retrieved from http://www.straininfo.net/.

TABLE S 4.2. Peak classes common to SSPs derived from five *A. cerevisiae* strains grown on AAM, DMS, YPM and GY agar[a].

	Peak class			
m/z values	AAM[b]	DMS[c]	YPM[d]	GY[e]
2079.40 ± 3.66	X			
2453.48 ± 3.96	X	X	X	X
2610.03 ± 4.09	X	X	X	
3122.41 ± 4.50	X	X	X	X
3625.49 ± 4.90	X	X		X
3703.30 ± 4.96	X	X		X
3885.30 ± 5.11	X			X
4162.94 ± 5.33				X
4565.33 ± 5.65				X
4855.27 ± 5.88	X			
4912.30 ± 5.93	X	X	X	X
4952.00 ± 5.96	X	X	X	
5090.62 ± 6.07	X			X
5225.61 ± 6.18	X	X	X	
5469.87 ± 6.38	X	X		X
6250.41 ± 7.00	X	X	X	X
6293.57 ± 7.03	X	X		X
6825.92 ± 7.46	X			
7062.08 ± 7.65	X	X	X	X
7256.65 ± 7.81	X	X	X	X
7412.25 ± 7.93	X	X	X	X
8330.88 ± 8.66	X	X		X
9317.74 ± 9.45				X
9716.15 ± 9.77	X			
9908.97 ± 9.93	X	X	X	X
10188.10 ± 10.15	X			

[a] Only the peak classes marked with an X are present in the respective medium, and the *m/z* values of the peak classes common to all of the SSPs are in bold (8/142 [6%])

[b] 23 (22%) out of 103 peak classes shared

[c] 16 (18%) out of 91 peak classes shared

[d] 11 (10%) out of 106 peak classes shared

[e] 18 (17%) out of 104 peak classes shared

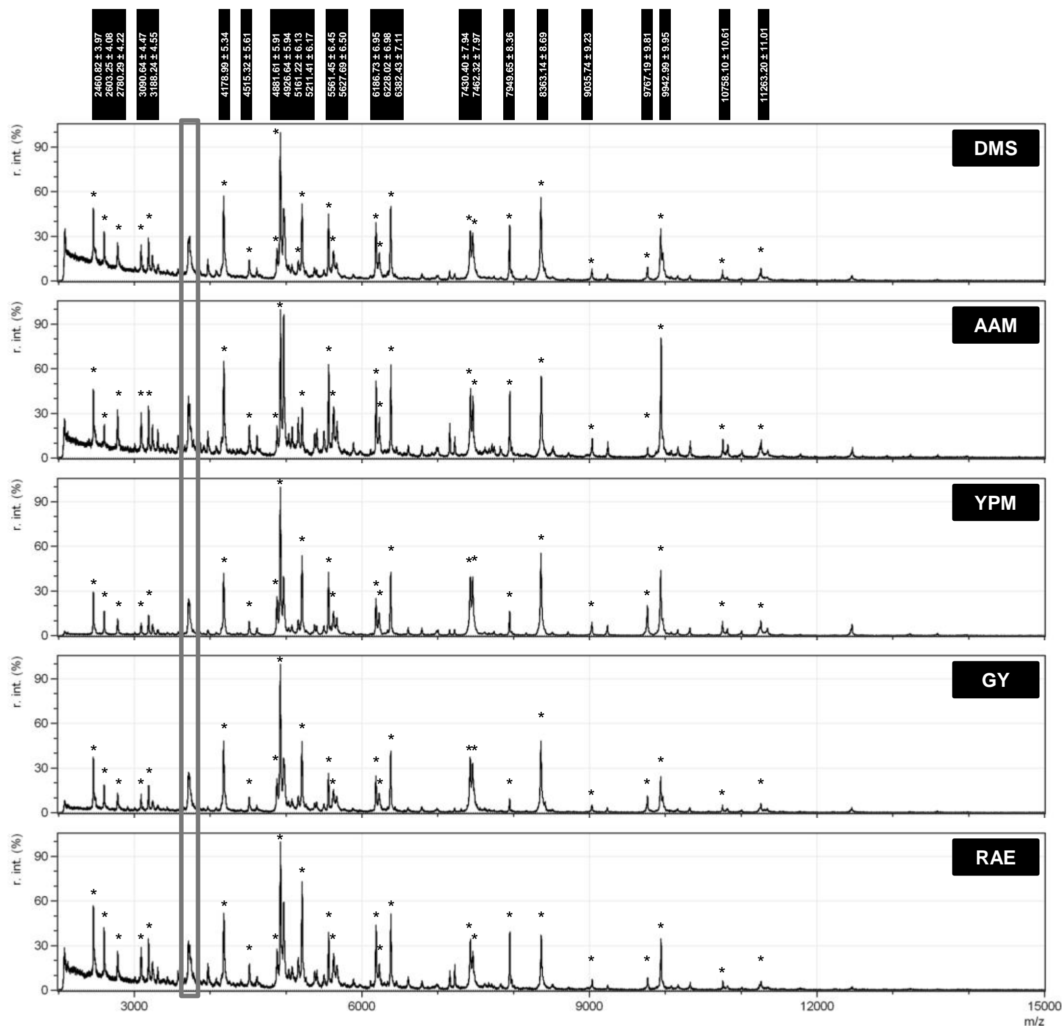

FIG S 4.1. Mass spectra generated from *G. oxydans* strain LMG 1406 grown on five different culture media. The common peak classes (25 [48%] out of 52) are indicated by asterisks and their corresponding *m/z* values are shown in black boxes at the top. The rectangle highlights the ambiguous double peak observed in all mass spectra as is further discussed within the manuscript (see **FIG S 4.3** in the supplementary material). r. int., relative intensity.

TABLE S 4.3. Strain-specific peak classes found in SSPs derived from five *A. cerevisiae* strains cultured on four growth media[a].

	m/z value for indicated peak class			
Strain	AAM	DMS	YPM	GY
LMG 1545		3390.86 ± 4.71		
		3545.56 ± 4.84		
	3987.20 ± 5.19	**3987.26 ± 5.19**		**3987.39 ± 5.19**
		4684.11 ± 5.75		
			4740.66 ± 5.79	**4741.76 ± 5.79**
		5638.46 ± 6.51		
		6895.26 ± 7.52		
			7168.12 ± 7.73	
	7980.43 ± 8.38	**7981.74 ± 8.39**		**7980.67 ± 8.38**
			8498.17 ± 8.80	
			9486.38 ± 9.59	**9486.92 ± 9.59**
			9678.32 ± 9.74	**9678.02 ± 9.74**
		11282.10 ± 11.03		
LMG 1599	2372.27 ± 3.90			
	3277.53 ± 4.62	**3277.27 ± 4.62**		**3277.80 ± 4.62**
				6479.92 ± 7.18
	6560.23 ± 7.25	**6559.38 ± 7.25**	**6559.68 ± 7.25**	**6561.02 ± 7.25**
	7127.58 ± 7.70	**7125.88 ± 7.70**	**7126.46 ± 7.70**	**7128.02 ± 7.70**
	8412.28 ± 8.73			
LMG 1625[T]				2138.37 ± 3.71
	5446.19 ± 6.36	**5446.26 ± 6.36**	**5446.28 ± 6.36**	**5448.23 ± 6.36**
				6926.59 ± 7.54
	10899.40 ± 10.72	**10899.50 ± 10.72**	**10900.40 ± 10.72**	**10902.30 ± 10.72**
LMG 1682			3041.47 ± 4.43	
				4748.45 ± 5.80
	5277.20 ± 6.22		**5276.76 ± 6.22**	
	5345.28 ± 6.28	**5345.71 ± 6.28**	**5346.98 ± 6.28**	**5345.99 ± 6.28**
				5800.40 ± 6.64
	5826.21 ± 6.66	**5826.41 ± 6.66**	**5827.02 ± 6.66**	**5826.21 ± 6.66**
	6441.78 ± 7.15			
				8152.66 ± 8.52
				9693.86 ± 9.76
				10077.30 ± 10.06
	10696.60 ± 10.56	**10697.90 ± 10.56**	**10700.10 ± 10.56**	**10699.10 ± 10.56**
			10727.70 ± 10.58	
LMG 1699	**2250.10 ± 3.80**			**2250.34 ± 3.80**
	2849.32 ± 4.28			
			2864.11 ± 4.29	
		3196.58 ± 4.56	**3196.52 ± 4.56**	**3196.67 ± 4.56**
	3941.24 ± 5.15			
	4229.34 ± 5.38			
	4300.51 ± 5.44	**4300.25 ± 5.44**	**4300.90 ± 5.44**	**4300.62 ± 5.44**
	5335.27 ± 6.27	**5335.03 ± 6.27**	**5335.19 ± 6.27**	**5335.56 ± 6.27**
		5906.59 ± 6.73	**5906.81 ± 6.73**	
	5712.78 ± 6.57			
	6210.96 ± 6.97			
	6365.91 ± 7.09	**6366.61 ± 7.09**	**6368.73 ± 7.09**	**6366.66 ± 7.09**
	6396.83 ± 7.12	**6398.98 ± 7.12**	**6398.55 ± 7.12**	**6399.63 ± 7.12**
	6573.83 ± 7.26	**6573.77 ± 7.26**	**6573.55 ± 7.26**	**6575.52 ± 7.26**
	6945.09 ± 7.56		**6946.15 ± 7.56**	
	7890.27 ± 8.31	**7890.00 ± 8.31**	**7888.95 ± 8.31**	**7890.39 ± 8.31**
	7916.58 ± 8.33			
	8461.93 ± 8.77			
	8511.24 ± 8.81			
			8660.28 ± 8.93	
			10503.50 ± 10.40	
	10675.40 ± 10.54	**10675.10 ± 10.54**	**10675.80 ± 10.54**	**10677.30 ± 10.54**
	11366.80 ± 11.09			
	12737.00 ± 12.19	**12737.40 ± 12.19**	**12738.80 ± 12.19**	**12738.30 ± 12.19**
	12870.20 ± 12.30	**12870.80 ± 12.30**		**12872.60 ± 12.30**

[a]The strain-specific peak classes common to the strain-specific SSPs of bacteria grown on two or three different culture media are in bold

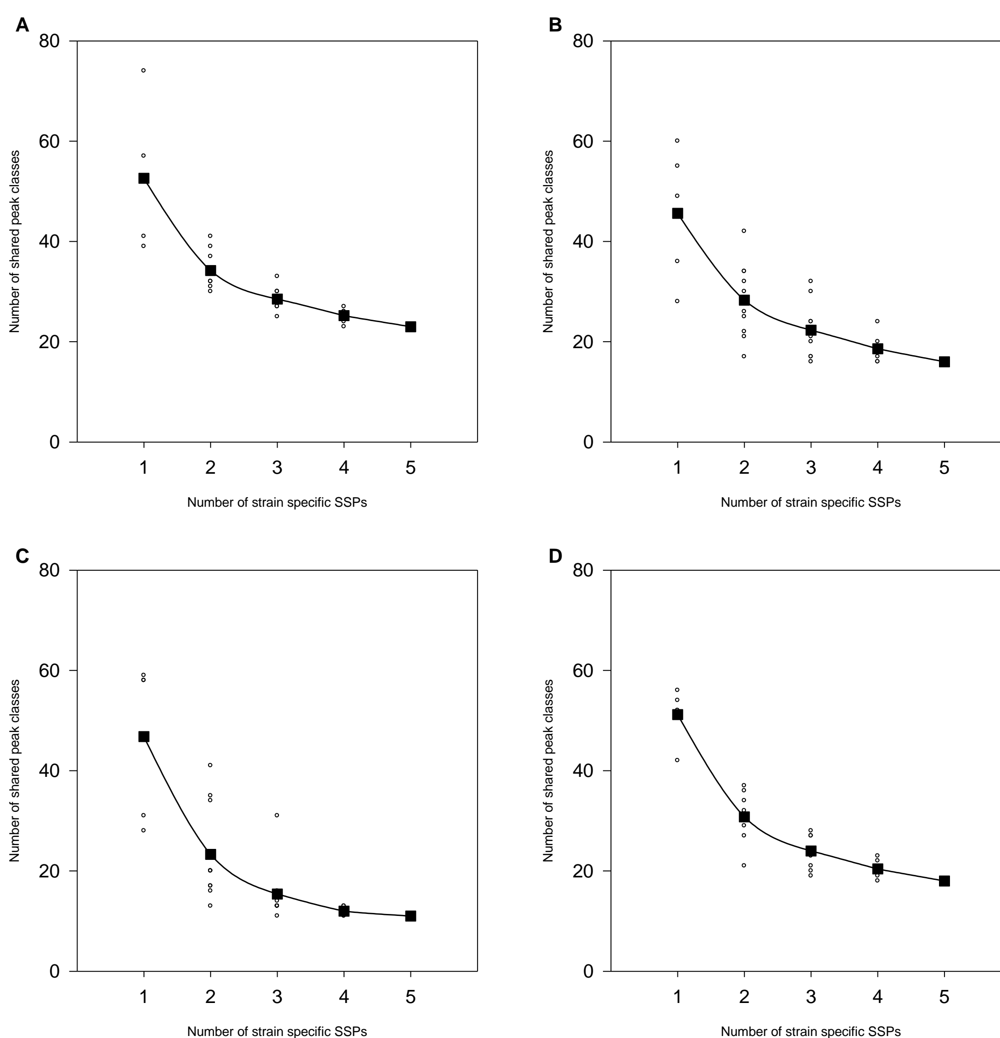

FIG S 4.2. The decrease in shared peak classes among SSPs of five *A. cerevisiae* strains grown on AAM (**A**), DMS (**B**), YPM (**C**), and GY agar (**D**). The number of shared peak classes is plotted as a function of the number of strain-specific SSPs sequentially added. Squares represent the average of shared peak classes per sequential addition of a strain-specific SSP.

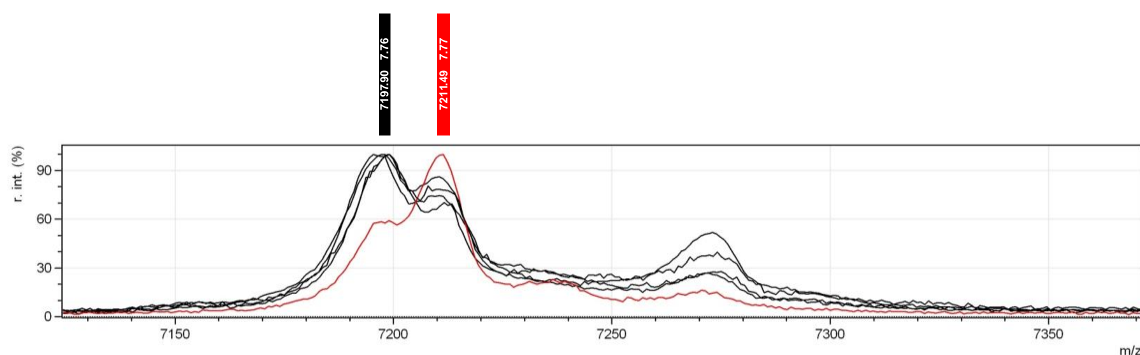

FIG S 4.3. A detailed representation of the spectrum shown in **FIG 4.7** in the manuscript focusing on the peak classes with *m/z* values of 7197.90 ± 7.76 and 7211.49 ± 7.77. Peak class *m/z* 7211.49 ± 7.77 is only present in the SSP derived from *A. pasteurianus* strain LMG 1262^T grown on YPM agar (red mass spectrum). A mass difference of approximately 14 Da ensured the incorrect assignment of that peak to the additional peak class (*m/z* 7197.90 ± 7.76, black box) present in the SSPs derived from the strain grown on the other four culture media (black mass spectra). r. int., relative intensity.

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5 Identification of beer spoilage bacteria using MALDI-TOF MS

5.1 Identification of bacteria isolated from spoiled beer and brewery samples using MALDI-TOF MS

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Author contributions: conceived and designed the experiments: ADW, FS, AVL and PV; performed the experiments and data analyses: ADW; contributed data analyses tools: FS, MA and KDB; wrote the manuscript: ADW; critically reviewed the manuscript: FS, MA, KDB, AVL and PV.

SUMMARY

Applicability of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for identification of beer-spoilage bacteria was examined. To achieve this, an extensive identification database was constructed comprising more than 4200 mass spectra, including biological and technical replicates derived from 273 acetic acid bacteria (AAB) and lactic acid bacteria (LAB), covering a total of 52 species, grown on at least three growth media. Sequence analysis of protein coding genes was used to verify aberrant MALDI-TOF MS identification results and confirmed the earlier misidentification of 34 AAB and LAB strains. In total, 348 isolates were collected from culture media inoculated with 14 spoiled beer and brewery samples. Peak-based numerical analysis of MALDI-TOF MS spectra allowed a straightforward species identification of 327 (94.0%) isolates. The remaining isolates clustered separately and were assigned through sequence analysis of protein coding genes either to species not known as beer-spoilage bacteria, and thus not present in the database, or to novel AAB species. An alternative, classifier-based approach for the identification of spoilage bacteria was evaluated by combining the identification results obtained through peak-based cluster analysis and sequence analysis of protein coding genes as a standard. In total, 263 out of 348 isolates (75.6%) were correctly identified at species level and 24 isolates (6.9%) were misidentified. In addition, the identification results of 50 isolates (14.4%) were considered unreliable, and 11 isolates (3.2%) could not be identified. The present study demonstrated that MALDI-TOF MS is well-suited for the rapid, high-throughput and accurate identification of bacteria isolated from spoiled beer and brewery samples, which makes the technique appropriate for routine microbial quality control in the brewing industry.

5.1.1 *Introduction*

Beer is a beverage with good microbiological stability because it contains almost no oxygen and nutrients for bacterial growth. In addition, low pH, high CO_2-content and the presence of ethanol and antibacterial hop compounds ensure microbiological stability [1-5]. Nevertheless, beer spoilage due to bacteria is a common problem in the brewing industry and causes important economic losses worldwide [6, 7]. The most problematic organisms are lactic acid bacteria (LAB) and acetic acid bacteria (AAB) which are the focus of the present study. The brewing process itself is prone to bacterial growth because of the nutrient-rich environment of wort which comprises fermentable sugars and amino acids, and vitamins produced by the pitching yeast during fermentation [8]. Low cell numbers (*i.e.*, 1 to 50 CFU per 100 to 250 mL sample) of a spoilage bacterium can cause visible turbidity [9], acidity and off-flavours [7]. At present, the hop-resistant lactic acid bacteria *Lactobacillus backii*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus coryniformis*, *Lactobacillus lindneri*, *Lactobacillus malefermentans*, *Lactobacillus parabuchneri*, *Lactobacillus plantarum*, *Pediococcus claussenii*, *Pediococcus damnosus* and *Pediococcus inopinatus* are generally regarded as the most problematic beer-spoilage microorganisms [10, 11]. The prevalence of strictly aerobic spoilage bacteria has decreased because of improved process technology resulting in lower oxygen levels during filling [8]. However, in aerobic environments, acetic acid bacteria, such as *Acetobacter* spp. and *Gluconobacter* spp., can prevail and form biofilms in which other spoilage bacteria are protected [12, 13]. During the last two decades, anaerobic Gram negative bacteria like *Pectinatus*, *Megasphaera* and *Zymophilus* species have gained importance as spoilage bacteria in the brewing industry [7, 8, 14, 15].

In order to anticipate or to prevent further spoilage, it is necessary to detect and identify detrimental bacteria in a fast and easy manner. Currently, beer-spoilage bacteria are primarily detected by plating on selective isolation media [16-20], an approach that is time-consuming and neither sensitive nor specific [21, 22]. Also, subsequent characterization and identification of spoilage bacteria is commonly carried out via phenotypic tests (Gram staining, cell morphology and sugar metabolism), but these tests are again time-consuming and lack specificity [21]. Consequently, quality control requires lengthy storage of beer, which increases brewing costs and demand for storage capacity [23].

Many culture-dependent and culture-independent detection and/or identification techniques for beer-spoilage bacteria have been developed. These include species-specific oligonucleotide probes [23-25], PCR-based methods [26-32], random amplified polymorphic DNA (RAPD) fingerprinting [33, 34], ribotyping [35-38], sodium dodecyl sulphate-polyacrylamide gel electrophoresis of whole-cell

proteins [39-41] and the use of monoclonal antibodies [42-44]. However, beer-spoilage bacteria are taxonomically diverse while most of these methods specifically target one or a narrow range of beer-spoilage species; furthermore other methods are time-consuming and therefore lack throughput capacity and do not facilitate real-time interventions.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has already been introduced as a high-throughput tool for species level identification in medical, environmental and food-related studies [45-50]. The present study aimed to evaluate MALDI-TOF MS as an alternative identification tool for the most prevalent beer-spoilage bacteria, by constructing an identification database and evaluating its applicability for the identification of novel spoilage bacteria. The accuracy of the identification results was verified using sequence analysis of protein coding genes. The latter approach has a superior taxonomic resolution to distinguish between closely related species [51, 52].

5.1.2 *Materials and Methods*

5.1.2.1 *Bacterial strains*

A total of 273 strains representing 52 species belonging to the groups of AAB (n = 50) and LAB (n = 223) (see **ANNEX 1**) which represent established or potential beer-spoilage bacteria were included in the database. These 273 strains included strains originating from spoiled beer or other brewery samples, but also from other niches where these same species occur in order to encompass the intraspecies phenotypic diversity wherever possible. The strains were obtained from the BCCM/LMG Bacteria Collection (http://www.belspo.be/bccm/; Ghent, Belgium), and from our own research collection and were cultured according to the providers' instructions, and on additional selective and non-selective growth media. Many of the strains were type and other taxonomically well-characterised reference strains; others were minimally characterised in previous studies. Some of the strains examined proved to be misclassified; therefore, the names of such misidentified strains will be written in square brackets below. In order to construct the identification database *Acetobacter* strains were grown on five different media: acetic acid medium (AAM) agar [53]; deoxycholate-mannitol-sorbitol (DMS) medium [54]; GY medium (5.0% (w/v) D-glucose, 1.0% (w/v) yeast extract and 1.5% (w/v) agar); GYAE medium (GY medium supplemented with 1.0% (v/v) acetic acid and 2.0% (v/v) ethanol) and YPM medium (0.5% (w/v) yeast extract, 0.3% (w/v) bacteriological peptone, 2.5% (w/v) D-mannitol, and 1.5% (w/v) agar). *Gluconobacter*, *Gluconacetobacter* and *Komagataeibacter* strains were cultured on the same media, with the exception of GYAE medium which was replaced by reinforced AE (RAE) medium (4.0% (w/v) D-glucose, 1.0% (w/v) yeast extract, 1.0% (v/v) acetic acid, 2.0% (v/v) ethanol, 1.0% (w/v) bacteriological peptone, 0.34% (w/v) $Na_2HPO_4.2H_2O$ and 0.15% (w/v) citric acid, a layer of 1.0% (w/v) agar is topped with a layer of medium containing 2.0% (w/v) agar) [55]. *Lactobacillus* and *Pediococcus* strains were cultured on three different media: de Man, Rogosa and Sharpe (MRS) medium (Oxoid), Nachweismedium für bierschädliche Bakterien (NBB) (1:1 lager pilsner beer:water) medium (Conda Pronadisa) and Raka-Ray medium (Oxoid).

5.1.2.2 *Sample preparation and MALDI-TOF MS data acquisition*

After resuscitating lyophilised and cryopreserved cultures, strains were subcultured twice on each growth medium and cell extracts were prepared from the subsequent 3rd, 4th and the 5th generation. Hence, variation induced during the adaptation to the growth medium was excluded and three biological replicates (*i.e.*, three generations) were included. Five mg of wet cells taken from one to ten single colonies were suspended into 300 µL Milli-Q water after which 900 µL pure ethanol was added. After centrifugation (3 minutes, 20817 × g, 4°C), 50 µL 70% formic acid and 50 µL acetonitrile were added to the bacterial cell pellet. After shaking vigorously and centrifugation (3 minutes, 20817 × g, 4°C), 1 µL of the supernatant (= the cell extract) was spotted in duplicate onto a MALDI-TOF MS stainless steel target plate to obtain two technical replicates. Immediately after drying, the spots were overlaid with 1 µL matrix solution, which consisted of 5 mg α-cyano-4-hydroxycinnamic acid dissolved in 1 mL acetonitrile/trifluoroacetic acid/Milli-Q water solvent (50:2:48). Prior to analysis the mass spectrometer was externally calibrated using a peptide mixture of adrenocorticotropic hormone (fragment 18-39) (Sigma-Aldrich), insulin (Sigma-Aldrich), ubiquitin (Sigma-Aldrich), cytochrome C (Sigma-Aldrich) and myoglobin (Sigma-Aldrich). A 4800 Plus MALDI TOF/TOFTM Analyzer (AB Sciex) was used in the linear mode and covered a mass range from 2 to 20 kDa. The mass spectrometer used a 200 Hz frequency tripled Nd:YAG laser, operating at a wavelength of 355 nm. Generated ions were accelerated at 20 kV through a grid at 19.2 kV into a 1.5 m, linear, field-free drift region towards the detector. For each spot, 40 sub-spectra each consisting of 50 laser shots, and from randomised positions within the spot were collected and presented as one spectrum (2000 laser shots in total), which will further be referred to as the spectral profile. The laser intensity was set between 4200 and 5700 procedure defined units. With every set of measurements, Bacterial Test Standard (Bruker Daltonics) was included as positive control.

5.1.2.3 *MALDI-TOF MS data analysis*

The mass spectra were retrieved as t2d files from the 4800 Plus MALDI TOF/TOF™ Analyzer via the 4000 Series Explorer software. Data Explorer 4.0-software (AB Sciex) was used to convert the t2d files into text files that were subsequently used as input files for the BioNumerics 7.1 software package (Applied Maths). A spectral profile was considered of acceptable quality if the absolute signal intensity of the highest peak was > 500 counts, if more than 5 peaks with a signal/noise ratio (S/N) > 20 were detected in the 3-20 kDa range and if there were no repetitive signals in the 2.1-3 kDa range. The import of the spectral data in BioNumerics 7.1 was conducted using an optimised preprocessing template including consecutive Continuous Wavelet Transform (CWT) noise estimation, a Savitsky-Golay filter smoothing and a baseline subtraction using the rolling disk algorithm [56]. After pre-processing, each peak with a S/N ratio ≥ 5 and an absolute intensity of at least 6 counts was annotated.

The peak-based data analysis matches all peaks in the mass spectra to a peak class using constant and linearly varying tolerance values of 2 *m/z* and 800 ppm, respectively [56]. The obtained dataset was converted into a binary character set and analyzed using the Dice coefficient and the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster algorithm. As a second approach, curve-based data analysis of mass spectra was performed using the Pearson product-moment correlation coefficient and the UPGMA cluster algorithm. The stability of the obtained cluster(s) was determined via re-sampling of the dataset with an automated Jackknife test [56]. This test uses the 'leave one out' principle, where one entry is removed from the dataset and is re-identified by calculating the average similarity towards each of the species-specific cluster(s). The percentage of true positive hits, *i.e.*, the

percentage of entries that were re-identified to the group they were originally assigned to, is a measure for the stability (consistency) of the species-specific cluster(s) [56] and for the identification potential of the method.

5.1.2.4 *Isolation of beer spoilage bacteria*

Fourteen spoiled beer or brewery samples were retrieved from Belgian breweries: nine samples from finished beer (eight bottles and one kegged), 4 samples collected from pitching yeast cultures and one wort sample (**TABLE 5.1**). Prior to isolation, all samples were checked microscopically for the presence of yeast cells and filtered. When yeast cells were present, the filtration was conducted over a set of three consecutive filters: 5 µm, 8 µm and 0.45 µm (cellulose nitrate, Whatman). Yeast cells were primarily retained on the first two filters, bacterial cells on the last. If no yeast cells were present, only the 0.45 µm filter was used to concentrate the bacterial cells. After filtration, the 0.45 µm filter was cut in three pieces which were submerged in three enrichment media: AAM broth to enrich AAB, MRS broth (Oxoid) to enrich LAB [20] and Nachweismedium für bierschädliche Bakterien (NBB) broth (1:1 lager pilsner beer/water) (Conda Pronadisa) to enrich both AAB and LAB. If yeast cells were initially present, cycloheximide and amphotericin B were added after sterilization to each enrichment broth in concentrations of 200 ppm and 5 ppm, respectively. After growth, enrichment broths were serially diluted to 10^{-6} in saline and plated onto the corresponding agar plates. All three media were incubated at 30°C, aerobically and anaerobically, with the exception of AAM agar plates which were only incubated aerobically. From the 14 spoiled beer and brewery samples, a total of 348 isolates including all different morphotypes were picked and analysed (**FIG 5.1**).

TABLE 5.1. Overview of the 14 spoiled beer and brewery samples (BS) examined.

Spoiled beer and/or brewery sample		Belgian brewery	Year of isolation
BS4	Final kegged pils beer	A	2010
BS5	Bottled dinner beer (alcohol content of 1.8% v/v)	B	2010
BS6	Bottled sweetened dinner beer (alcohol content of 2.25% v/v)	B	2010
BS7	Fruity wheat beer (alcohol content of 4.3% v/v)	A	2010
BS9	Spoiled pitching yeast culture	B	2012
BS10	Spoiled pitching yeast culture	B	2012
BS11	Spoiled pitching yeast culture	B	2012
BS12	Bottled dinner beer (alcohol content of 1.5% v/v)	A	2012
BS13	Spoiled wort	C	2012
BS14	Bottled beer (alcohol content of 6.6% v/v)	A	2012
BS15	Bottled beer (alcohol content of 7.5% v/v)	A	2012
BS16	Bottled beer (alcohol content of 7.5% v/v)	A	2012
BS36	Spoiled pitching yeast culture	A	2013
BS37	Bottled dinner beer (alcohol content of 1.5% v/v)	A	2013

5.1.2.5 *Identification of beer spoilage bacteria*

For each isolate, one cell extract was prepared from the third generation culture only and mass spectra were generated as described above. Mass spectra of two technical replicates per isolate were combined into a summary spectral profile (SSP). To obtain this SSP, a peak matching analysis was conducted with constant and linearly varying tolerance values of 1 *m/z* and 800 ppm, respectively [56]. The minimum peak detection rate was set at 100%, implying that each summary peak occurred in both profiles of the technical replicates, thus minimizing the impact of technically induced variations [56]. Subsequently, the signal intensity for each data point in the SSP was calculated by averaging the respective signal intensities from the technical replicates.

The identification of the 348 novel isolates was first achieved through comparison of their SSPs with the 4200 reference mass spectra from 273 strains belonging to 52 AAB and LAB species present in the identification database using the binary peak-based cluster analysis. Isolates were assigned to species based on their position in the obtained UPGMA cluster analysis. Alternatively, three classifiers were created based on the pairwise similarity values obtained after peak-based analysis of all reference mass spectra (*i.e.*, the 4200 mass spectra described above), each with an own scoring method (*i.e.*, basic similarity, balanced similarity and weighted average similarity). The species-specific groups defined by peak-based cluster analysis and evaluated using the Jackknife test will be referred to below as classes. The classifier based on basic similarity searches the maximum similarity value between an isolate's SSP and all mass spectra in the database, and then assigns the isolate to the class containing the mass spectrum with this maximum pairwise similarity value. The balanced similarity classifier scores the match between an isolate's SSP and the class it is compared to by determining a weighted sum of the maximum (S_{max}) and average (S_{avg}) similarities to all mass spectra within that class [56] as defined by

$$\text{Score} = w\,S_{max} + (1-w)\,S_{avg}$$

in which the maximum similarity weight parameter (*w*) was set at 0.75.

The weighted average similarity classifier determines the weighted sum of the similarity values (S_i) between an isolate's SSP and each of the class member spectra as defined by

$$\text{Score} = \frac{\sum_{i=1}^{n} w_i S_i}{\sum_{i=1}^{n} w_i}\text{, with } (0 \le S_i \le 1) \text{ and } w_i = e^{-F(1-S_i)}.$$

The weight factor *F* was set at 50 [56].

5.1.2.6 *Sequence analysis of protein coding genes*

MALDI-TOF MS identifications of AAB and LAB strains were verified using protein coding gene sequence analysis. To this end, DNA was prepared using the protocol described by Niemann, *et al.* [57]. For the identification of LAB, amplification and sequencing of the gene encoding for phenylalanyl-tRNA synthase alpha subunit (*pheS*) was performed as described by Naser, *et al.* [52] but with optimised annealing temperatures between 52-55°C to avoid aspecific amplicons. AAB were identified via amplification and sequencing of *rpoB* (the β-subunit of the RNA polymerase), *dnaK* (encoding the heat-shock protein DnaK) and/or *groEL* (encoding a 60 kDa chaperonin protein) [51]. All sequencing reactions were purified using the BigDye® XTerminatorTM Purification Kit according to the standard protocol (AB Sciex). Electrophoresis of sequence reaction products was performed using an ABI Prism 3100 Genetic Analyzer (AB Sciex). The generated sequences were analyzed using BLAST analyses [58]. The list of reference strains and isolates analyzed as such can be found in **TABLE 5.2** and **TABLE 5.3**, respectively.

5.1.3 *Results and Discussion*

5.1.3.1 *Construction of the MALDI-TOF MS identification database*

A total of 4248 good quality mass spectra generated for 273 reference strains representing 52 AAB and LAB species were included in the MALDI-TOF MS identification database (see **ANNEX 1**). To encompass intraspecies phenotypic diversity, strains isolated from different niches were included wherever possible [59, 60]. Furthermore, although we previously demonstrated that growth medium-induced effects did not influence differentiation at species level ([60]; **CHAPTER 4**), we preferred to grow all reference strains on multiple culture media to include and anticipate medium-induced variation in the MALDI-TOF MS identification database as this may affect the bacterial protein expression pattern [60-62] and hence alter the generated MALDI-TOF mass spectra [60, 63, 64] (**CHAPTER 4**).

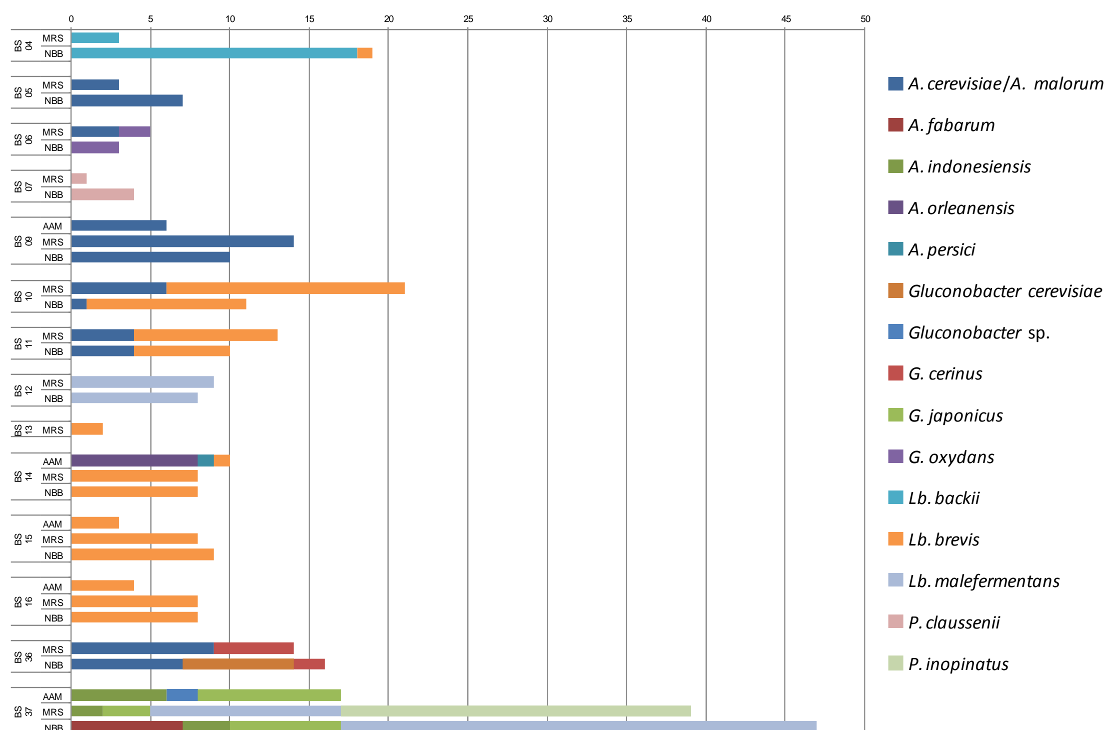

FIG 5.1. Overview of the isolates collected from 14 spoiled beer and brewery samples, and their identification result. BS, Beer Sample; values shown on the x-axis represent the number of isolates collected from MRS, NBB and AAM agar.

The performance of a MALDI-TOF MS identification database largely depends on the correct classification of the reference strains examined. The mass spectra generated from the AAB and LAB strains were analysed separately using a curve- and a peak-based algorithm in order to evaluate the

taxonomic classifications of all reference strains. When using the curve-based algorithm, species were not nicely differentiated in distinct clusters as spectra of eleven species were split into multiple clusters. In addition, twelve clusters comprised mass spectra of two or more species (data not shown). In contrast, a much clearer species level differentiation was obtained using the peak-based algorithm which was therefore used to verify the classification of each of the examined reference strains. In total, 34 reference strains did not cluster with the other strains of their respective species (see **ANNEX 1**) and their classification was verified using sequence analyses of protein encoding genes (**TABLE 5.2**). In bacterial taxonomy and identification it is well known that the resolution of 16S rRNA sequence analysis is insufficient to discriminate between closely related AAB and LAB species [51, 52], although this approach has been commonly used for tentative identification of novel AAB and LAB isolates [65-67]. Protein encoding genes however have a higher taxonomic resolution and different genes were reported useful for the accurate species level identification of different groups of AAB and LAB species [51, 52, 65-67] In the present study we used the *pheS* gene sequence for the identification of LAB species [52, 67], and *dnaK*, *rpoB* and/or *groEL* gene sequences for AAB species [51, 66], as taxonomic studies demonstrated their valid use for this purpose.

TABLE 5.2. Results of the *rpoB*, *dnaK* and *pheS* sequence analyses of the reclassified reference strains.

					Type strain with highest pairwise sequence similarity to query sequence		
	Query strain	**Partially sequenced gene**	**Accession number**	**Former species name**	**Corrected species name**	**Percentage sequence similarity**	**Accession number**
AAB	LMG 1698	*rpoB*	**KF910100**	*A. pasteurianus*	*A. malorum*	97.8%	**KF537504**
	LMG 1743	*rpoB*	**KF910106**	*G. cerinus*	*G. thailandicus*	98.3%	**FN391803**
	LMG 1678	*rpoB*	**KF910111**	*G. cerinus*	*G. japonicus*	99.4%	**HG329610**
	LMG 1678	*dnaK*	**KF910094**	*G. cerinus*	*G. japonicus*	99.1%	**HG329573**
	LMG 1701	*dnaK*	**KF910095**	*A. pasteurianus*	*A. fabarum*	98.8%	**HG329536**
LAB	LMG 11771	*pheS*	**KF910135**	*Lb. parabuchneri*	*Lb. brevis*	99.0%	**AM087680**
	LMG 11974	*pheS*	**KF910134**	*Lb. buchneri*	*Lb. brevis*	98.9%	**AM087680**
	LMG 18940	*pheS*	**KF910115**	*Lb. perolens*	*Lb. brevis*	99.1%	**AM087680**
	LMG 12000	*pheS*	**KF910137**	*Lactobacillus sp.*	*Lb. buchneri*	100.0%	**AM087681**
	LAB 285	*pheS*	**KF910130**	*Lb. brevis*	*Lb. collinoides*	99.5%	**AM087730**
	LAB 1446	*pheS*	**KF910136**	*Lb. perolens*	*Lb. harbinensis*	99.0%	**HQ419067**
	LMG 18938	*pheS*	**KF910131**	*Lb. perolens*	*Lb. harbinensis*	99.2%	**HQ419067**
	LMG 7934	*pheS*	**KF910114**	*Lb. brevis*	*Lb. hilgardii*	99.8%	**AM087698**
	LMG 7935	*pheS*	**KF910113**	*Lb. brevis*	*Lb. hilgardii*	99.3%	**AM087698**
	R-21110	*pheS*	**KG910141**	*Lb. parabuchneri*	*Lb. paracasei*	99.5%	**AM087710**
	LAB 352	*pheS*	**KF910127**	*Lb. plantarum*	*Lb. pentosus*	97.8%	**AM087713**
	LAB 1159	*pheS*	**KF910125**	*Lb. buchneri*	*Lb. plantarum*	99.6%	**FR775943**
	LMG 22108	*pheS*	**KF910148**	*Lb. brevis*	*Lb. plantarum*	99.6%	**FR775943**
	LAB 1192	*pheS*	**KF910124**	*Lb. perolens*	*Lb. rossiae*	90.0%	**AM087768**
	LAB 1193	*pheS*	**KF910128**	*Lb. perolens*	*Lb. rossiae*	89.3%	**AM087768**
	LMG 26011	*pheS*	**KF910126**	*P. damnosus*	*P. acidilactici*	99.2%	**AM749814**

The peak-based cluster analysis of 1361 mass spectra generated from 50 AAB reference strains revealed that mass spectra of 16 out of 18 AAB species examined were grouping in species-specific clusters (**FIG 5.2**). Mass spectra of *Acetobacter cerevisiae* and *Acetobacter malorum* strains were grouped in a single cluster (**FIG 5.2**). These two species are very closely related and that they can only be differentiated by sequence analysis of the 16S-23S rRNA gene internal transcribed spacer region [68]. In addition, some AAB reference strains clustered aberrantly (**FIG 5.2**). [*Gluconobacter cerinus*] strains LMG 1678 (a beer isolate) and LMG 1743 (a rotting pear isolate) grouped separately from the other AAB reference strains comprised in our identification database (**FIG 5.2**). The former was identified as *Gluconobacter japonicus*, a species not regarded as a beer spoilage bacterium, and the latter as *Gluconobacter thailandicus* (**TABLE 5.2**). Similarly, [*Acetobacter pasteurianus*] strains LMG 1549, LMG 1587, LMG 1597, LMG 1604 and LMG 1698 were reassigned to the *A. cerevisiae/A. malorum* group according to MALDI-TOF MS and this was confirmed through sequence analysis of strain LMG 1698 (**FIG 5.2**; **TABLE 5.2**). Furthermore, [*A. pasteurianus*] LMG 1701 (beer wort) and [*Acetobacter lovaniensis*] LMG 24630 (kefir grains) were reassigned to *Acetobacter fabarum* (**FIG 5.2**; **TABLE 5.2**), a species also isolated from an industrial spontaneously fermented lambic beer (West Flanders, Belgium) (FS and PV, unpublished data) and from American coolship ale (USA) [69]. Finally, the mass spectra generated from [*Komagataeibacter xylinus*] LMG 25 did not cluster with any of the AAB reference strains included in this study (**FIG 5.2**). Similar results were recently obtained in the study of Andrés-Barrao *et al*. [45] who reported that LMG 25 belongs to *Komagataeibacter swingsii*.

The peak-based analysis of 2887 mass spectra generated from 223 LAB reference strains generally revealed that mass spectra of most species examined were grouping in species-specific clusters; except for the mass spectra of the closely related *Lb. plantarum* and *Lactobacillus paraplantarum* which grouped together in a single, intermixed cluster (**FIG 5.3**). Mass spectra of *Lactobacillus collinoides/Lactobacillus paracollinoides* (data not shown) and *Lb. buchneri/Lb. parabuchneri* (**FIG 5.3**) grouped in dispersed, but species-specific clusters. The growth medium used may have contributed to this dispersion effect, but a similar result was obtained when only spectra generated after growth on a single growth medium were compared (data not shown). Some reference strains again clustered aberrantly (see **ANNEX 1**; **FIG 5.3**). *Lb. brevis*, a major beer spoilage bacterium, was represented by 60 strains and most mass spectra grouped together in a single cluster. However, [*Lb. brevis*] strains LAB 285 and LMG 22108 grouped with *Lb. collinoides* and *Lb. plantarum/Lb. paraplantarum* strains, respectively (**FIG 5.3**), and these identification results were confirmed by *pheS* sequence analysis (**TABLE 5.2**). Strains known as "*Lb. brevis* subsp. *gravesensis*" (LMG 7934) and "*Lb. brevis* subsp. *otakiensis*" (LMG 7935) [68] were also grouping separately from all other LAB reference strains (**FIG 5.3**) and were assigned to *Lactobacillus hilgardii* by *pheS* sequence analysis

(**TABLE 5.2**). [*Lb. parabuchneri*] LMG 11771, [*Lb. buchneri*] LMG 11974 and [*Lactobacillus perolens*] LMG 18940 grouped within the *Lb. brevis* cluster and this identification was confirmed by *pheS* sequence analysis (**FIG 5.3**; **TABLE 5.2**). Similarly, *Lactobacillus* sp. strain LMG 12000 was identified as *Lb. buchneri*, [*Lb. parabuchneri*] strains R-21110 and R-21121 were reassigned to *Lactobacillus paracasei*, [*Lb. perolens*] strains LAB 1446 and LMG 18938 were identified as *Lb. harbinensis*, [*Lb. plantarum*] strains LAB 351 and LAB 352 were identified as *Lactobacillus pentosus*, and both [*Lb. buchneri*] strain LAB 1159 and [*Lb. paracasei*] strain LAB 541 were reassigned to *Lb. plantarum* (Fig. 2; Table 2). Interestingly, [*Lb. perolens*] strains LAB 1192 and LAB 1193 (both soft drink isolates) were reassigned to *Lactobacillus rossiae* (**FIG 5.3**; **TABLE 5.2**), a strongly slime forming bacterium recovered previously from a German yeast culture containing wheat beer and isolated from biofilms in a brewery's filling hall [10].

Also some *Pediococcus* reference strains proved to be classified incorrectly. [*P. damnosus*] strain LMG 26011 and [*P. inopinatus*] strain LMG 26012 were reclassified to *Pediococcus acidilactici* (**FIG 5.3**; **TABLE 5.2**), an organism encountered less frequently in the brewing environment compared to *P. damnosus* and *P. inopinatus* [3, 9]. [*P. damnosus*] strains LAB 1330, LAB 1331 and LAB 1453 were reassigned to *P. claussenii* (**FIG 5.3**), a potential beer spoilage bacterium which causes ropiness when present in packaged beer due to excessive production of exopolysaccharides [71, 72]. Finally, as described earlier [*Pediococcus lolii*] LMG 25667^T was identified as *P. acidilactici* ([73]; **CHAPTER 5.2**).

5.1.3.1 *Application of the MALDI-TOF MS identification database to identify unknown bacteria*

Species level identification of microbial contaminants in beer and brewery samples is of utmost importance in the brewing industry and a broad diversity in hop resistance and spoilage potential has been observed among strains within the same species [74-76]. However, hop resistance is not a stable characteristic and a decrease in resistance can occur after passaging hop-resistant strains in a hop-deprived environment [5, 28, 77]. Furthermore, this characteristic can be easily transmitted among strains via horizontal gene transfer [77]. Therefore, species level identification of potential spoilage bacteria is an important first step in microbial quality control regardless of the spoilage capabilities of the microorganism isolated.

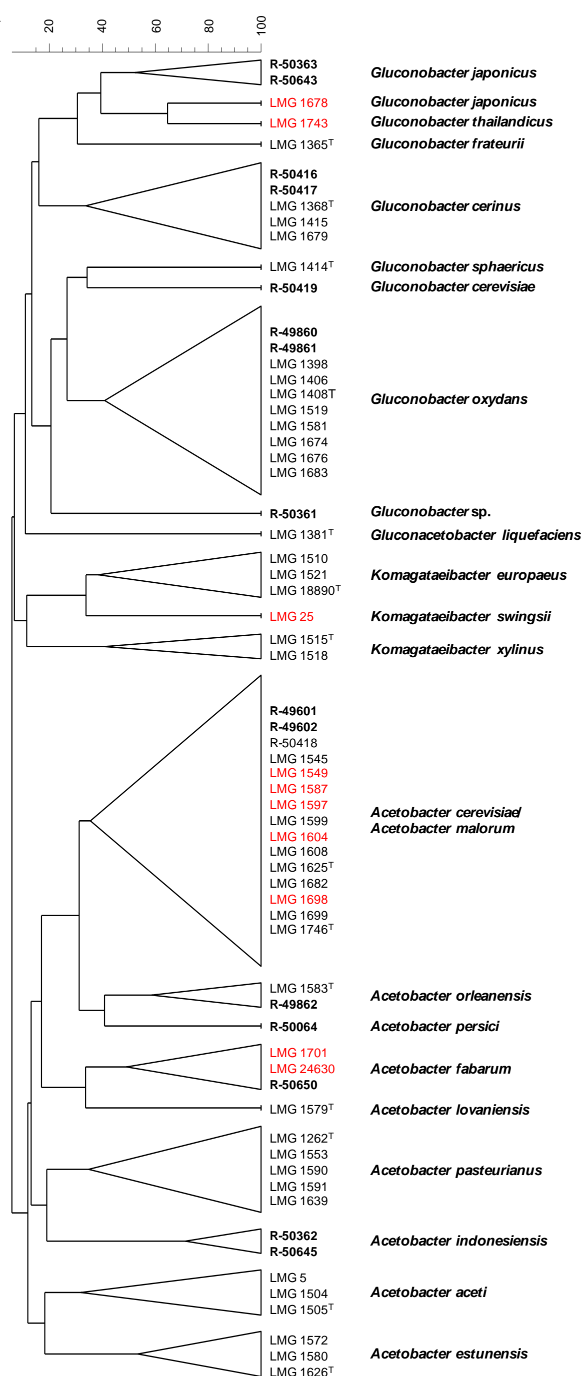

FIG 5.2. Peak-based cluster analysis using the binary Dice coefficient and UPGMA cluster algorithm of a selection of mass spectra of 50 AAB reference strains and of cluster representatives of novel spoilage isolates examined in the present study (isolate numbers are highlighted in bold character type). Strain numbers of reclassified reference strains are shown in red.

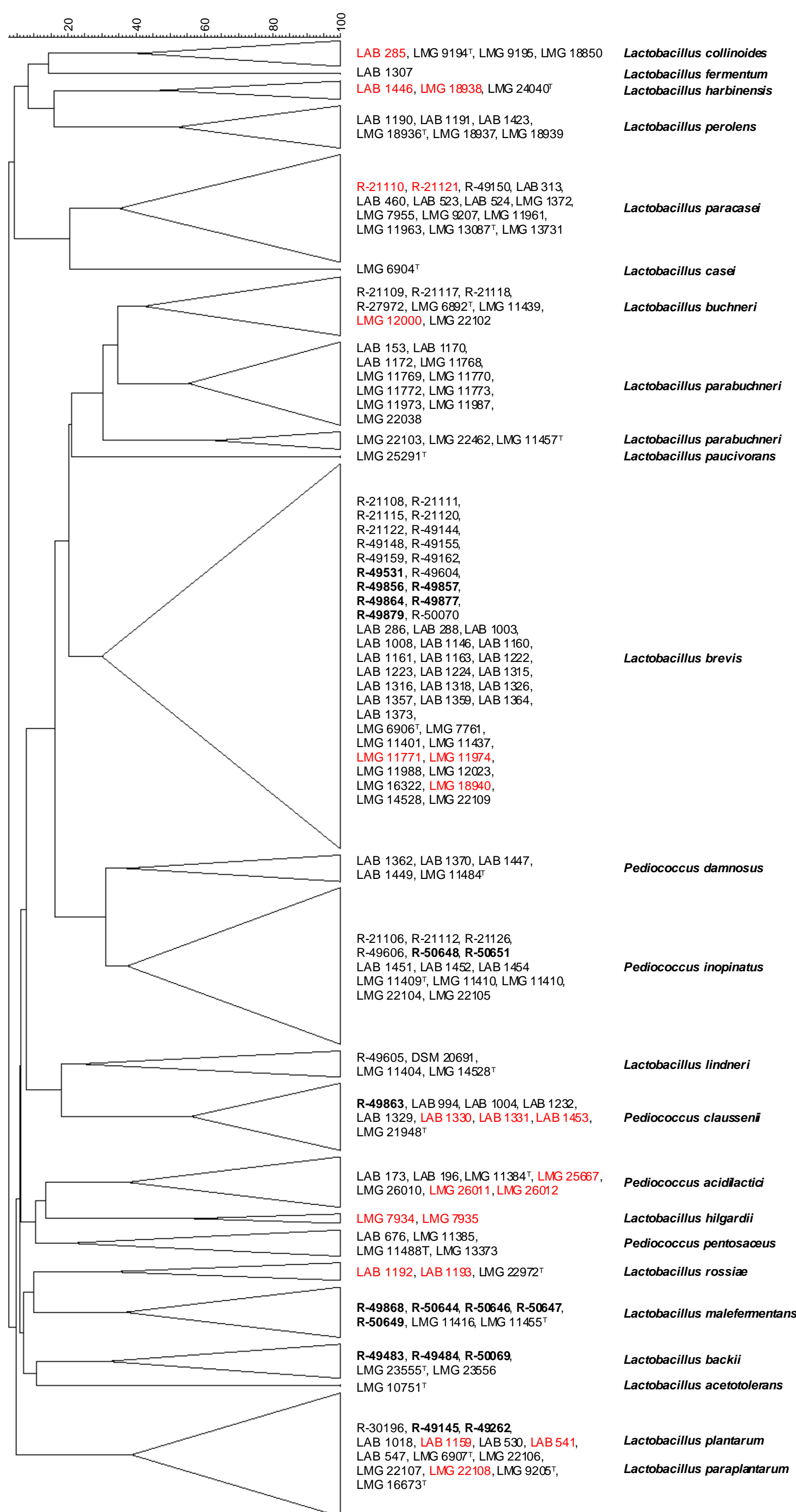

FIG 5.3. Peak-based cluster analysis using the binary Dice coefficient and UPGMA cluster algorithm of a selection of mass spectra generated from 223 LAB reference strains and of cluster representatives of novel spoilage isolates from the present study (isolate numbers are highlighted in bold character type). Strain numbers of reclassified reference strains are shown in red.

TABLE 5.3. Identification results of 32 isolates representing 15 cluster of 348 novel beer spoilage isolates from the present study. The type strain which had the highest sequence similarity with respect to the query sequence is annotated in the designated column.

					Type strain with highest pairwise % sequence similarity to query sequence		
Cluster representatives	**Strain**	**BS**	**Gene**[*]	**Accession no**[¥]	**Strain identified as**		**Accession no**
Cluster 1 74 isolates							
Acetobacter cerevisiae /	R-49601	10	*rpoB*	**KF910097**	*A. cerevisiae*	98.3%	**KF537492**
Acetobacter malorum[1]	R-49602	11	*rpoB*	**KF910098**	*A. cerevisiae*	98.3%	**KF537492**
Cluster 2 7 isolates							
Acetobacter fabarum	R-50650	37	*dnaK*	**KF910092**	*A. fabarum*	98.5%	**HG329542**
Cluster 3 11 isolates							
Acetobacter indonesiensis	R-50362	37	*rpoB*	**KF910108**	*A. indonesiensis*	97.4%	**KF537503**
	R-50645	37	*rpoB*	**KF910109**	*A. indonesiensis*	97.4%	**KF537503**
Cluster 4 8 isolates							
Acetobacter orleanensis	R-49862	14	*rpoB*	**KF910101**	*A. orleanensis*	99.4%	**KF537507**
Cluster 5 1 isolates							
Acetobacter persici	R-50064	14	*rpoB*	**KF910096**	*A. persicus*	98.7%	**KF537531**
Cluster 6 7 isolates							
Gluconobacter cerevisiae	R-50419	36	*dnaK*	**KF910090**	*G. kondonii*	98.4%	**HG329571**
		36	*groEL*	**HG329605**	*G. kanchanaburiensis*	97.2%	**HG329598**
		36	*rpoB*	**KF910104**	*G. kondonii*	89.0%	**HG329607**
Cluster 7 2 isolates							
Gluconobacter sp.	R-50361	37	*dnaK*	**KF910093**	*G. uchimurae*	97.9%	**HG329581**
		37	*rpoB*	**KF910110**	*G. roseus*	95.2%	**HG329613**
Cluster 8 7 isolates							
Gluconobacter cerinus	R-50416	36	*rpoB*	**KF910102**	*G. cerinus*	98.2%	**FN391790**
	R-50417	36	*dnaK*	**KF910091**	*G. cerinus*	98.0%	**FN391644**
	R-50417	36	*rpoB*	**KF910103**	*G. cerinus*	98.1%	**FN391790**
Cluster 9 19 isolates							
Gluconobacter japonicus	R-50363	37	*rpoB*	**KF910105**	*G. japonicus*	98.9%	**HG329615**
	R-50643	37	*rpoB*	**KF910107**	*G. japonicus*	99.0%	**HG329615**
Cluster 10 5 isolates							
Gluconobacter oxydans	R-49860	6	*rpoB*	**KF910112**	*G. oxydans*	99.9%	**FN391799**
	R-49861	6	*rpoB*	**KF910110**	*G. oxydans*	99.9%	**FN391799**
Cluster 11 21 isolates							
Lactobacillus backii	R-49483	4	*pheS*	**KF910133**	*Lb. backii*	99.6%	**AB769496**
	R-49484	4	*pheS*	**KF910132**	*Lb. backii*	99.7%	**AB769496**
	R-50069	4	*pheS*	**KF910147**	*Lb. backii*	99.5%	**AB769496**
Cluster 12 100 isolates							
Lactobacillus brevis[2]	R-49531	11	*pheS*	**KF910129**	*Lb. brevis*	89.9%	**AM087680**
	R-49856	15	*pheS*	**KF910143**	*Lb. brevis*	99.0%	**AM087680**
	R-49857	15	*pheS*	**KF910144**	*Lb. brevis*	99.0%	**AM087680**
	R-49864	10	*pheS*	**KF910155**	*Lb. brevis*	98.9%	**AM087680**
	R-49877	16	*pheS*	**KF910146**	*Lb. brevis*	98.9%	**AM087680**
	R-49879	13	*pheS*	**KF910156**	*Lb. brevis*	98.9%	**AM087680**
Cluster 13 59 isolates							
Lactobacillus malefermentans	R-49868	12	*pheS*	**KF910142**	*Lb. malefermentans*	99.8%	**AM263505**
	R-50644	37	*pheS*	**KF910149**	*Lb. malefermentans*	99.7%	**AM263505**
	R-50646	37	*pheS*	**KF910150**	*Lb. malefermentans*	99.7%	**AM263505**
	R-50647	37	*pheS*	**KF910151**	*Lb. malefermentans*	99.7%	**AM263505**
	R-50649	37	*pheS*	**KF910152**	*Lb. malefermentans*	99.7%	**AM263505**
Cluster 14 5 isolates							
Pediococcus claussenii	R-49863	7	*pheS*	**KF910145**	*P. claussenii*	100.0%	**AM899832**
Cluster 15 22 isolates							
Pediococcus inopinatus	R-50648	37	*pheS*	**KF910153**	*P. inopinatus*	100.0%	**AM899821**
	R-50651	37	*pheS*	**KF910154**	*P. inopinatus*	100.0%	**AM899821**

BS = Beer Sample (see **TABLE 5.1**)
[1] also retrieved from BS5, BS6, BS9 and BS36
[2] also retrieved from BS4 and BS14
[*] Partially sequenced
[¥] Query sequence

A total of 348 isolates were retrieved from 14 spoiled beer and brewery samples (**TABLE 5.1**; **FIG 5.1**). The SSPs generated from these 348 isolates were compared using the curve-based algorithm and UPGMA cluster analysis (data not shown). This approach allowed a rapid assessment of the diversity among the isolates. Fifteen distinct profiles corresponding with different clusters (referred to as clusters 1 through 15) were present and for each of the clusters one or six representative isolates were chosen based on the cluster heterogeneity observed (**TABLE 5.3**) for confirmation of their identification through sequence analysis (see below). The 348 isolates' SSPs were subsequently

added to the AAB and LAB reference strains peak-based cluster analyses and the spectral dataset was re-analysed. Isolates were assigned to species based on their position in the obtained UPGMA cluster analysis (**FIG 5.2**; **FIG 5.3**) and these identifications were consistently verified through *pheS*, *rpoB* or *dnaK* sequence analysis (**TABLE 5.3**).

The 74 isolates comprised in cluster 1 were identified as *A. cerevisiae*/*A. malorum* (**TABLE 5.3**; **FIG 5.2**) and were collected in two breweries from four spoiled brewer's pitching yeast cultures and from two different types of bottled dinner beers with respective alcohol contents of 1.8% v/v and 2.25% v/v (**TABLE 5.1**; **TABLE 5.3**). Besides *A. cerevisiae*/*A. malorum*, *Gluconobacter oxydans* (cluster 10, 5 isolates) was also isolated from the latter dinner beer which was artificially sweetened whereas the former was not (**TABLE 5.1**; **TABLE 5.3**; **FIG 5.2**). *A. fabarum* (cluster 2, 7 isolates) was also isolated from a spoiled bottled dinner beer together with other AAB and LAB species (see below; **TABLE 5.3**). Several other AAB species, *i.e.*, *Acetobacter orleanensis* (cluster 4, 8 isolates), *G. cerinus* (cluster 8, 7 isolates) and *G. japonicus* (cluster 9, 19 isolates) were identified as well (**TABLE 5.3**; **FIG 5.2**). In contrast, the SSPs of 21 Gram negative isolates comprised in clusters 3, 5, 6 and 7 formed separate clusters not matching with any of the reference strains and therefore, their identification was inconclusive based on MALDI-TOF MS (**FIG 5.2**). *rpoB* or *dnaK* sequence analyses identified cluster 3 (11 isolates) and cluster 5 (1 isolate) isolates as *Acetobacter indonesiensis* and *Acetobacter persici*, respectively (Table 3), which are AAB species previously not regarded as beer spoilage bacteria. More remarkably, *rpoB* or *dnaK* sequence analyses failed to identify cluster 6 (7 isolates) and cluster 7 (2 isolates) isolates and extensive polyphasic taxonomic analyses demonstrated that isolates from both clusters represent potentially novel *Gluconobacter* species ([65] and AW and PV, unpublished data). The name *Gluconobacter cerevisiae* was recently proposed for the former taxon which was also isolated from two lambic breweries ([72]; **CHAPTER 5.3**).

AAB prevail in aerobic environments in the brewery environment (*e.g.*, dispensing system, yeast propagation tanks) producing biofilms [13] and causing spoilage [7]. More recently, their prevalence decreased and they are often not considered a genuine threat in modern brewery industry [11]. This clearly contrasts with the isolation of ten AAB species from eight different samples [*i.e.*, four spoiled brewer's pitching yeast cultures, three spoiled bottled dinner beers and one bottled beer with an alcohol content of 6.6% v/v, all collected from two out of three breweries examined (**TABLE 5.1**; **TABLE 5.3**)]. Some of these AAB species have not been reported as potential beer spoilage species and were therefore initially not included in the MALDI-TOF MS identification database. These include *A. indonesiensis* and *G. japonicus* which were both isolated together with other AAB and LAB species from a spoiled bottled dinner beer (alcohol content of 1.5% v/v) (**FIG 5.1**; **TABLE 5.3**), and *A. persici* which was isolated together with *A. orleanensis* from a spoiled blond ale type beer with

refermentation in the bottle (alcohol content of 6.6% v/v) (**FIG 5.1**; **TABLE 5.3**). Such bacteria will be missed by detection and identification kits currently used in the brewery industry which focus primarily on beer spoiling LAB and some strict anaerobic genera, *i.e.*, *Megasphaera* and *Pectinatus*. Our present data suggest that these species should not be neglected as potential beer spoilage bacteria, as these may cause off-flavours, turbidity and ropiness [11, 73, 74].

The largest cluster of spoilage isolates (cluster 12, 100 isolates) was identified as *Lb. brevis* (**TABLE 5.3**), the most common beer spoilage bacterium [80]. Similar to *A. cerevisiae*/*A. malorum*, *Lb. brevis* was well-represented in 7 out of 14 spoiled beer and brewery samples examined (**FIG 5.1**; **TABLE 5.3**). Other LAB species were identified as well, *i.e.*, *Lb. backii* (cluster 11, 21 isolates), *Lb. malefermentans* (cluster 13, 59 isolates), *P. claussenii* (cluster 14, 5 isolates) and *P. inopinatus* (cluster 15, 22 isolates) (**FIG 5.1**; **FIG 5.3**; **TABLE 5.3**). The latter two species are regarded to be potential beer spoilage bacteria as growth of these species occurs mostly in weak beers (*i.e.*, beers with low alcohol content, higher pH, low bitter units) [30]. *P. inopinatus* was isolated from a spoiled bottled dinner beer with a final alcohol content of 1.5% v/v as is in correspondence with literature (**TABLE 5.1**; **TABLE 5.3**). Similarly, *P. claussenii* was retrieved from a spoiled fruity wheat beer with an alcohol content of 4.3% v/v (**TABLE 5.1**; **TABLE 5.3**). *Lb. backii* recently emerged as an obligate beer spoilage bacterium in the brewing environment [80-82] which was confirmed in the present study by the isolation of *Lb. backii* along with *Lb. brevis* from spoiled kegged beer (**FIG 5.1**; **TABLE 5.1**; **TABLE 5.3**). *Lb. malefermentans* has been isolated from a top fermented beer [83] and was more recently recovered from beer bottles found in a 170-year-old shipwreck discovered in the Baltic Sea off the Åland Isles [84]. In the present study, *Lb. malefermentans* was isolated from two different batches of contaminated bottled dinner beer obtained from the same brewery with a one year interval (**FIG 5.1**; **TABLE 5.1**; **TABLE 5.3**). Isolates from both samples had indistinguishable DNA fingerprints as determined through RAPD analysis (data not shown) suggesting that *Lb. malefermentans* is a resident spoilage bacterium of this brewery.

In conclusion, 327 out of 348 isolates (94.0%) from 14 spoiled beer and brewery samples were identified using the peak-based cluster analysis approach. The remaining isolates clustered separately and could either be assigned to species that were not recognised as (potential) beer spoilage bacteria before or to novel AAB species altogether. The inclusion of reference strains of each of these missing reference species in the MALDI-TOF MS identification database allowed a straightforward identification of these isolates (data not shown).

5.1.3.2 *Alternative identification approach based on classifiers*

A peak-based classifier-based approach was investigated as an alternative, high-throughput identification approach. The SSPs of the 348 isolates were matched against all (> 4200) AAB and LAB reference mass spectra using three different scoring approaches referred to as, classifiers [56]. Per classifier, scores were generated between isolates and the best matching reference strains. The identification results for all 348 isolates obtained through peak-based cluster analyses and sequence analyses of protein coding genes allowed the determination and delineation of a score cut-off. The highest number of correct identifications was obtained when the first hit of each classifier had a score of more than 60% and the difference between the highest score and the runner-up was more than 6.5%. If this difference was lower than 6.5% the result was considered unreliable. In total 263 out of 348 isolates (75.6%) were correctly identified this way. In contrast, 24 isolates (6.9%) were misidentified although the first hit of each classifier was identical and higher than 60% and the difference between the highest two scores of each classifier was more than 6.5%. The identification results of 50 isolates (14.4%) were considered unreliable, and 11 isolates (3.2%) had scores lower than 60% and were thus not assigned to a species present in the MALDI-TOF MS identification database. For example, the seven isolates of cluster 6 representing the novel species *G. cerevisiae* were erroneously assigned to *Gluconobacter sphaericus* (although their identification scores were >75%). Similarly, fourteen *Lb. malefermentans* isolates were erroneously identified as *Lb. paracasei*. Previous studies demonstrated that in order to obtain reliable identification results several reference strains per species, preferentially isolated from different niches, should be included to sufficiently cover the intraspecies diversity [59, 60, 80-82]. In our MALDI-TOF MS identification database, *Lb. malefermentans* was represented by two strains only, compared to 15 for *Lb. paracasei*. Including additional *Lb. malefermentans* reference strains might therefore result in an increase of reliable and correct classifier-based identifications.

The classifier-based approach is a far more rapid tool for the identification of unknowns, but in the present study about 7% of the isolates was misidentified and about 18% of the strains were not or not conclusively identified. We therefore prefer to use the peak-based cluster analysis for future analysis of unknown bacteria, due to its accurate performance. Nevertheless, the classifier-based approach could initially be used as a rapid screening tool for a large number of isolates.

5.1.4 *Conclusion*

The present study demonstrated that MALDI-TOF MS is a well-suited identification tool for bacteria isolated from spoiled beer and brewery samples. Its capacity for rapid, high-throughput analysis (about 200 isolates can be easily analysed in a working day starting from the extract preparation), its low consumable cost (less than € 3 per isolate) and the reliable identifications make this technique appropriate for routine quality control in the brewing industry. Moreover, novel beer-spoilage species or other established beer-spoilage bacteria can easily be added to the identification database without adjusting the sample preparation or data acquisition methods and the identification database can easily be extended to include other established beer-spoilage bacteria, like strict anaerobes. Yet, the initial investment cost and subsequent maintenance costs of the apparatus are high, which render its purchase and use in small and medium-sized brewery companies problematic. It seems therefore more appropriate to adopt MALDI-TOF MS in food-related reference laboratories and to provide these low-cost identification services to the brewing industry.

Species level identification of potential spoilage microorganisms is of utmost importance for the brewery industry although not all strains share similar beer spoiling capabilities [88, 89]. Nevertheless, species level identification enables the estimation of the potential impact of spoilage and the prevention of further product deterioration and future spoilage. Unfortunately, enrichment and isolation of the spoilage bacteria are required prior to MALDI-TOF MS analysis [61], as too few bacterial cells are present in the spoiled beer and brewery samples to allow direct detection (and identification) through MALDI-TOF MS [90-92]. Moreover, the beer matrix and the presence of multiple species in a single sample could hamper direct detection as well. At present, this technique seems therefore more suitable for identification of isolates rather than early detection of beer-spoilage bacteria.

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GENE SEQUENCE DATA

The Genbank accession numbers for sequences generated in this study are KF910090-KF910095 for the *dnaK* sequences; KF910096-KF910112 for the *rpoB* sequences; KF910113-KF910156 for the *pheS* sequences and HF329605 for the *groEL* sequence.

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5.2 *Pediococcus lolii* DSM 19927^T and JCM 15055^T represent a *Pediococcus acidilactici* strain

Redrafted from Wieme AD, Cleenwerck I, Van Landschoot A, Vandamme P. (2012) *Pediococcus lolii* DSM 19927^T and JCM 15055^T represent a *Pediococcus acidilactici* strain. International Journal of Systematic and Evolutionary Microbilogy 62: 3105-3108

Author Contributions: conceived and designed experiments: ADW and PV; performed experiments and data analyses: ADW; data analysis of the FAFLP fingerprinting patterns executed by IC; wrote the manuscript: ADW; critically reviewed the manuscript: IC, AVL and PV.

SUMMARY

Strain NGRI 0510Q^T, isolated from ryegrass silage, was recently classified as a representative of a novel *Pediococcus* species, *Pediococcus lolii* Doi *et al.* (2009). It was deposited in the DSMZ and JCM culture collections as DSM 19927^T and JCM 15055^T, respectively. A polyphasic taxonomic study, including matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry, *pheS* and 16S rRNA gene sequence analysis, fluorescent amplified fragment length polymorphism and DNA-DNA hybridization, was used to prove that both subcultures of the type, and only, strain of this species represent are strains of *Pediococcus acidilactici*.

In 2009, Doi and colleagues reported on a novel strain belonging to the genus *Pediococcus*, NGRI 0510Q^T, for which they proposed the name *Pediococcus lolii* [1]. The description was based on a single strain isolated from ryegrass silage that was deposited in the DSMZ and JCM culture collections as DSM 19927^T and JCM 15055^T, respectively. Their study revealed that the strain exhibited distinct phenotypic characteristics, divergent sequences of the 16S rRNA gene and the 16S-23S rRNA intergenic spacer region, and low rates of DNA-DNA hybridization in comparison with the type strains of *Pediococcus acidilactici* DSM 20284^T (= LMG 11384^T) and *Pediococcus pentosaceus* DSM 20336^T (= LMG 11488^T).

The present study was initiated upon analysis of the *P. lolii* type strain accessioned from the JCM culture collection, JCM 15055^T (= LMG 25667^T), by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) which failed to discriminate *P. lolii* LMG 25667^T from *P. acidilactici* strains. Subsequently DSM 19927^T (= LMG 27029^T) was accessioned from the DSMZ culture collection and the polyphasic taxonomic study described below was performed.

All strains were grown on MRS agar (Oxoid) at 28°C in anaerobic atmosphere, except strain *P. acidilactici* LMG 11384^T which was cultured in an aerobic atmosphere. Prior to MALDI-TOF MS analysis, strains were subcultured twice. Five to ten mg of wet cells were suspended into Milli-Q water comprising 75% pure ethanol. Subsequently, formic acid and acetonitrile were added in a 1:1 (v/v) ratio to the bacterial cell pellet. After shaking vigorously, 1 µL of the supernatant (= the cell extract) was spotted onto a MALDI-TOF MS stainless steel target plate. Spots were overlaid with 1 µL matrix, which consisted of 5 mg α-cyano-4-hydroxycinnamic acid (α-CHCA) dissolved in 1 mL acetonitrile/trifluoroacetic acid/Milli-Q water solvent (50:2:48). Prior to analysis, the mass spectrometer was externally calibrated using a peptide mixture of adrenocorticotropic hormone (fragment 18-39) (Sigma-Aldrich), insulin (Sigma-Aldrich), ubiquitin (Sigma-Aldrich), cytochrome C (Sigma-Aldrich) and myoglobin (Sigma-Aldrich). A 4800 Plus MALDI TOF/TOF™ Analyzer (AB Sciex, USA) was used in linear mode and covered a mass range of 2-20 kDa. The mass spectrometer used a 200 Hz frequency tripled Nd:YAG laser, operating at a wavelength of 355 nm. Generated ions were accelerated at 20 kV through a grid at 19.2 kV into a short, linear, field-free drift region onto the detector. For each spot, 50 subspectra for each of 40 randomized positions within the spot were collected and presented as one main spectrum. The laser intensity was set between 4300 and 5100 procedure defined units. Spectral profiles were retrieved via the 4000 Series Explorer software (AB Sciex, USA) and objectively scored for several parameters (signal quality, intensity and the number of peaks). Data Explorer 4.0-software (AB Sciex, USA) was used to convert the profiles into .txt files to import them into a BioNumerics 5.1 database (Applied Maths, Belgium). Spectral profiles were compared using the Pearson product-moment correlation coefficient and a dendrogram was built

using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA)-cluster algorithm (data not shown). Both subcultures, LMG 25667^T and LMG 27029^T, clustered together with the *P. acidilactici* reference strains present in the database. The database used included a set of 36 *Pediococcus* reference strains representing all established species, examined previously by means of multilocus sequence analysis (MLSA) [2]. The MALDI-TOF MS profiles of both LMG 25667^T and LMG 27029^T were indistinguishable from those of 13 *P. acidilactici* reference strains (data not shown). **FIG 5.4** was generated using the mMass 5.1.0-software [3] and shows the high similarity between the MALDI-TOF MS profiles of both *P. lolii* subcultures and those of *P. acidilactici* LMG 11384^T, in contrast with that of *P. pentosaceus* LMG 11488^T.

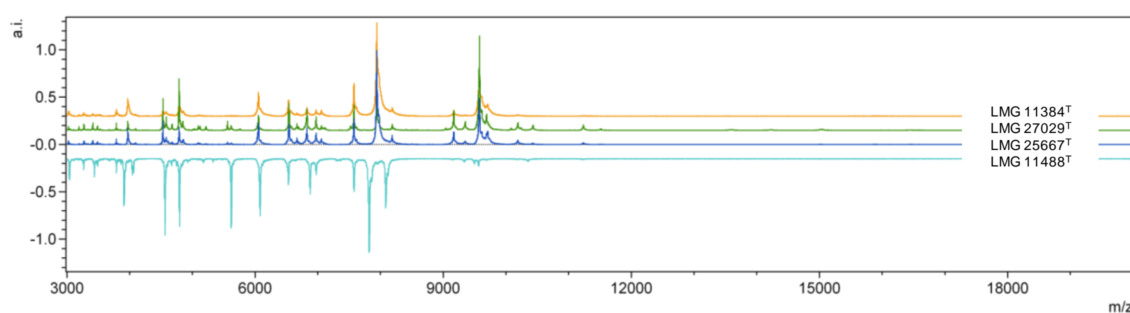

FIG 5.4. Comparison of the MALDI-TOF mass spectra of both *P. lolii* subcultures LMG 25667^T and LMG 27029^T, *P. acidilactici* LMG 11384^T and *P. pentosaceus* LMG 11488^T using the mMass 5.1.0 software [3]. a.i., absolute intensity.

Reliable identification of *Pediococcus* strains and many other lactic acid bacteria is complicated by their ambiguous response in traditional physiological tests and methods. Also the sequence divergence of the 16S rRNA gene fails to discriminate between closely related species and therefore, the use of protein-coding gene sequence data for the determination of genomic relatedness at the species and genus levels is more appropriate [4, 5]. MLSA data of the genes encoding the alpha subunit of phenylalanyl-tRNA synthase (*pheS*), RNA polymerase (*rpoA*) and ATP synthase (*atpA*) were generated by De Bruyne *et al.* [2] as a superior approach for species level identification of pediococci. In that scheme, the *phe*S gene is the most variable gene. Therefore, the *pheS* gene was amplified and sequenced as described previously [2]. The MEGA package version 5.05 [6] was used to align and analyse the *pheS* sequences of LMG 25667^T, LMG 27029^T and all sequences of the type strains of all established *Pediococcus* species. The neighbor-joining, maximum-parsimony and maximum-likelihood methods were used to analyse the sequences. The statistical reliability of the tree topologies was established by bootstrapping analysis based on 1000 tree replicates. The neighbor-joining tree and maximum-parsimony tree revealed topologies similar to those obtained in a phylogenetic tree based on the maximum-likelihood method (**FIG 5.5**). The pairwise similarity

matrix revealed that the strain *P. lolii* LMG 25667^{T} has 100.0, 97.7 and 82.0% sequence similarity with *P. lolii* LMG 27029^{T}, *P. acidilactici* LMG 11384^{T} and *P. pentosaceus* LMG 11484^{T}, respectively. The high degree of similarity between the *P. lolii* and *P. acidilactici* strains indicates that these strains represent the same species.

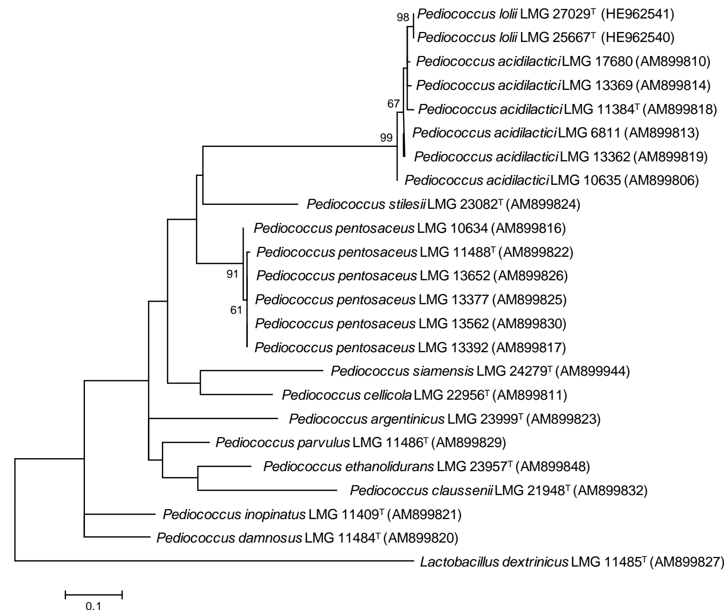

FIG 5.5. Maximum-likelihood tree based on *pheS* gene sequences showing the phylogenetic relationships of the two subcultures of *P. lolii.* Bootstrap percentage values (>50), based on 1000 replications, are shown at branch points. The substitution model used is the Tamura 3-parameter model and the aligned sequences have a length of 344 bp. The bar indicates 10% sequence divergence.

Similarly, fluorescent amplified fragment length polymorphism (FAFLP) profiles of *P. lolii* LMG 25667^{T} and LMG 27029^{T} were generated as described previously and compared with FAFLP profiles of *Pediococcus* reference strains generated previously [2, 7, 8]. The resulting electrophoretic patterns were tracked and normalized using the Gene Mapper 4.0 software package (Applera Co.) and normalized tables of peaks were transferred into the BioNumerics software package, version 5.1 (Applied Maths, Belgium). The FAFLP fingerprints of LMG 25667^{T} and LMG 27029^{T} proved to be similar to those from *P. acidilactici* strains (**FIG 5.6**).

Genomic DNAs of strain *P. lolii* LMG 25667^{T}, *P. acidilactici* LMG 11384^{T} and *P. pentosaceus* LMG 11488^{T} were extracted and purified as described by Marmur *et al*. [9] and modified by Stackebrandt and Kandler [10]. DNA-DNA hybridizations were performed using the microplate method, with photobiotin for labeling the DNA [11], as modified by Goris *et al.* [12]. The mean DNA-DNA hybridization value of strain LMG 25667^{T} with *P. acidilactici* LMG 11384^{T} and *P. pentosaceus* LMG 11488^{T} were 87% (reciprocal values were 82% and 92%) and 19% (reciprocal values were 18% and 19%), respectively, confirming that strain *P. lolii* LMG 25667^{T} is a strain of *P. acidilactici*.

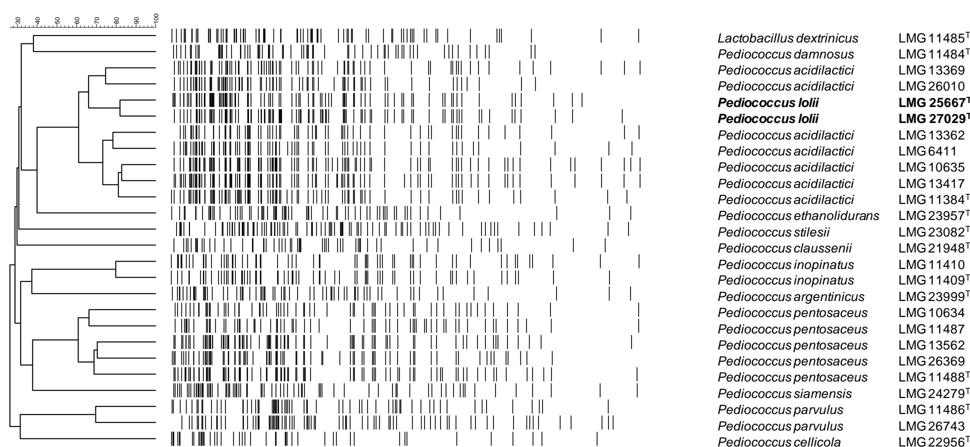

FIG 5.6. FAFLP patterns and dendrogram based on the UPGMA linkage of Dice coefficients (Dice tolerance: 0.15%, range: 40-580bp) (expressed as percentages for convenience) of *P. lolii* LMG 25667^T and LMG 27029^T and the reference strains of all established species of the genus *Pediococcus*.

Finally, complete 16S rRNA gene sequence analysis was performed as described previously [13] for both *P. lolii* subcultures LMG 25667^T and LMG 27029^T to check their authenticity. The 16S rRNA gene sequence of LMG 25667^T and LMG 27029^T were identical and differed only in 6 nt (99.3% sequence similarity) from the sequence deposited by Doi *et al*. [1]. Furthermore they were 99.5% and 98.3% similar to those of *P. acidilactici* DSM 20284^T (AJ305320) and *P. pentosaceus* DSM 20336^T (AJ305321), respectively.

On the basis of the evidence presented, we conclude that the two *P. lolii* NGRI 0510Q^T subcultures deposited in the DSMZ and JCM culture collections as DSM 19927^T (= LMG 27029^T) and JCM 15055^T (= LMG 25667^T) belong to *P. acidilactici.* Whether *P. lolii* should be considered a junior heterotypic synonym of *P. acidilactici* depends on the availability of biological material corresponding with the original description of *P. lolii* by Doi *et al*. (2009).

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GENE SEQUENCE DATA

The GenBank/EMBL/DDBJ accession number for the *pheS* gene sequences for LMG 25667^T and LMG 27029^T, reported in this paper are HE962540 and HE962541, respectively.

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5.3 *Gluconobacter cerevisiae* sp. nov. isolated from the brewery environment

Redrafted from: Wieme AD[¥], Spitaels F[¥], Balzarini T, Cleenwerck I, Van Landschoot A, De Vuyst L, Vandamme P. (2014) *Gluconobacter cerevisiae* sp. nov. isolated from the brewery environment. International Journal of Systematic and Evolutionary Microbilogy 64, 1134-1141. [¥]These authors contributed equally to this work, and are therefore considered joint first authors.

Author contributions: conceived and designed the experiments: ADW, FS and PV; performed the experiments: ADW and FS; determination of the production of keto-D-gluconic acids: TB and FS; analysed the data: ADW and FS; wrote the manuscript: ADW and FS; critically reviewed the manuscript: IC, AVL, LDV and PV.

SUMMARY

Three strains, LMG 27748^T, LMG 27749 and LMG 27882 with identical MALDI-TOF mass spectra were isolated from samples from the brewery environment. Analysis of the 16S rRNA gene sequence of strain LMG 27748^T revealed that the taxon it represents was closely related to the type strains of *Gluconobacter albidus* (100% sequence similarity), *Gluconobacter kondonii* (99.9%), *Gluconobacter sphaericus* (99.9%) and *Gluconobacter kanchanaburiensis* (99.5%). DNA-DNA hybridization experiments towards the type strains of these species revealed moderate DNA relatedness values (39-65%). The three strains used D-fructose, D-sorbitol, *meso*-erythritol, glycerol, L-sorbose, ethanol (weakly), sucrose and raffinose as the sole carbon source for growth (weak growth on the latter two carbon sources was obtained for strains LMG 27748^T and LMG 27882). The strains were unable to grow on glucose-yeast extract medium at 37°C. They produced acid from *meso*-erythritol and sucrose, but not from raffinose. D-Gluconic acid, 2-keto-D-gluconic acid and 5-keto-D-gluconic acid were produced from D-glucose, but not 2,5-diketo-D-gluconic acid. These genotypic and phenotypic characteristics distinguish strains LMG 27748^T, LMG 27749 and LMG 27882 from species of the genus *Gluconobacter* with validly published names and, therefore, we propose to classify them formally as representatives of a novel species, *Gluconobacter cerevisiae* sp. nov., with LMG 27748^T (=DSM 27644^T) as the type strain.

The genus *Gluconobacter* belongs to the family *Acetobacteraceae* within the class α-Proteobacteria and currently comprises 13 validly named species. *Gluconobacter* strains oxidize glucose to gluconic acid [1, 2] rather than ethanol to acetic acid, differentiating them from most acetic acid bacteria (AAB) [2, 3]. They are unable to oxidize acetate to carbon dioxide and water [4]. Strains of the species of the genus *Gluconobacter* thus prefer carbohydrates as carbon sources, whereas other AABs such as members of the genus *Acetobacter* thrive in alcohol-rich environments [5]. Strains of the species of the genus *Gluconobacter* are able to grow in highly concentrated sugar solutions and at low pH values [6]. This capacity for growth can be detrimental, for instance when it leads to spoilage of lager or ale beers, soft drinks, wines and ciders, but beneficial to the production of vinegar, red sour ales and lambic beers [1, 3, 5, 7-10].

Strain LMG 27748^T was isolated during a study of the fermentation process of acidic lambic beers. The latter beers are the product of a spontaneous fermentation, which progresses for at least two years in wooden casks. Strain LMG 27748^T was isolated on acetic acid medium (AAM), an AAB enrichment medium that consists of 1.0% (w/v) D-glucose, 0.5% (v/v) ethanol, 1.5% (w/v) peptone, 1.5% (w/v) agar, 0.8% (w/v) yeast extract and 0.3% (v/v) acetic acid [11]. The medium was adjusted to a pH of 3.5 and supplemented with 5 ppm amphotericin B and 200 ppm cycloheximide to prevent fungal growth. Isolates grown on AAM were subjected to matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) as described previously [12]. MALDI-TOF MS was shown useful for the identification of AAB involved in the production of vinegar [3] and was used as a dereplication tool in the present study. A total of 14 AAB isolates obtained from two different lambic breweries (an industrial and a traditional type located 74 km apart in Belgium) and a spoiled brewer's yeast starter culture of a third brewery displayed identical mass spectra that differed from those of established AAB species, which suggested a unique taxonomic position (data not shown).

Random amplified polymorphic DNA (RAPD) analysis of a selection of six AAB isolates representing the three breweries was performed as described by Williams *et al.* [13], using primers RAPD-270 and RAPD-272 [14]. The results revealed three RAPD patterns, corresponding with the three breweries (**FIG 5.7**) and thus indicated the presence of three genetically distinct strains. Subsequently, one isolate from each brewery was chosen for further analyses: strain LMG 27748^T representing isolates of the industrial lambic brewery, strain LMG 27749 originating from the spoiled brewer's yeast starter culture and strain LMG 27882 isolated in a traditional lambic brewery.

The 16S rRNA gene sequence of strain LMG 27748^T was determined as described previously [15]. EzBioCloud analysis [16] of this 16S rRNA gene sequence revealed similarity to those of *Gluconobacter albidus* NBRC 3250^T (100%), *Gluconobacter kondonii* NBRC 3266^T (99.9%),

Gluconobacter sphaericus NBRC 12467^T (99.9%) and *Gluconobacter kanchanaburiensis* BCC 15889^T (99.5%) (pairwise similarity values in parentheses). All 16S rRNA gene sequences were aligned using the SILVA Incremental Aligner (SINA v1.2.11) (http://www.arb-silva.de/aligner/) [17], with the corresponding SILVA SSURef 115 database [18], and phylogenetic trees were reconstructed using the MEGA 5.2 software package [19]. Tree topologies were analysed statistically using 1000 bootstrapping replications. The maximum-likelihood and maximum-parsimony method trees (data not shown) showed the same topology as the neighbour-joining method tree (**FIG 5.8**).

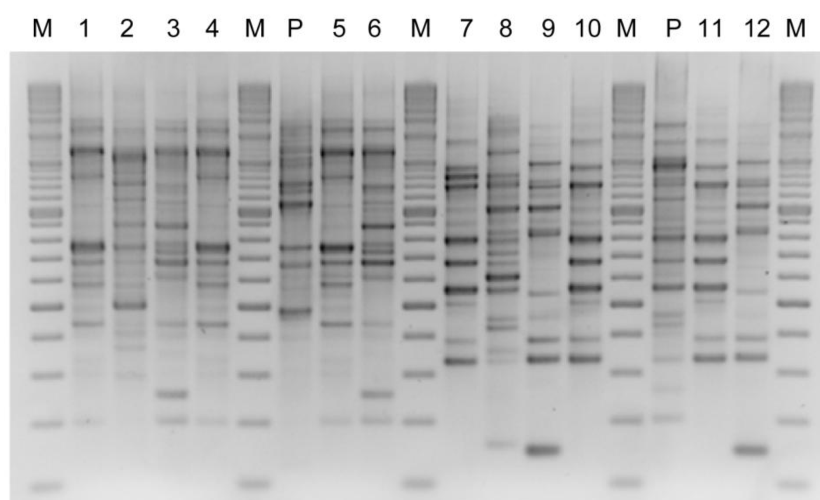

FIG 5.7. Patterns of the RAPD analysis of six representative AAB isolates using primers RAPD-270 (**1-6**) and RAPD-272 (**7-12**). Lanes: **1/7,** LMG 27748^T; **2/8,** LMG 27882; **3/9,** LMG 27749; **4/5/10/11,** two additional isolates from an industrial lambic brewery; **6/12,** additional isolate from a spoiled brewer's yeast starter culture. **M** denotes the size marker and **P** represents a positive control sample.

Because of the limited taxonomic resolution of the 16S rRNA gene in the AAB group of bacteria, the 16S-23S rRNA gene internal transcribed spacer (ITS) sequence of strain LMG 27748^T was determined using the same protocol as used for the 16S rRNA gene sequence [15]. The 16S-23S rRNA gene ITS primers 16S-23S-ITS-1F 5′-TGCGGCTGGATCACCTCCT-3′ (positions 1522–1540 on the 16S rRNA gene, *Escherichia coli* numbering) and 16S-23S-ITS-2R 5′-GTGCCAAGGCATCCACCG-3′ (positions 38–22 on the 23S rRNA gene, *E. coli* numbering) were used. BLAST analysis [20] of the 16S-23S rRNA gene ITS sequence revealed that the LMG 27748^T ITS sequence was similar to that of *Gluconobacter kondonii* NBRC 3266^T (96.0%) and *Gluconobacter albidus* NBRC 3250^T (94.0%) (pairwise similarity values in parentheses). Phylogenetic trees were reconstructed using the MEGA 5.2 software package [19]. Tree topologies were analysed statistically using 1000 bootstrapping replications. The maximum-likelihood and maximum-parsimony trees (data not shown) showed the same topology as the neighbour-joining method tree (see **FIG S 5.1** in the supplementary material).

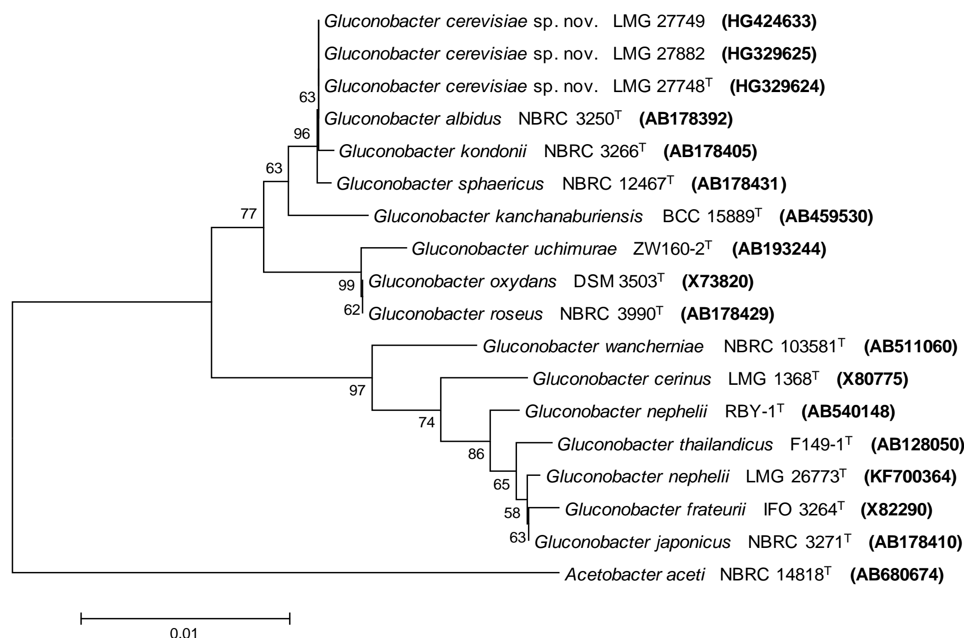

FIG 5.8. Neighbour-joining tree based on nearly full-length 16S rRNA gene sequences (1363 bp) showing the phylogenetic relationship of isolates LMG 27748^T, LMG 27749 and LMG 27882 and of the type strains of all species of the genus *Gluconobacter* with validly published names. *Acetobacter aceti* NBRC 14818^T (= LMG 1504^T) was used as an outgroup. Evolutionary distances were computed using the maximum composite likelihood method [40]. Sequence accession numbers are given in parentheses. Bootstrap percentages (≥ 50%) are shown next to the branch points. Bar, 1% sequence divergence.

Additionally, the phylogenetic position of the taxon represented by strains LMG 27748^T, LMG 27749 and LMG 27882 was analysed using partial sequences of the housekeeping genes *dnaK* (encoding the chaperone protein DnaK), *groEL* (encoding a 60-kDa chaperonin) and *rpoB* (encoding the DNA-directed RNA polymerase beta subunit) [21]. Sequences of type and additional taxonomic reference strains of the genus *Gluconobacter* were determined to make a comprehensive multilocus sequence analysis MLSA dataset for the entire genus. Sequences of at least 654, 534 and 510 nt were generated for *dnaK*, *groEL* and *rpoB*, respectively. All gene sequences were aligned at the amino acid level using the MEGA 5.2 software [19]. Trees were reconstructed using the maximum-likelihood model. A discrete gamma distribution was used to model evolutionarily rate differences among sites and the rate variation model allowed for some sites to be evolutionarily invariable. Tree topologies were analysed statistically using 1000 bootstrapping replications. Numerical analysis of the individual (see **FIG S 5.2**, **FIG S 5.3** and **FIG S 5.4** in the supplementary material) and concatenated (**FIG 5.9**) gene sequences revealed that strains LMG 27748^T, LMG 27749 and LMG 27882 could be clearly differentiated from their nearest neighbours, *G. kondonii* LMG 1367^T t1 and *G. albidus* LMG 1356^T. The concatenated MLSA data revealed that most species of the genus *Gluconobacter* were

well separated, with the exception of *G. nephelii* LMG 26773^T that grouped with *G. japonicus* strains. A pairwise comparison of the 16S rRNA gene sequence of *G. nephelii* LMG 26773^T with that of *G. nephelii* RBY-1^T (AB540148) revealed a sequence similarity value of 99.8%, *i.e.*, a difference of 3 out of 1410 nt. With *G. japonicus* NBRC 3271^T (AB178410) a sequence similarity value of 99.9% was found, *i.e.*, a difference of 1 out of 1406 nt. *G. nephelii* strain RBY-1^T was originally deposited as NBRC 106061^T in the NITE Biological Resource Center (NBRC, Japan) and *G. nephelii* strain LMG 26773^T is a direct subculture of the *G. nephelii* strain NBRC 106061^T culture. The 16S rRNA gene sequences of the subcultures LMG 26773^T and NBRC 106061^T (16S rRNA gene sequence retrieved from the NBRC website, http://www.nbrc.nite.go.jp/NBRC2/NBRCCatalogueDetailServlet?ID=NBRC&CAT=00106061) are fully identical, suggesting that LMG 26773^T and NBRC 106061^T represent the same strain. Therefore, it is likely that the sequence of RBY-1^T with accession number AB540148 contains sequencing errors or that the biological material that was deposited in the NBRC culture collection does not correspond to strain RBY-1^T [22] (**FIG 5.8**).

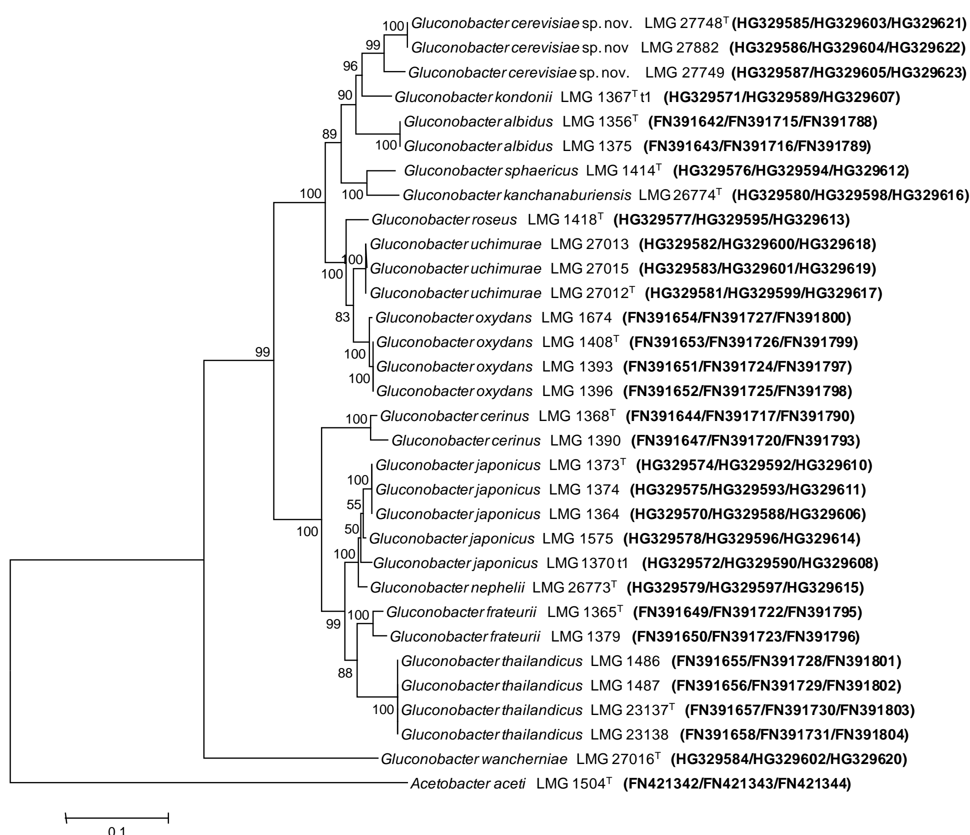

FIG 5.9. Maximum-likelihood tree based on concatenated *dnaK*, *groEL* and *rpoB* gene sequences (a total of 1698 bp) showing the phylogenetic relationship of isolates LMG 27748^T, LMG 27749 and LMG 27882 and all species of the genus *Gluconobacter* with validly published names. *Acetobacter aceti* LMG 1504^T (= NBRC 14818^T) was used as an outgroup. The substitution model used was the General Time Reversible model [41]. Sequence accession numbers for *dnaK*, *groEL* and *rpoB* gene sequences are given in parentheses in that order. Bootstrap percentages (≥ 50%) are shown next to the branch points. Bar, 10% sequence divergence.

DNA-DNA hybridizations were performed between strains LMG 27748^T and LMG 27749 and the type strains of their nearest phylogenetic neighbours as described previously [23]. DNA-DNA hybridization values are presented as means of reciprocal reactions (A×B and B×A, values are indicated between parentheses), where each reciprocal reaction was performed at least in three-fold. Strains LMG 27748^T and LMG 27749 showed 80% (81% and 80%) DNA-DNA relatedness. The DNA-DNA relatedness between strain LMG 27748^T and the type strains of its nearest phylogenetic neighbours was 65% (66% and 64%) towards *G. kondonii* LMG 1367^T t1, 54% (60% and 49%) towards *G. albidus* LMG 1356^T, 45% (56% and 36%) towards *G. sphaericus* LMG 1414^T and 41% (52% and 30%) towards *G. kanchanaburiensis* LMG 26774^T. The DNA G+C content of strains LMG 27748^T and LMG 27749 were determined as described previously [23] and were 58.0 mol% and 57.7 mol% respectively.

The phenotypic characteristics of strains LMG 27748^T, LMG 27749 and LMG 27882 were determined as described previously [24]. Type strains of closely related AAB (*G. albidus* LMG 1356^T, *G. kondonii* LMG 1367^T t1, *G. sphaericus* LMG 1414^T and *G. kanchanaburiensis* LMG 26774^T) were included as positive or negative controls. For microscopy and morphological examination of colonies, strains were grown aerobically on AAM agar at 28°C for 48 h. The biochemical characteristics tested included a Gram-stain reaction, analysis of catalase and oxidase activities, growth on 0.3% D-fructose, D-sorbitol, *meso*-erythritol, glycerol, sucrose, raffinose, L-sorbose or ethanol as the sole carbon sources, growth at 37°C on GY agar (5% D-glucose, 1% yeast extract and 1.5% agar). In addition, acid production from 1% *meso*-erythritol, sucrose and raffinose was determined as described previously [25], results are shown in the species description.

For testing the production of 2-keto-D-gluconic acid and 5-keto-D-gluconic acid, cells were grown as described by Gosselé *et al.* [26] and the presence of both keto-D-gluconic acids was determined as described by Spitaels *et al*. [27]. All three strains produced D-gluconic acid, 2-keto-D-gluconic acid and 5-keto-D-gluconic acid, but not 2,5-diketo-D-gluconic acid (data not shown).

Strains LMG 27748^T, LMG 27749 and LMG 27882 could be differentiated from *G. kondonii*, *G. albidus*, *G. sphaericus* and *G. kanchanaburiensis* by means of multiple biochemical characteristics, such as acid production from sucrose and raffinose and growth on ethanol as sole carbon source (**TABLE 5.4**). The biochemical test results did not always correspond to published data. The utilization of L-sorbose and raffinose by *G. kondonii* LMG 1367^T t1 was as reported by Yukphan *et al.* [28] (positive for L-sorbose and negative for raffinose) and differed from results reported by Malimas *et al.* [29, 30]. Similarly, acid production from maltose (absent) and growth on D-arabitol (present) by *G. cerinus* NBRC 3267^T as reported by Malimas *et al.* [29], Tanasupawat *et al.* [31] and Yukphan *et al.* [28] contradicted results reported by Tanasupawat *et al.* [32]. In addition, Kommanee *et al.* [22] reported

both characteristics as present in *G. cerinus* strains. These discrepant test results were obtained using the same test procedures [25, 33-38]. Therefore, these biochemical tests appear to reproduce poorly as observed previously by Yukphan *et al.* [39] and the inclusion of sufficient and appropriate control strains is warranted when performing them.

Numerical comparison of the MALDI-TOF mass spectra of strains LMG 27748^T, LMG 27749 and LMG 27882, and those of reference strains of their nearest phylogenetic neighbours by means of the Pearson product-moment correlation coefficient allowed a very straightforward separation of these taxa. As described above, the three strains displayed indistinguishable spectra that could be differentiated from those of species of the genus *Gluconobacter* with validly published names by the consistent presence of five biomarker peaks characterised by *m/z* values of 3253.41 ± 4.60, 4912.38 ± 5.93, 6371.16 ± 7.10, 6506.29 ± 7.21 and 9171.97 ± 9.34 (**FIG 5.10**); some of these peaks were present in the mass spectra of strains of other species of the genus *Gluconobacter*, but never all five simultaneously.

In conclusion, the present polyphasic study provides taxonomic data demonstrating that the taxon represented by strains LMG 27748^T, LMG 27749 and LMG 27882 could be differentiated, by means of multiple genotypic [*i.e.*, 16S-23S rRNA gene ITS sequence analysis (see **FIG S 5.1** in the supplementary material), MLSA (**FIG 5.9**) and DDH] and phenotypic characteristics [*i.e.*, MALDI-TOF MS analysis (**FIG 5.10**), acid production and growth on several carbon sources (**TABLE 5.4**)] using various methodologies, from its nearest phylogenetic neighbours. We, therefore, propose to assign these strains to a novel species, *Gluconobacter cerevisiae* sp. nov., with LMG 27748^T (=DSM 27644^T) as the type strain.

TABLE 5.4. Differential characteristics for *Gluconobacter cerevisiae* and the type strains of the validly named *Gluconobacter* species. Taxa: 1, LMG 27748^{T}; 2, LMG 27749; 3, LMG 27882; 4, *G. albidus* (LMG 1356^{T}); 5, *G. kondonii* (LMG 1367^{T}); 6, *G. sphaericus* (LMG 1414^{T}); 7, *G. kanchanaburiensis* (LMG 26774^{T}); 8, *G. uchimurae* (ZW 160-2^{T}); 9, *G. oxydans* (NBRC 14819^{T}); 10, *G. roseus* (NBRC 3990^{T}); 11, *G. wancherniae* (BCC 15775^{T}); 12, *G. cerinus* (NBRC 3267^{T}); 13, *G. japonicus* (NBRC 3271^{T}); 14, *G. frateurii* (NBRC 3264^{T}); 15, *G. thailandicus* (BCC 14116^{T}); 16, *G. nephelii* (NBRC 106061^{T}). Data for taxa 1-3 were generated in this study. +, positive; -, negative; w, weakly positive; vw, very weakly positive; ND, not determined. Data taken from: [a]Tanasupawat *et al*. [32]; [b]this study; [c]Malimas *et al*. [43]; [d]the original species description; [e]Malimas *et al*.[30]; [f]Kommanee *et al*. [22].

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Water-soluble brown pigment[a*]	-	-	-	-	-	+	+	+	-	-	+	-	-	-	-	-
2,5-Diketo- D-gluconic acid production[a]	-	-	-	-	-	+	+	+	-	-	+	-	-	-	-	-
Growth at 37°C[a]	-	-	-	-	-	-	-	+	+	-	-	-	-	-	w	-
Acid production from:																
Sucrose[a]	+	+	+	+	-[b]	+	-[b]	-	-	+	w	+	w	-	-	w
Raffinose[c]	-	-	-	+	+	+	w	-[a]	w	+	vw[d]	w	+	+	w	+[f]
meso-erythritol[e]	+	+	+	+[b]	+	-[b]	w	ND	+	-	-	+	+	w	+	+
Growth on:																
D-Fructose[f]	+	+	+	+[b]	+	+[b]	w[d]	ND	+	+[d]	+[d]	+	+[c]	+	+	+
D-Sorbitol[f]	+	+	+	+[b]	+[b]	+[b]	+[d]	ND	+	vw[d]	+[d]	+	+[c]	+	-	+
Glycerol[f]	+	+	+	+[b]	+	-[b]	+[d]	ND	+	vw[d]	+[d]	+	+[c]	+	+	+
Sucrose[f]	w	+	w	+[b]	-[b]	vw[d]	+[d]	ND	-	+[d]	-[d]	w	+[c]	+	+	+
Meso-erythritol[e]	+	+	+	+[b]	+	-[b]	+[d]	+[a]	w	-[d]	w	+	+	-	w	w
Raffinose[c]	w	+	w	+	-[b]	-	w[d]	ND	-	+[d]	-[d]	-	w	+	w	+[f]
L-Sorbose[c]	+	+	+	-	+[b]	-	+	ND	-	-	+[d]	-	+	-	-	+[f]
Ethanol	w	w	w	-[d]	-[e]	-[d]	w[d]	-[a]	ND	-[d]	vw[d]	+[d]	-[c]	ND	ND	-[f]
G+C (%)[a]	58	57.7	ND	60	59.8	59.5	59.5	60.5	60.3	60.5	56.6	55.9	56.4	55.1	55.8	57.2

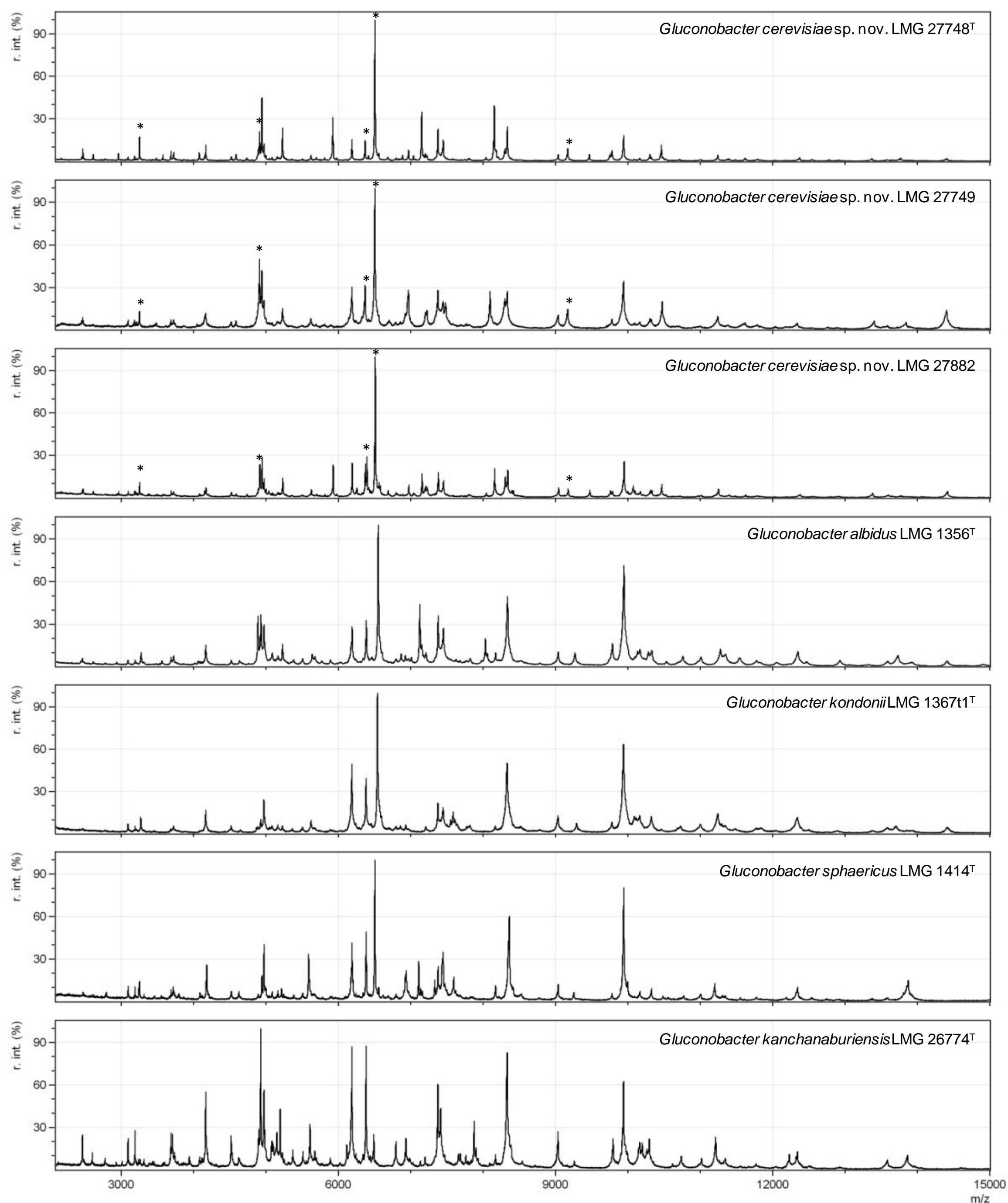

FIG 5.10. MALDI-TOF mass spectra of *Gluconobacter cerevisiae* sp. nov. and its close phylogenetic neighbours. Asterisks indicate the set of five peaks (*m/z* 3253.41 ± 4.60, 4912.38 ± 5.93, 6371.16 ± 7.10, 6506.29 ± 7.21 and 9171.97 ± 9.34) by which the strains could be differentiated from the species of the genus *Gluconobacter* with validly published names. The profiles are visualised using mMass 5.5.0 [42].

DESCRIPTION OF *GLUCONOBACTER CEREVISIAE* SP. NOV.

Gluconobacter cerevisiae (ce.re.vi'si.a.e. L. fem. gen. n. *cerevisiae* of beer, referring to the source from which the three cultures have been isolated)

Cells are Gram-stain negative, non-motile rods and are approximately 1 µm × 2-3 µm long. Cells occur separately or in pairs. Catalase activity is exhibited, but no oxidase activity. After 48 h of incubation on AAM agar at 28°C colonies are round, rough, brownish beige and slightly raised, with a diameter of approximately 1-2 mm. D-Gluconic acid is produced from D-glucose as well as 2-keto-D-gluconic acid and 5-keto-D-gluconic acid. Able to grow on D-fructose, D-sorbitol, *meso*-erythritol, glycerol, L-sorbose and ethanol (weakly) as the sole carbon source. Growth on sucrose and raffinose as the sole carbon source is variable, ranging from weak (LMG 27748^T and LMG 27882) to strong (LMG 27749). Unable to grow on glucose-yeast extract medium at 37°C. Acid is produced from *meso*-erythritol and sucrose, but not from raffinose.

The type strain is strain LMG 27748^T (=DSM 27644^T), which was isolated from fermenting lambic beer. The DNA G+C content of strain LMG 27748^T is 58.0 mol%.

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GENE SEQUENCE DATA

The Genbank/EMBL accession numbers for sequences generated in this study are HG329624, HG329625, HG424633 and KF700364 for the 16S rRNA gene sequences; HG424630-HG424632 for the 16S-23S ITS gene sequence; HG329570-HG329587 for the *dnaK* gene sequences; HG329588-HG329605 for the *groEL* gene sequences and HG329606-HG329623 for the *rpoB* gene sequences.

SUPPLEMENTARY MATERIAL

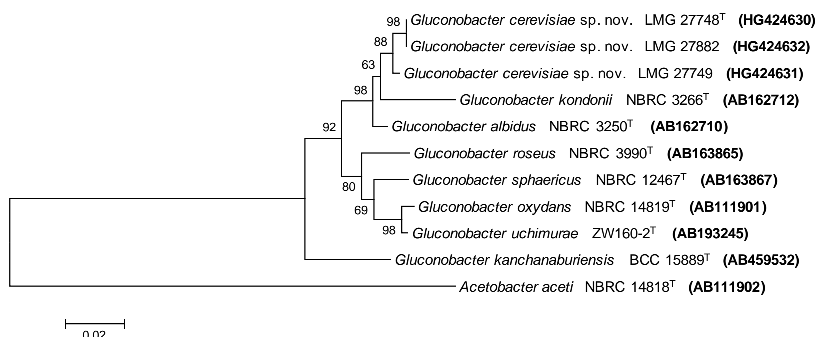

FIG S 5.1. Neighbour-joining tree based on 16S–23S rRNA gene ITS sequences (627 bp) showing the phylogenetic relationship of strains LMG 27748^T, LMG 27749 and LMG 27882 and of their closest phylogenetic neighbours. *Acetobacter aceti* NBRC 14818^T (= LMG 1504^T) was used as an outgroup. Evolutionary distances were computed using the maximum composite likelihood method [40] and are expressed as the number of base substitutions per site. Sequence accession numbers are given between brackets. Bootstrap percentages (≥ 50%) are shown next to the branch points. Bar, 2% sequence divergence.

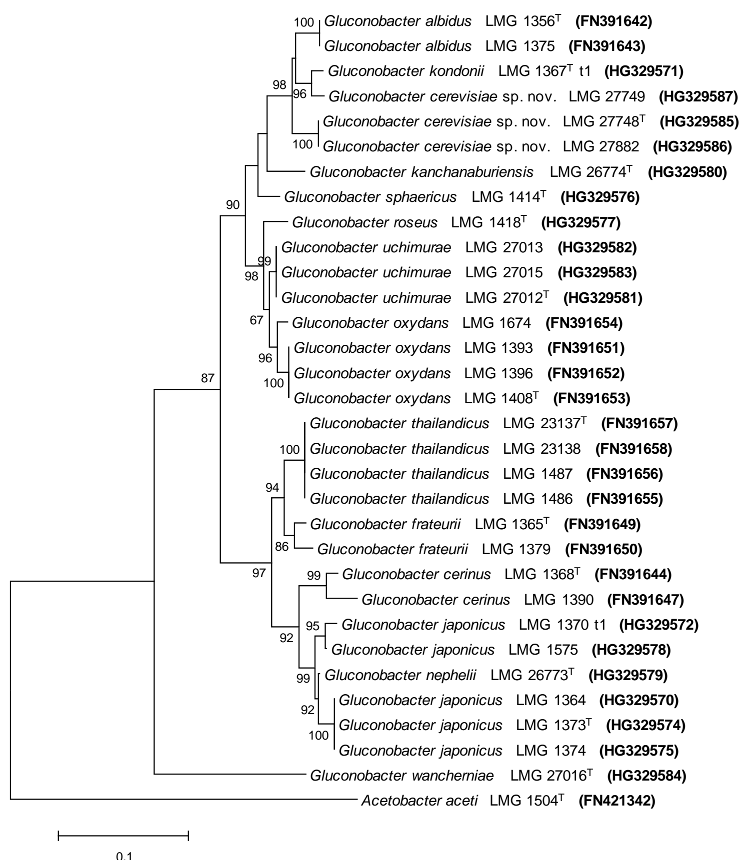

FIG S 5.2. Maximum-likelihood tree based on *dnaK* gene sequences (654 bp) showing the phylogenetic relationships of strains LMG 27748^T, LMG 27749 and LMG 27882 and all species of the genus *Gluconobacter* with validly published names. *Acetobacter aceti* LMG 1504^T (= NBRC 14818^T) was used as an outgroup. The substitution model used was the General Time Reversible model [41]. Sequence accession numbers for the *dnaK* gene sequences are given between brackets. Bootstrap percentages (≥ 50%) are shown next to the branch points. Bar, 10% sequence divergence.

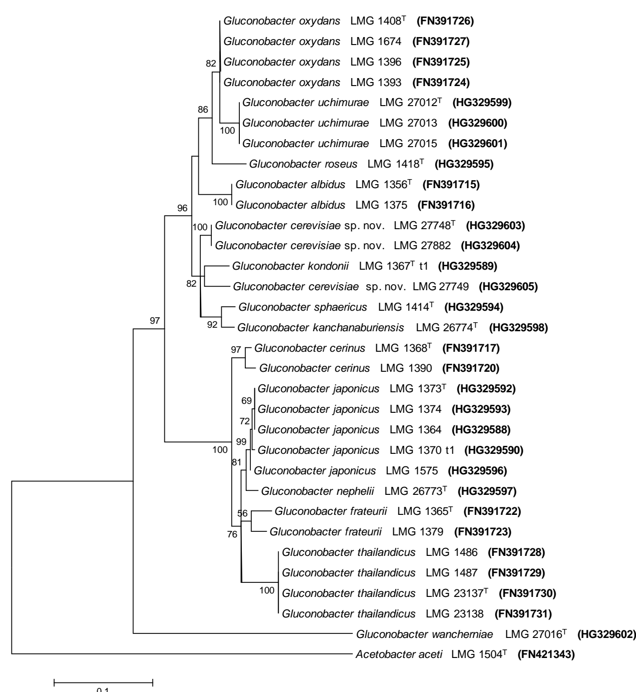

FIG S 5.3. Maximum-likelihood tree based on *groEL* gene sequences (534 bp) showing the phylogenetic relationships of strains LMG 27748^T, LMG 27749 and LMG 27882 and all species of the genus *Gluconobacter* with validly published names. *Acetobacter aceti* LMG 1504^T (= NBRC 14818^T) was used as an outgroup. The substitution model used was the General Time Reversible model [41]. Sequence accession numbers for the *groEL* gene sequences are given between brackets. Bootstrap percentages (≥ 50%) are shown next to the branch points. Bar, 10% sequence divergence.

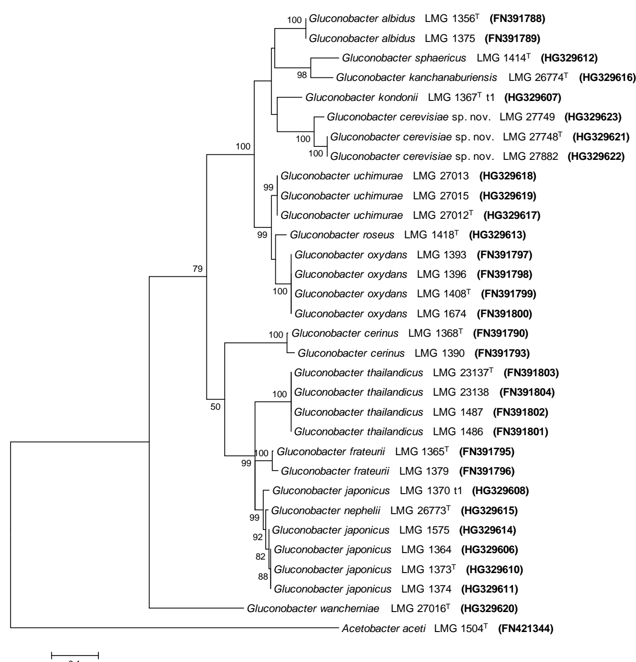

FIG S 5.4. Maximum-likelihood tree based on *rpoB* gene sequences (510 bp) showing the phylogenetic relationships of strains LMG 27748^T, LMG 27749 and LMG 27882 and all species of the genus *Gluconobacter* with validly published names. *Acetobacter aceti* LMG 1504^T (= NBRC 14818^T) was used as an outgroup. The substitution model used was the General Time Reversible model [41]. Sequence accession numbers for the *rpoB* gene sequences are given between brackets. Bootstrap percentages (≥ 50%) are shown next to the branch points. Bar, 10% sequence divergence.

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6 Direct detection and identification of bacteria in enrichment cultures of spoiled beer and brewery samples using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry: a proof of concept

Wieme AD, Spitaels F, Van Landschoot A, Vandamme P. Direct detection and identification of bacteria in enrichment cultures of spoiled beer and brewery samples using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry: A proof of concept. (*In preparation*).

Author contributions: conceived and designed the experiments: ADW, AVL and PV; performed the experiments and data analyses: ADW and FS, wrote the manuscript: ADW, critically reviewed the manuscript: FS, AVL and PV.

SUMMARY

The present study investigated the applicability of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) for the identification of bacteria directly in enrichment cultures of spoiled beer and brewery samples. The bacteria present in such samples were collected using a filtration procedure and were subsequently inoculated in enrichment broth. Mass spectra generated from these enrichment cultures enabled a tentative identification of the spoilage bacteria. However, beer spoilage commonly involves multiple contaminating strains and/or species, and the identification of spoilage bacteria in mixtures may be extremely challenging because of peak suppression effects that are further confounded by the identity and ratio of the microorganisms present. Therefore, the preliminary identification requires confirmation through cultivation and MALDI-TOF MS based dereplication and identification of the bacteria present in the enrichment cultures.

6.1 Introduction

Species level identification of spoilage microorganisms is of utmost importance for the brewing industry even though not all strains of the same species share similar beer spoiling capabilities [1-3]. Nevertheless, species level identification enables the estimation of the impact of bacterial spoilage and subsequently the prevention of further product deterioration and future spoilage. It is demonstrated that matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) can be applied as a rapid and high-throughput tool for the identification of beer spoilage bacteria (**CHAPTER 5.1**). However, microorganisms in spoiled beer and brewery samples must first be enriched before potential contaminants can be isolated and subsequently identified by MALDI-TOF MS, as the numbers of bacterial cells that are initially present in such samples are too low [4-7]. The MALDI-TOF MS cell detection limit is in the range of 10^3 to 10^8 CFU/mL and depends on the type of sample, the sample preparation procedure, the data analysis method and the type of instrumentation [4, 5, 7-12].

Sample preparation procedures have been optimised permitting the direct identification of microorganisms in clinical specimens like blood and urine through MALDI-TOF MS [13, 14]. Interfering matrix compounds are mostly removed by incorporating washing, centrifugation or filtration steps into the sample preparation protocol prior to cell extraction [11, 13-15]. Here too, the MALDI-TOF MS cell detection limit proved to be a major hurdle and enrichment procedures of microorganisms in clinical specimens remain a necessity [15]. Also the co-occurrence of multiple microorganisms in samples where a specific microorganism's presence is examined, challenges its direct identification [13, 14, 16, 17].

The use of species-specific oligonucleotide probes [18-20], PCR-based methods [21-27] or of monoclonal antibodies [28-30], may allow simultaneous detection and identification of multiple species present in a spoiled beer or brewery sample. However, these methods are rather expensive and time-consuming, lack throughput capacity and target a narrow range of beer spoilage species only. The present study investigated the effect of the (beer) matrix on the mass spectra generated, determined the lowest cell concentration required to obtain good quality mass spectra and examined the applicability of MALDI-TOF MS to detect and identify bacteria directly in enrichment cultures of spoiled beer and brewery samples with or without yeast cells present. Moreover, the suitability of MALDI-TOF MS for the analysis of polymicrobial spoiled beer samples was assessed by means of mixed axenic cultures.

6.2 Materials and Methods

6.2.1 *Bacterial strains*

Four strains of well-known beer spoilage microorganisms were selected, *i.e.*, *Lactobacillus brevis* strains LMG 11401 and LMG 16322, *Lactobacillus lindneri* strain LMG 11404 and *Pediococcus claussenii* strain LMG 21948^T. Each of these strains was originally isolated from spoiled beer samples ([31]; http://www.belspo.be/bccm/). The strains used were obtained from the BCCM/LMG Bacteria Collection (http://www.belspo.be/bccm/; Ghent, Belgium) and were cultured aerobically according to the provider's instructions onto de Man, Rogosa and Sharpe (MRS) medium (Oxoid). After resuscitating lyophilised cells, strains were subcultured twice prior to further analysis.

6.2.2 *Sample preparation and MALDI-TOF MS data acquisition and analysis*

The cell extract preparation procedure described previously [32] for the analysis of axenic cultures was initially used. In brief, five mg of wet cells was suspended in 300 µL Milli-Q water, after which 900 µL pure ethanol was added [33]. After centrifugation (3 min, 20817 × *g*, at 4°C), 50 µL 70% formic acid and 50 µL acetonitrile were added to the bacterial cell pellet. After shaking vigorously and centrifugation (3 min, 20817 × *g*, at 4°C), 1 µL of the supernatant was spotted in duplicate onto a MALDI-TOF MS stainless steel target plate to obtain two technical replicates. Immediately after drying, the spots were overlaid with 1 µL matrix solution, which consisted of 5 mg α-cyano-4-hydroxycinnamic acid (α-CHCA) dissolved in 1 mL acetonitrile/trifluoroacetic acid/Milli-Q water solvent (50:2:48). Subsequently, MALDI-TOF MS data acquisition, data import in the BioNumerics 7.1 software (Applied Maths N.V., Belgium) and mass spectral quality control was performed as described previously [32].

Data were analysed using a curve-based and peak-based approach as described in **CHAPTER 5.1**. In brief, the peak-based data analysis matched all peaks in the mass spectra to a peak class using constant and linearly varying tolerance values of 2 *m/z* and 800 ppm, respectively [34]. The obtained dataset was subsequently converted into a binary character set and analysed using the Dice coefficient and the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster algorithm. The curve-based data analysis of mass spectra was performed using the Pearson product-moment correlation coefficient and the UPGMA cluster algorithm.

6.2.3 *The influence of the beer matrix on MALDI-TOF mass spectra*

Strains *Lb. brevis* LMG 16322 and LMG 11401 were used to determine the influence of the beer matrix on the mass spectra generated. In the standard procedure, an amount of five mg of wet bacterial cells was suspended in 300 µL lager pilsner beer prior to extraction. The effect of different amounts of cells suspended in beer on the mass spectra generated was subsequently examined by suspending 600 mg of wet cells into 3 mL lager pilsner beer (= twelve times more than in the standard procedure) after which a dilution series was prepared corresponding with 12× to 0.2× the standard cell amount (**TABLE 6.1**). Cell extracts were prepared from each of these dilutions. Also, the cell extraction protocol described above was modified by incorporating two consecutive washing steps in Milli-Q water prior to cell extraction to remove beer matrix compounds: after centrifugation for 3 min at 20817 × *g* at 4°C, the bacterial cell pellet was resuspended in 300 µL Milli-Q water, and this was repeated after another centrifugation for 3 minutes at 5000 × *g* (at 4°C). This altered protocol was used for the further study unless specifically mentioned. Blank extracts were also prepared from Milli-Q water or lager pilsner beer without bacterial cells.

TABLE 6.1. The dilution series prepared to assess the influence of the beer matrix in correlation with the cell amount present on the mass spectra generated. The master suspension was prepared by suspending 600 mg of wet cells into 3 mL of lager pilsner beer (*i.e.*, 12× the standard cell amount for MALDI-TOF MS analysis).

Dilution	Master suspension (µL)	Lager pilsner beer (µL)
12×	300	0
10×	250	50
8×	200	100
6×	150	150
4×	100	200
2×	50	250
1×	25	275
0.4×	10	290
0.2×	5	295

6.2.4 *Determination of the lowest cell concentration required*

The sensitivity of MALDI-TOF MS was determined using 2 mL suspensions of *P. claussenii* strain LMG 21948^T and *Lb. brevis* strain LMG 16322 (**TABLE 6.2**). Cell suspensions with an optical density of 1 at 590 nm (OD_{590}) were prepared for both strains and cell numbers were determined by plating 50 µL of each dilution (*i.e.*, 10^0 to 10^{-7}) of a tenfold dilution series in saline in triplicate onto MRS agar. Colonies on plates comprising 25 to 250 CFU were counted after five days of aerobic incubation at 30°C and the CFU/mL was calculated.

TABLE 6.2. Suspensions containing different cell numbers were prepared from a suspension with an OD_{590} of 1 in order to determine the lowest cell concentration required for MALDI-TOF MS identifications.

Dilution	OD_{590} 1 suspension (µL)	Saline (µL)
2×	2000	0
1.8×	1800	200
1.6×	1600	400
1.4×	1400	600
1.2×	1200	800
1×	**1000**	**1000**
0.8×	800	1200
0.6×	600	1400
0.4×	400	1600
0.2×	200	1800

6.2.5 *Analysis of monomicrobial spoiled brewery samples with or without yeast cells present*

Four beer and brewery samples (A to D) were examined. Sample A was a spoiled dinner beer with an alcohol content of 1.5% v/v. Sample B was collected from spoiled wort. Both samples C and D were spoiled top fermented beers with an alcohol content of 7.5% v/v and contained yeast cells due to refermentation in the bottle. Microscopic analysis revealed the presence of bacteria in each of these samples. An enrichment step was performed by incubating the spoiled sample in double concentrated Nachweismedium für bierschädliche Bakterien (NBB) broth medium (Conda Pronadisa) in a 1:1 ratio. The spoilage bacteria of each of these samples were isolated and identified during a previous study using MALDI-TOF MS and state of the art taxonomic methods (**CHAPTER 5.1**). A monoculture of *Lactobacillus malefermentans* was isolated from sample A while monocultures of *Lb. brevis* were isolated from the three remaining samples. The mass spectra of the axenic cultures were thus available for comparison in the present study.

Fifty mL of a 3 days old enrichment culture of samples A and B were used for the preparation of cell extracts. In order to separate the yeast fraction from the bacterial fraction several procedures were tested using 7 days old enrichment cultures of samples C and D (**FIG 6.1**). In procedure 1 yeast cells in 100 mL enrichment culture were allowed to settle down at low temperature (3 h at 4°C) after which a maximum of enrichment culture was collected without disturbing or collecting the yeast cells. This fraction of the enrichment culture was centrifuged (3 min, 20817 × *g*, at 4°C), and the cell pellet was used for the cell extract preparation. Procedure 2 consisted of three subsequent centrifugation steps at a low speed [35] (20 min, 10 × *g*, at 4°C) starting with 50 mL enrichment culture. After each centrifugation step, the supernatant was collected and the cell pellet was re-suspended in 25 mL saline. The supernatants were combined and then centrifugated at high speed (3 min, 5000 × *g*, at 4°C) to spin down the bacterial cells. The final cell pellet was used for extract preparation. In procedure 3, three cellulose nitrate filters (5 µm, 8 µm and 0.45 µm [Whatman]) were stacked and 100 mL of enrichment culture was filtered through this series of three filters under negative pressure. The yeast cells were primarily captured on the 5 µm and to some extent on the 8 µm filters while the bacterial cells were collected on the 0.45 µm filter. The 0.45 µm filter was submerged in saline and gently vortexed to release the bacterial cells. The filter was subsequently removed and the cell pellet was collected after centrifugation (3 min, 5000 × *g*, at 4°C). Similarly, in procedure 4 the same filtration steps were applied to collect the bacterial cell fraction from 100 ml of the original spoiled sample. However, the 0.45 µm filter with the bacterial cells was submerged in NBB broth and incubated at 30°C for 3 days. Cells present in 2 mL of the enrichment culture were subsequently collected by centrifugation (3 min, 20817 × *g*, at 4°C) for extract preparation.

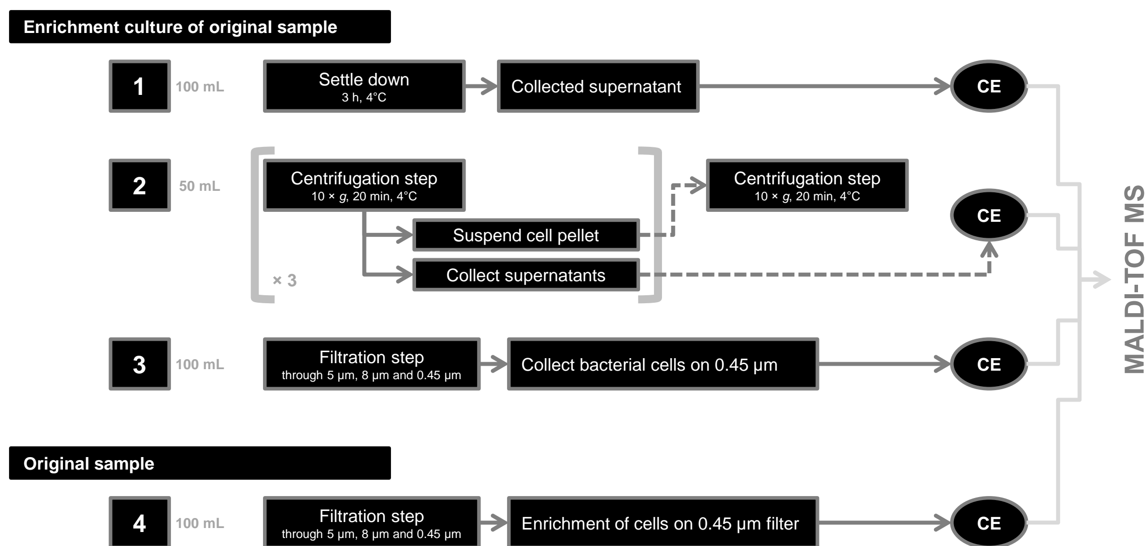

FIG 6.1. Scheme of the four procedures tested to separate the yeast fraction from the bacterial fraction.

6.2.6 *Analysis of mixtures of suspensions of axenic cultures*

The effect of mixed cultures on the mass spectra generated was determined using *P. claussenii* strain LMG 21948^T, *Lb. brevis* strain LMG 16322 and *Lb. lindneri* strain LMG 11404. Per strain, a suspension with an OD_{590} of 1 was prepared and cell counts were determined as described above. Two mL mixtures of different amounts of two strains were prepared as outlined in **TABLE 6.3**.

TABLE 6.3. Mixed cultures were prepared by mixing the suspensions of two strains with an OD_{590} of 1 in different proportions.

	OD_{590} 1 suspension (µL)	
Mixture	Strain A	Strain B
10|0	2000	0
9|1	1800	200
8|2	1600	400
7|3	1400	600
6|4	1200	800
5|5	1000	1000
4|6	800	1200
3|7	600	1400
2|8	400	1600
1|9	200	1800
0|10	0	2000

6.3 Results and Discussion

6.3.1 *The beer matrix strongly influences the mass spectra generated*

The beer matrix comprises several compounds like proteins, carbohydrates, and ions that can interfere during MALDI-TOF MS desorption and ionisation, and consequently suppress ionisation of bacterial proteins [12, 14, 36]. To investigate this, mass spectra were generated from bacterial suspensions prepared in lager pilsner beer. The mass spectrum generated from 5 mg of wet cells suspended in 300 µL lager pilsner beer was highly similar to that of the lager pilsner beer without bacterial cells (**FIG 6.2**) and did not share peaks with the mass spectrum of bacterial cells suspended in Milli-Q water (**FIG 6.2**). A set of repetitive peaks in the mass range of *m/z* 9000 to 12000 was observed in both the mass spectra of lager pilsner beer and in that of *Lb. brevis* cells suspended in 300 µL lager pilsner beer (**FIG 6.2**). However, the (bacterial) peak suppression effect decreased with increasing bacterial cell amounts (**FIG 6.3**) and the mass spectra of the suspensions with the highest cell amount resembled best that of the cells suspended in Milli-Q water. The extraction protocol was modified by incorporating two washing steps using Milli-Q water prior to cell extraction. The mass spectra then better resembled those generated from reference strains present in the MALDI-TOF

MS identification database (**FIG 6.4**; **FIG 6.5**); the same effect was also noticed at lower bacterial cell amounts (**FIG 6.6**). Therefore the two additional washing steps using Milli-Q water were used in the remainder of the present study unless mentioned specifically.

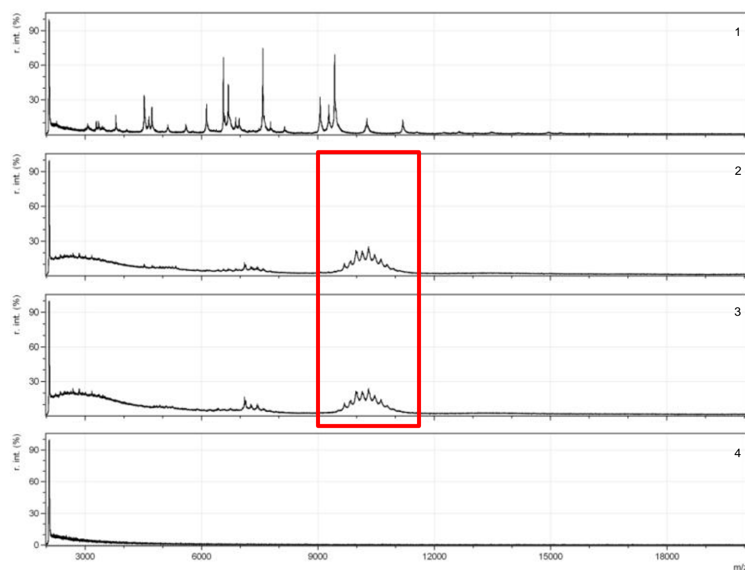

FIG 6.2. Mass spectra derived from 5 mg of wet cells of *Lb. brevis* strain LMG 16322 suspended in 300 µL Milli-Q water (**1**) and in 300 µL lager pilsner beer (**2**), of lager pilsner beer (**3**) and of Milli-Q water (**4**). A set of repetitive peaks in the mass range of *m/z* 9000-12000 is indicated inside the rectangle. r. int., relative intensity.

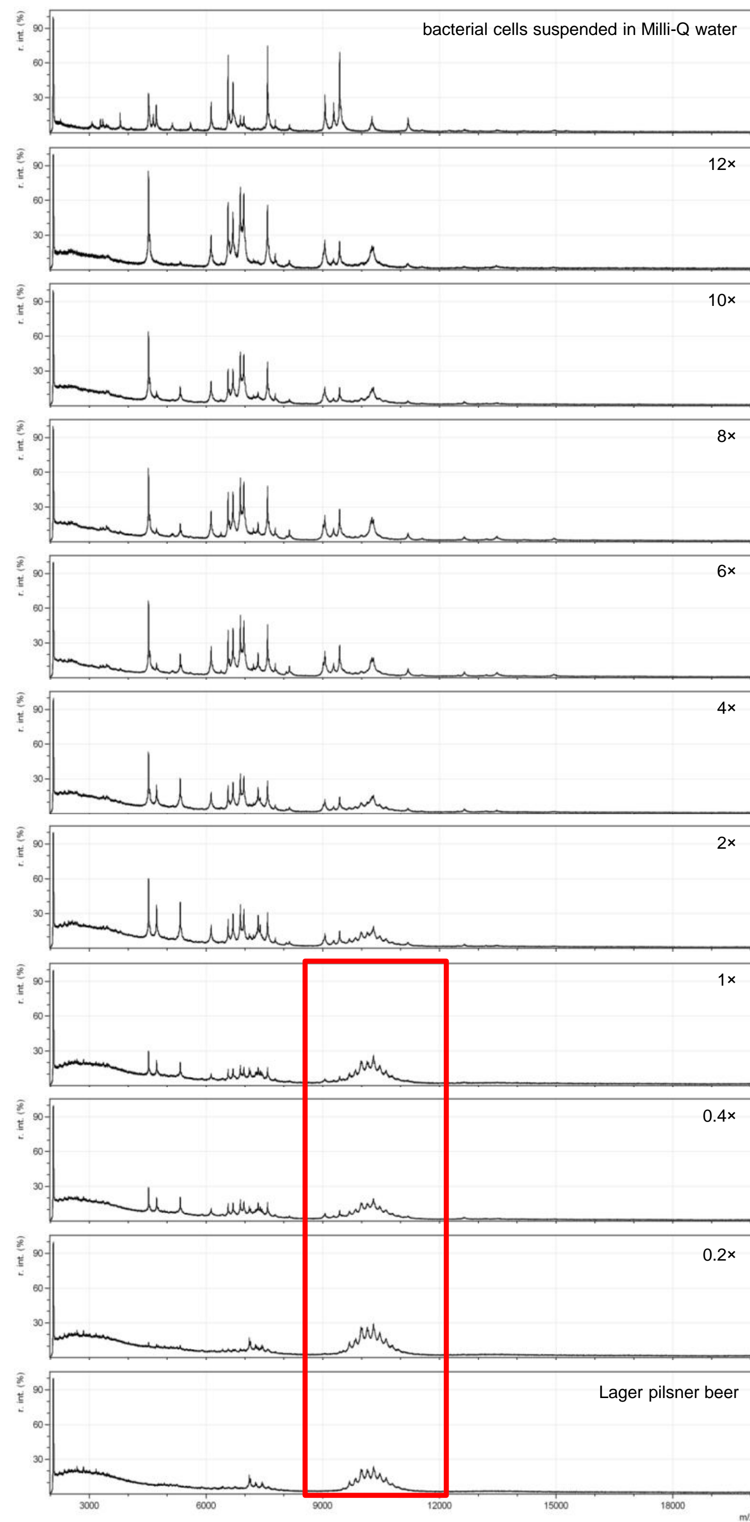

FIG 6.3. Mass spectra derived from a dilution series of *Lb. brevis* strain LMG 16322 cells in lager pilsner beer, ranging from 12× to 0.2× a standard cell amount 5 mg/300 µL. Mass spectra generated from 5 mg of wet cells of *Lb. brevis* strain LMG 16322 suspended in Milli-Q water and from the lager pilsner beer are shown as well. A set of repetitive peaks in the mass range of *m/z* 9000-12000 is indicated inside the rectangle. r. int., relative intensity.

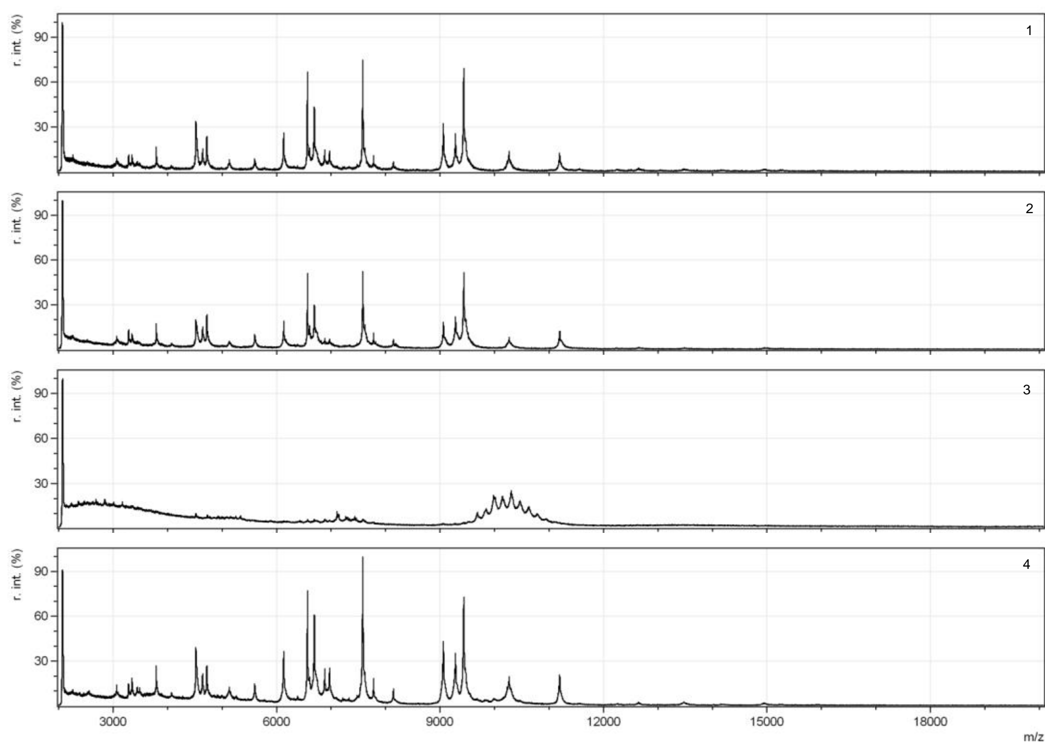

FIG 6.4. Mass spectra generated from 5 mg wet cells of *Lb. brevis* strain LMG 16322 cells suspended in 300 µL Milli-Q water (**1** and **2**) and 300 µL lager pilsner beer (**3** and **4**) without (**1** and **3**) and with (**2** and **4**) two additional washing steps in Milli-Q water. r. int., relative intensity.

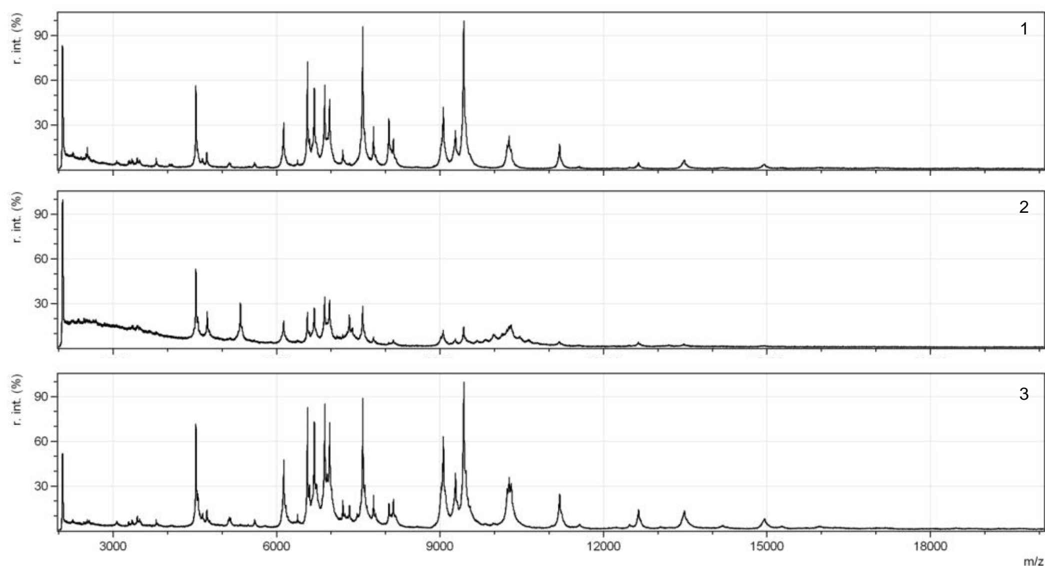

FIG 6.5. Mass spectra generated from the 4× dilution of *Lb. brevis* strain LMG 16322 cells in Milli-Q water (**1**) and lager pilsner beer (**2** and **3**) without (**1** and **2**) and with (**3**) two additional washing steps in Milli-Q water. r. int., relative intensity.

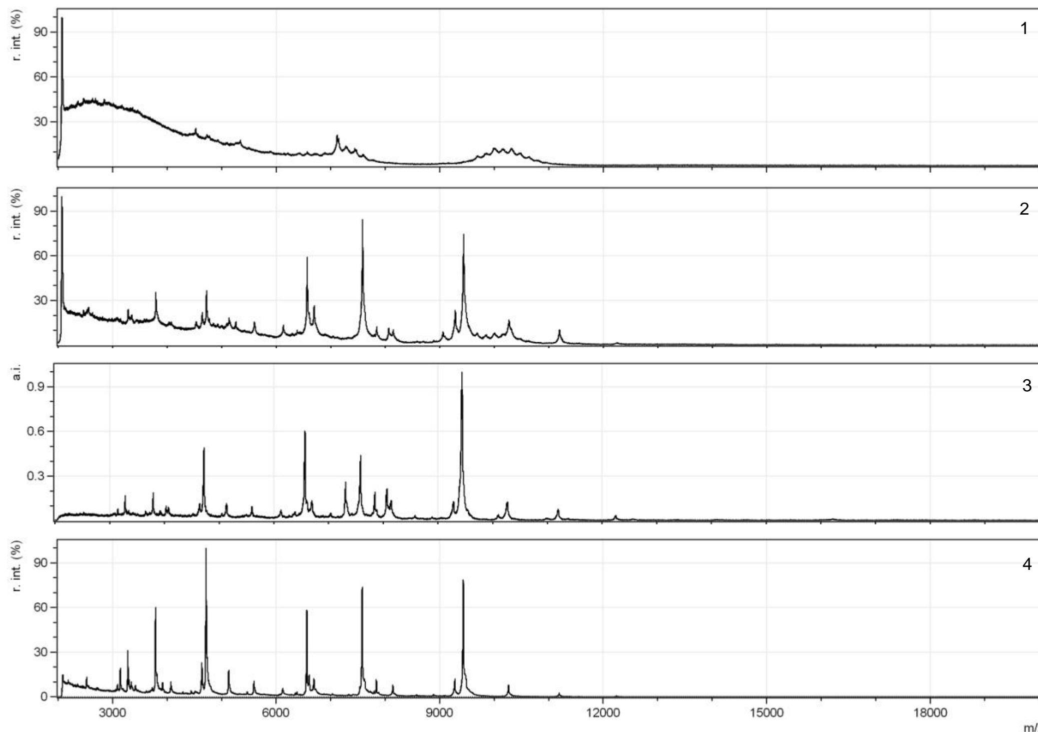

FIG 6.6. Mass spectra generated from the 0.4× dilution of *Lb. brevis* strain LMG 11401 cells in lager pilsner beer (**1** and **2**) and Milli-Q water (**3**); and of 5 mg cells suspended in 300 µL Milli-Q water (**4**) without (**1**, **3** and **4**) and with (**2**) two additional washing steps in Milli-Q water. r. int., relative intensity.

6.3.2 *Direct detection of spoilage bacteria requires a sufficiently high cell concentration for MALDI-TOF MS analysis and necessitates an enrichment of microorganisms present in spoiled beer and brewery samples*

Spoiled beer and brewery samples mostly contain limited numbers of bacterial cells. Therefore, the effect of increasing cell concentrations on the mass spectra generated was determined using *P. claussenii* strain LMG 21948^T and *Lb. brevis* strain LMG 16322. The quality of the mass spectra generated from *P. claussenii* LMG 21948^T was strongly affected at cell concentrations below approximately 2×10^7 CFU/mL (*i.e.*, 0.05×), due to an increase of repetitive background signals and decrease of data richness (*i.e.*, number of peaks detected) (**FIG 6.7**).

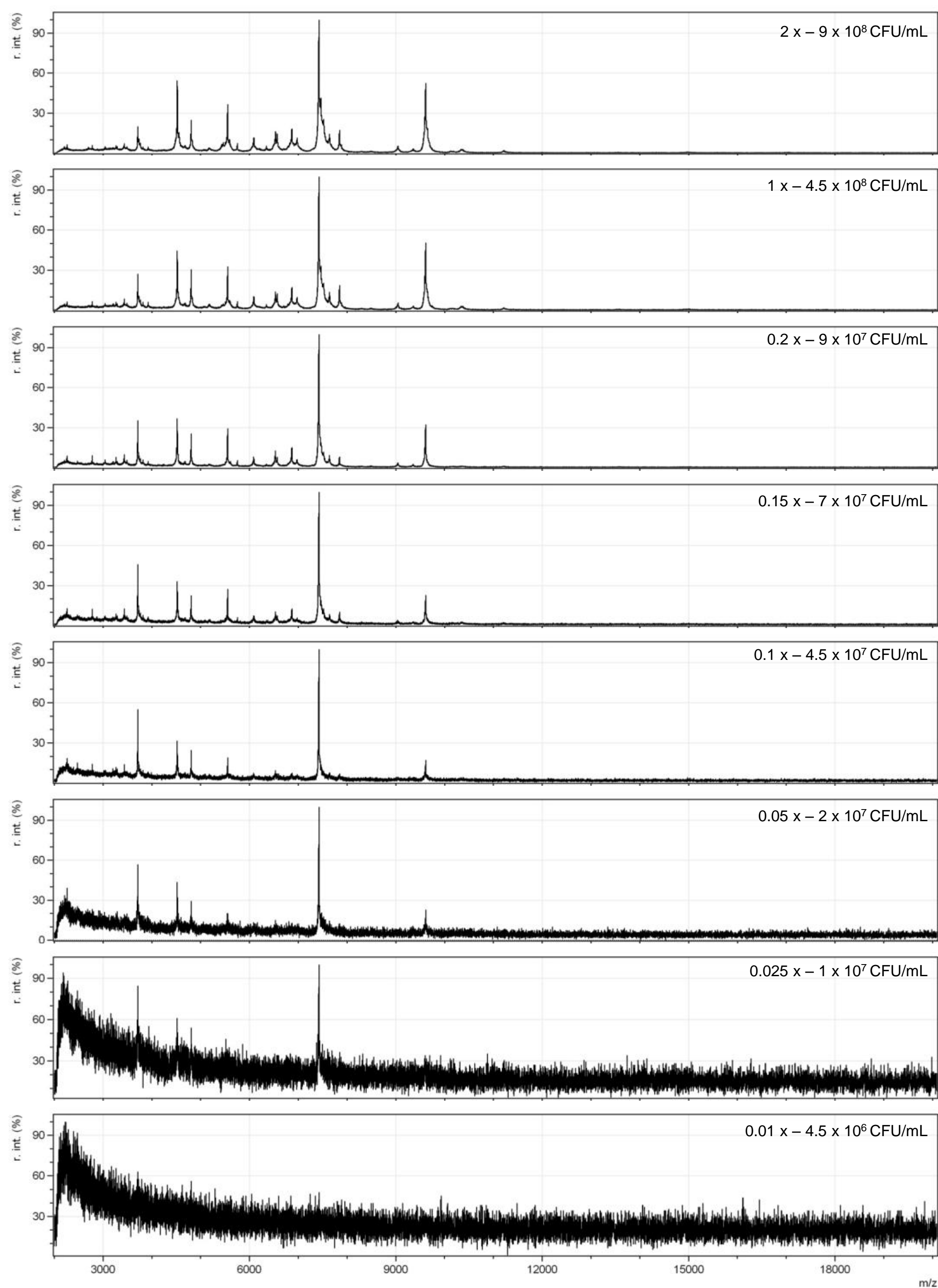

FIG 6.7. Mass spectra generated from a dilution series of *P. claussenii* strain LMG 21948^T with two additional washing steps in Milli-Q water. The dilution and the corresponding CFU/mL values are shown for each spectrum. A decrease in mass spectral quality was visually observed starting from the 0.05 × dilution (corresponding to 2 × 10^7 CFU/mL), as background peaks became more prominent. r. int., relative intensity.

The peak-based cluster analysis revealed that mass spectra generated from the 0.15× to 2× suspensions (of about 2×10^7 to 9×10^8 CFU/mL) grouped together, and were separated from those of dilutions with a lower cell number (0.01× to 0.1×) (**FIG 6.8A**). In contrast, the lowest cell concentration required of *Lb. brevis* LMG 16322 was higher compared to that of *P. claussenii* LMG 21948^T, as the mass spectral quality visually decreased below a cell concentration lower than approximately 7×10^7 CFU/mL (data not shown). Moreover, the peak-based cluster analysis grouped the mass spectra generated from the 0.15× to 2× suspensions (*i.e.*, 1×10^8 to 1.4×10^9 CFU/mL) separately from mass spectra generated from the 0.01× to 0.1× suspensions (**FIG 6.8B**). The number of peaks detected in the mass spectra decreased with lowering cell numbers. No peaks were detected in the mass spectra derived from the 0.01× suspension (about 4.5×10^6 CFU/mL) or from the 0.05× (about 3.5×10^7 CFU/mL) suspensions of strains *P. claussenii* LMG 21948^T and *Lb. brevis* LMG 16322, respectively (**FIG 6.8**). Based on a visual inspection and peak-based cluster analysis, the minimal cell concentration required to generate mass spectra that were still useful for species level MALDI-TOF MS identification was determined at approximately 5×10^7 to 1×10^8 CFU/mL, which corresponds with previous reports [5, 7, 9-12]. The number of cells of spoilage microorganisms in most spoiled beer and brewery samples is very low (*i.e.*, 1 to 50 CFU/100 to 250 mL sample) [37-40]; hence an enrichment step will be required to obtain mass spectra suitable for MALDI-TOF MS based identification.

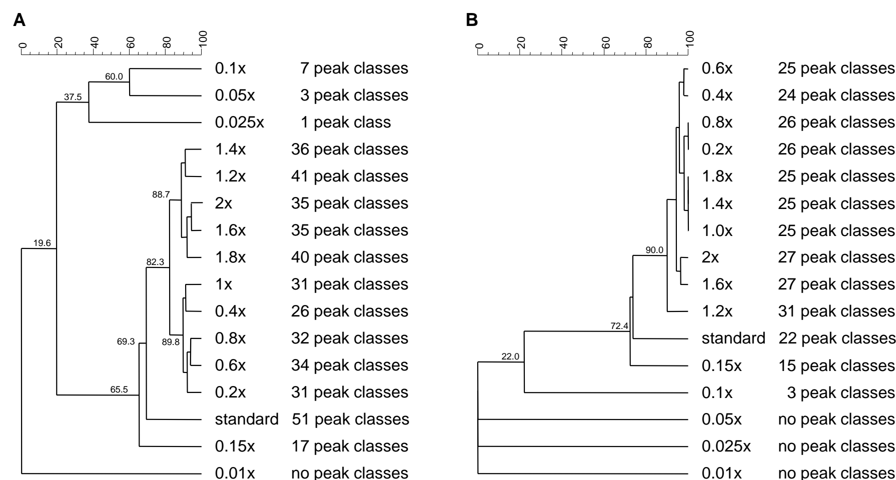

FIG 6.8. Peak-based cluster analysis using the binary Dice coefficient and UPGMA cluster algorithm of mass spectra generated from a dilution series of (**A**) *P. claussenii* strain LMG 21948^T and (**B**) *Lb. brevis* strain LMG 16322. The mass spectrum generated from 5 mg of wet cells per 300 µL Milli-Q water for both strains was included in the cluster analysis (and was labelled as 'standard'). The number of peak classes detected in each mass spectrum is shown.

6.3.3 *MALDI-TOF MS is well-suited for the detection and identification of a bacterium present in enrichment cultures of monomicrobially spoiled beer and brewery samples with or without yeast cells present*

Microscopic analysis of sample A revealed the presence of bacterial cells only. The mass spectrum generated from a 50 mL enrichment culture of sample A was similar to that of strain R-49868, the spoilage bacterium previously isolated from sample A, and to that of *Lb. malefermentans* strains LMG 11455^T, a reference strain present in our MALDI-TOF MS identification database (**FIG 6.9**). Curve-based cluster analysis revealed that mass spectra generated from the enrichment culture and R-49868 grouped together with a high similarity (**FIG 6.10**).

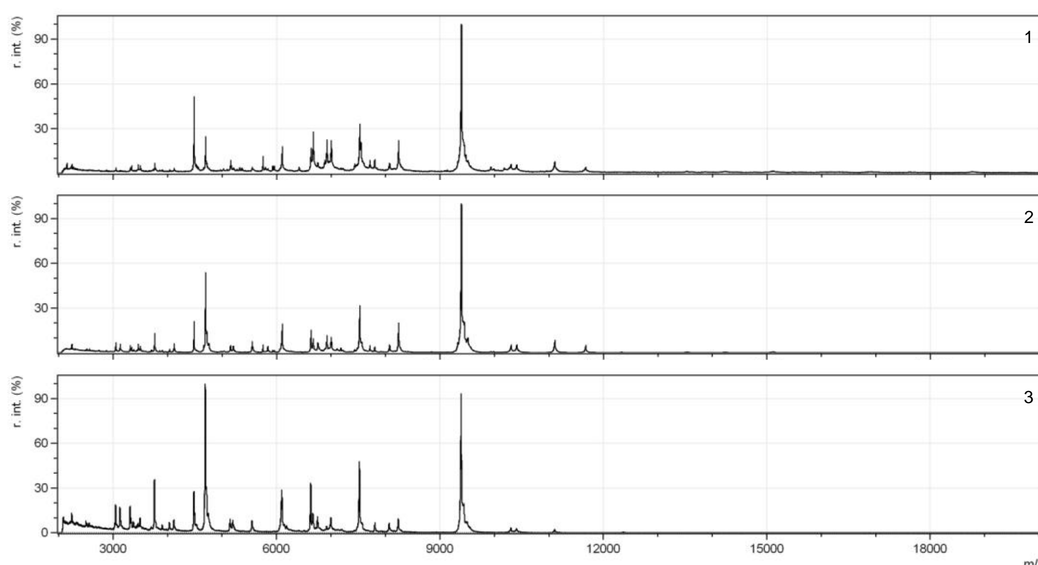

FIG 6.9. Mass spectra generated from 50 mL of enrichment culture of sample A (**1**); from *Lb. malefermentans* strain R-49868, the isolate previously obtained from sample A (**2**); and from *Lb. malefermentans* strain LMG 11455^T (**3**), a reference strains present in the MALDI-TOF MS identification database. r. int., relative intensity.

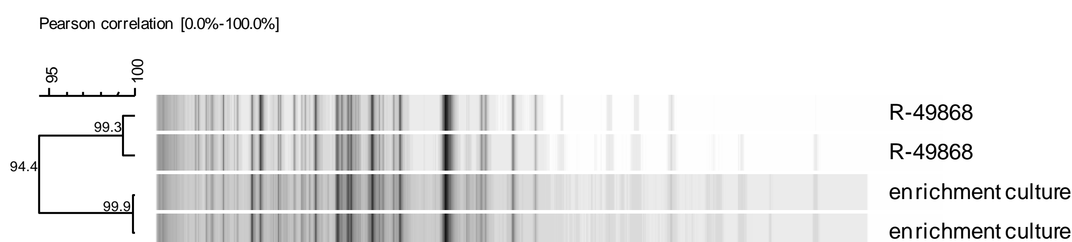

FIG 6.10. The curve-based cluster analysis using the Pearson product-moment correlation coefficient and UPGMA cluster algorithm of mass spectra of technical replicates derived from enrichment culture of sample A and *Lb. malefermentans* strain R-49868, an isolate obtained from sample A.

Microscopic analyses of samples B to D revealed besides bacterial cells also the presence of mould (sample B) and yeast cells (sample C and D) which are known to interfere during desorption and ionization, and consequently suppress ionization of bacterial proteins [12, 14]. Hence, different sample treatment procedures were tested to retain the bacterial fraction for further analysis. The mass spectra generated directly from a 50 mL enrichment culture of sample B were clearly ill-suited for further analysis due to a decrease in the mass spectral quality (*i.e.*, increase in repetitive background signals and poor data richness) (**FIG 6.11**). Procedure 1 and 2 were however ill-suited to separate the threadlike structure of the fungal mycelium from the bacterial cells. The use of procedure 3 to remove the mould cells improved the mass spectrum generated (**FIG 6.11**); however, the mass spectral quality was best when procedure 4 was used which yielded mass spectra highly similar to those obtained from *Lb. brevis* strain R-49879, the spoilage strain isolated from sample B, and from *Lb. brevis* strain LMG 16322 (a reference strain) (**FIG 6.11**).

The mass spectra generated from a 2 mL enrichment culture of sample C did not resemble that obtained from the isolate collected from sample C (strain awI16_9, **FIG 6.12**). Therefore, procedures 1 through 4 were applied to retain the bacterial fraction. The mass spectrum generated from the supernatant recovered using procedure 1 resembled that obtained from the *Lb. brevis* isolate awI16_9 (**FIG 6.12**). Procedure 2 failed to properly separate the yeast and bacterial fractions in the enrichment culture as microscopic analysis of the combined supernatants revealed the presence of numerous young buds of yeast cells. Not unexpectedly the mass spectrum obtained using this cumbersome procedure was not comparable with that generated from the axenic culture of strain awI16_9 (**FIG 6.12**). Procedure 3 proved even more ill-suited as the yeast cells fouled the pores of the filters and the amount of bacterial cells entrapped on the 0.45 µm filter was insufficient to generate mass spectra of good quality (data not shown). The mass spectrum generated using procedure 4 was of high quality and most comparable with that of the *Lb. brevis* isolate awI16_9 (**FIG 6.12**) which was confirmed by curve-based cluster analysis (**FIG 6.13**). Similarly, mass spectra generated using the latter procedure generated a mass spectrum of sample D that was highly similar to that obtained from *Lb. brevis* strain R-49856, which was previously isolated from sample D (**FIG 6.14**).

These results demonstrated that procedure 4 (based on a filtration procedure coupled to an enrichment step) is well-suited for the detection and identification of bacteria present in the enrichment cultures of spoiled beer and brewery samples containing yeast cells.

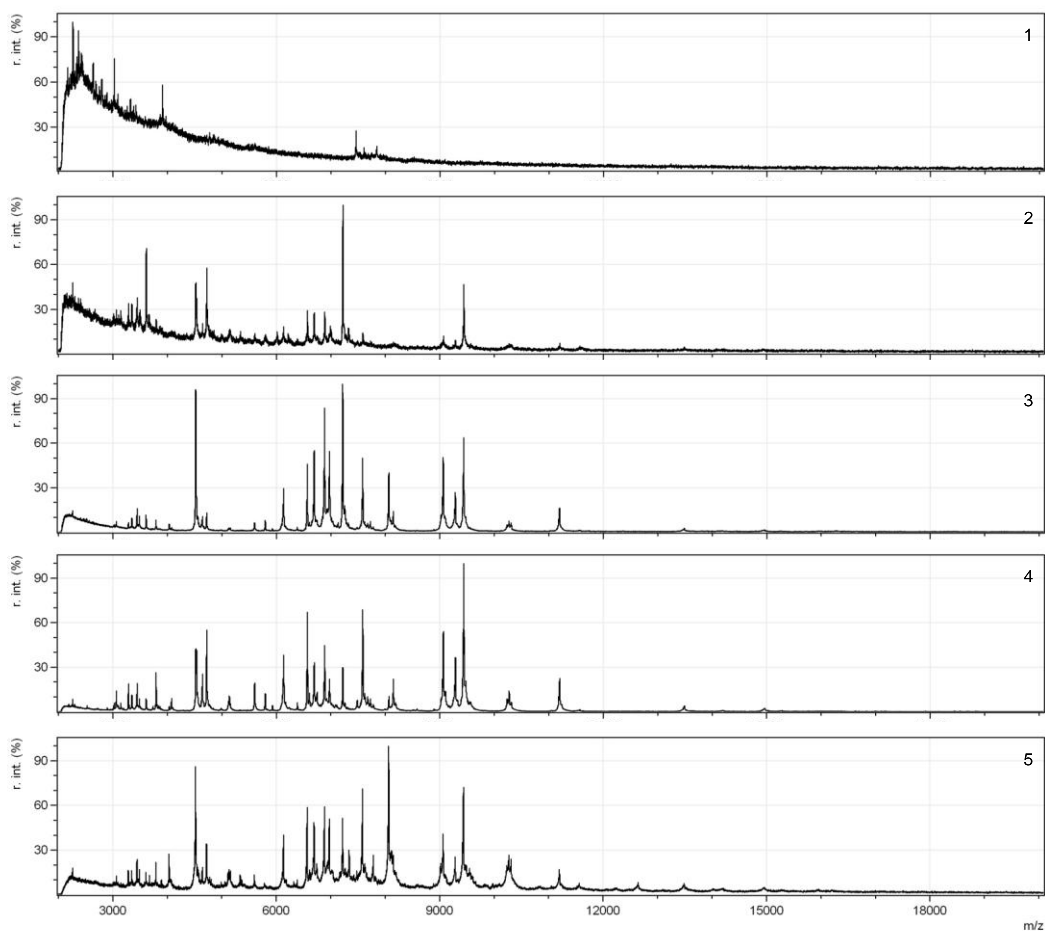

FIG 6.11. Mass spectra generated from cells extracted from 50 mL of the enrichment culture sample B (**1**). The presence of moulds resulted in mass spectra with poor quality. Therefore, mass spectra of the enrichment culture were generated using procedure 3 (**2**) and 4 (**3**) to enable separation of the mould fraction from the bacterial fraction of the enrichment culture. Mass spectra generated from *Lb. brevis* strain R-49879 (**4**), and from *Lb. brevis* strain LMG 16322 (**5**) are shown. r. int., relative intensity.

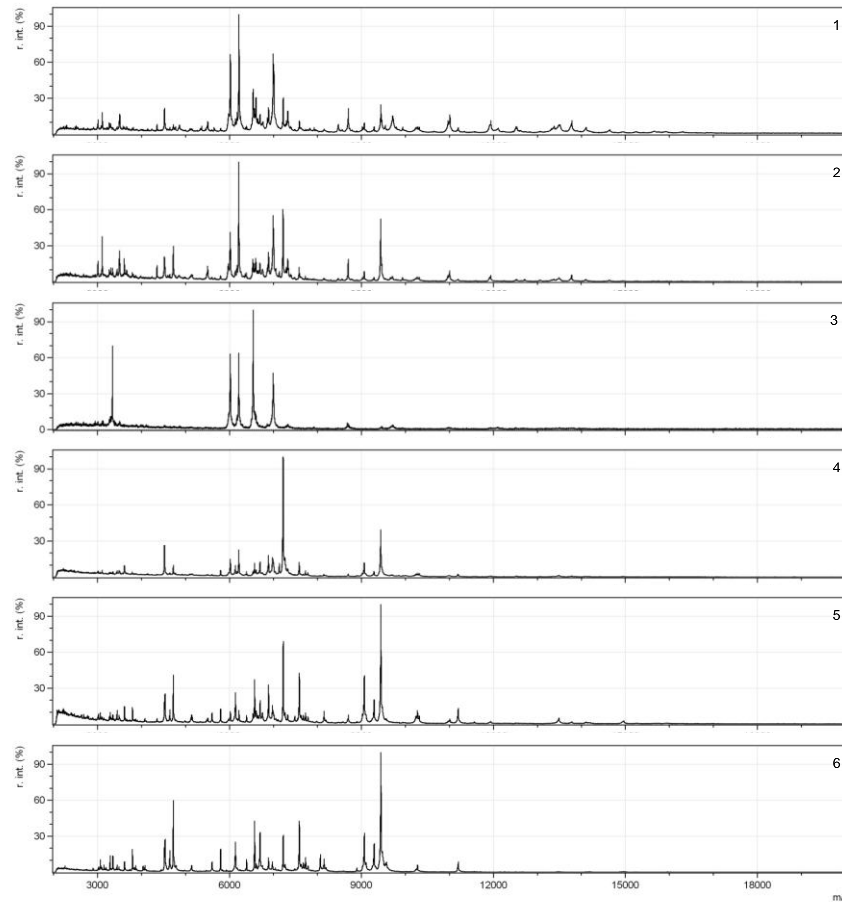

FIG 6.12. Mass spectra generated from 2 mL of the enrichment culture of sample C (**1**). The abundantly present yeast cells resulted in mass spectra that did not resemble that generated from *Lb. brevis* strain awI16_9, an isolate previously obtained from sample C (**6**). Similarly, mass spectra generated from the resulting supernatant (**2**) and the cell pellet (**3**) when procedure 2 was applied did not resemble that of the isolate's mass spectrum. The mass spectra generated when procedure 1 (**4**) and procedure 4 (**5**) were applied were similar to the isolate's mass spectrum (**6**). r. int., relative intensity.

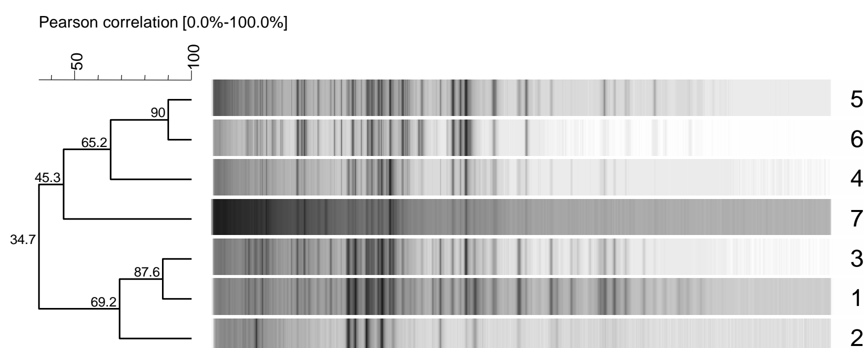

FIG 6.13. The curve-based cluster analysis using the Pearson product-moment correlation coefficient and UPGMA cluster algorithm of mass spectra derived from 2 mL of the enrichment culture of sample C (**1**). This mass spectrum grouped closely together with those obtained when cell extracts were prepared from the cell pellet (**3**) and the supernatant (**2**) obtained when procedure 2 was applied. The mass spectrum obtained from *Lb. brevis* strain awl16_9, an isolate previously obtained from sample C (**6**), grouped together with high similarity with that obtained when procedure 4 (**5**) was used. The mass spectrum generated when procedure 3 (**7**) was used had a poor mass spectral quality en clustered separately from the isolate's mass spectrum. Also, the mass spectrum obtained from the supernatant when procedure 1 (**4**) was applied clustered separately from that generated from *Lb. brevis* strain awl16_9 (**6**).

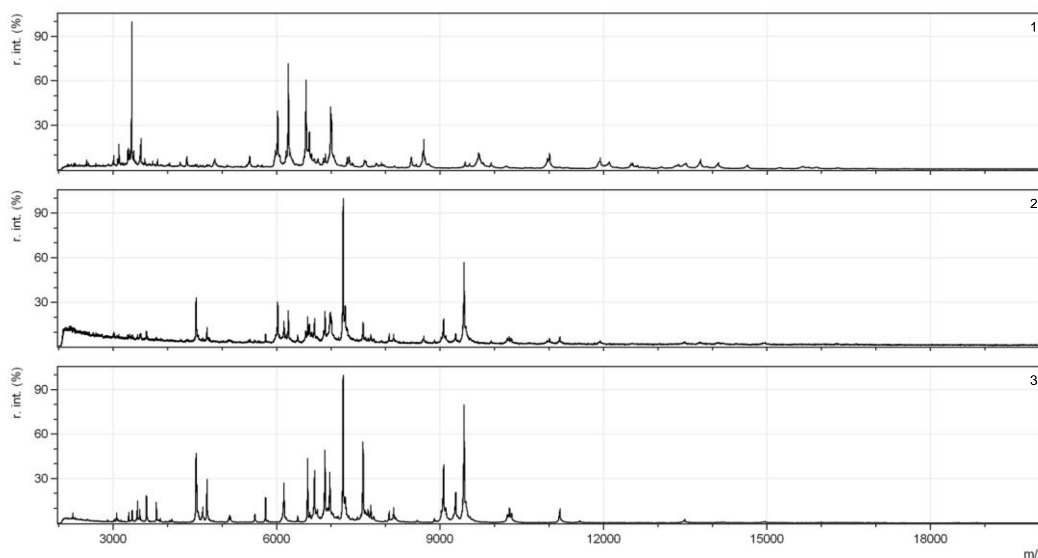

FIG 6.14. Mass spectra generated from 2 mL of the enrichment culture of sample D (**1**). The abundantly present yeast cells resulted in mass spectra that did not resemble that generated from *Lb. brevis* strain R-49856, an isolate previously obtained from sample D (**3**). The mass spectrum obtained when procedure 4 (**2**) was used was similar to that generated from the isolate (**3**). r. int., relative intensity.

6.3.4 *The analysis of mixtures of spoilage bacteria*

The MALDI-TOF MS technique has been successfully applied for the direct identification of contaminants present in enrichment cultures of clinical specimens like blood and urine, but such identifications were challenged when specimens were contaminated with more than one microorganism [12, 15, 41]. In the brewery industry, it is common that multiple strains belonging to different species simultaneously cause beer spoilage [42] (**CHAPTER 5.1**). We therefore investigated the MALDI-TOF mass spectra of mixtures of several pairs of spoilage bacteria as outlined in **TABLE 6.3**.

Analysis of the mass spectra of mixtures of *P. claussenii* LMG 21948^T and *Lb. brevis* LMG 16322 revealed that the most abundant peak classes (*e.g.*, *m/z* 4806.06 ± 4.97, 5553.01 ± 6.44, 7423.33 ± 7.94 and 9609.87 ± 9.69) present in the mass spectrum derived from the axenic culture of LMG 21948^T persisted in the mass spectra generated of each of the mixtures (**FIG 6.15**). An expected decrease in peak signal intensity with a decreasing proportion of LMG 21948^T cells was observed (**FIG 6.15**). Some of the less abundant peaks present in the mass spectrum of the axenic suspension of LMG 21948^T persisted also in the mass spectra generated for each of the mixtures (*e.g.*¸ *m/z* 2777.24 ± 4.22, 3712.61 ± 4.97, 5751.41 ± 6.60 and 6089.95 ± 6.87), while others were increasingly masked with a decreasing proportion of LMG 21948^T cells (*e.g.*, *m/z* 5435.80 ± 6.35, 5463.58 ± 6.37, 7519.84 ± 8.02 and 9044.69 ± 9.24). The most abundant peak classes present in the mass spectrum of the axenic culture of LMG 16322 (*e.g.*, *m/z* 6134.36 ± 6.91, 6886.01 ± 7.51, 7584.96 ± 8.07 and 9439.46 ± 9.55) persisted in the mass spectra generated from the mixtures with *P. claussenii* LMG 21948^T and a similar decrease in peak signal intensity with a decreasing proportion of LMG 16322 cells was observed (**FIG 6.15**). The abundant peak class with *m/z* 4520.62 ± 5.62 was dominantly present in the mass spectra obtained from all cell mixtures but this peak was also present in the spectra of each of the axenic cultures (**FIG 6.15**; data not shown).

The most abundant peak classes present in the mass spectrum generated from the axenic culture of *Lb. brevis* LMG 16322 persisted in all mass spectra generated of mixtures with *Lb. lindneri* LMG 11404 (**FIG 6.16**). However, a decrease in peak signal intensity with an increasing proportion of LMG 11404 was less apparent (**FIG 6.16**) indicating that the most abundant peak classes of axenic cultures may not always persist in mass spectra of such mixed cultures. For instance, the abundant peak class *m/z* 5334.42 ± 6.27 which is present in the mass spectrum of the axenic culture of LMG 11404 was masked in the spectrum generated upon mixing with LMG 16322 (**FIG 6.16**). Other abundant peak classes (*e.g.*, *m/z* 4806.71 ± 5.85 and 9611.20 ± 9.69) that were able to persist in the mass spectra of certain mixtures were gradually masked with an increasing proportion of other cells (**FIG 6.16**).

Similarly, the most abundant peak classes present in the mass spectrum generated from the axenic culture of LMG 11404 were suppressed in mass spectra of mixtures with an increasing cell concentration of LMG 21948^T (data not shown). Colony counts of the axenic suspensions with an OD_{590} of 1 revealed that approximately 5 times and 3.5 times more CFU/mL were detected in the suspensions of *Lb. brevis* LMG 16322 (6.9×10^8 CFU/mL) and *P. claussenii* LMG 21948^T (4.5×10^8 CFU/mL), respectively, compared to that of *Lb. lindneri* LMG 11404 (1.3×10^8 CFU/mL) which likely contributed to the suppression effect observed in mass spectra of mixed suspensions with the latter [14, 43, 44].

The identification of microbes in mixtures may therefore be extremely challenging, considering these peak suppression effects which are confounded by the identity and proportions of the individual microorganisms present in the mixture. We subsequently compared the mass spectra generated from the mixed cell suspensions with those of axenic well-characterised reference strains present in the MALDI-TOF MS identification database (**CHAPTER 5.1**) using the peak-based cluster analysis approach. Some spectra of mixed cell suspensions simply clustered separately in the numerical analysis; others grouped in species-specific clusters among spectra from axenic cultures revealing species level identification of, probably, the most abundant microorganism present. For example, the mixtures containing cells of *P. claussenii* LMG 21948^T and *Lb. brevis* LMG 16322 in a ratio of 9:1 and 1:9 grouped together with *P. claussenii* and *Lb. brevis*, respectively, and it could therefore be assumed that this contaminant was present in the mixed suspension.

The analysis of the mass spectra obtained from the enrichment cultures of samples A through D revealed similar results, *i.e.*, the spectra of these monocultures grouped in the respective *Lb. malefermentans* and *Lb. brevis* species-specific clusters. A comparison of peak classes present in the 540 mass spectra of 60 *Lb. brevis* reference strains with those in the spectrum generated from the enrichment culture of sample B showed that each of the peak classes observed in the latter spectrum also occurred in those of the *Lb. brevis* reference strains. A similar comparison of peak classes in the spectra of the enrichment cultures of samples C and D revealed six and eight peak classes, respectively, that were not present in those of *Lb. brevis* reference strains. These peak classes were also not observed in other spoilage or non-spoilage bacteria in our database suggesting they represent strain-specific markers, or possibly growth medium components. In contrast, a comparison of peak classes in the spectrum of the enrichment culture of sample A revealed that 39 out of 77 peak classes detected could not be linked to a peak present in the mass spectra generated from the two *Lb. malefermentans* reference strains that are included in our database, and that 20 out of these 39 peak classes were present in mass spectra generated from multiple LAB reference strains. Comparison of the mass spectra of (axenic cultures of) *Lb. malefermentans* reference strains with

those of *Lb. malefermentans* strains isolated previously from a spoiled sample (**CHAPTER 5.1**), and that of the enrichment culture revealed that only seven peak classes present in the mass spectrum of the enrichment culture of sample A were not present in those of the four *Lb. malefermentans* strains and none of the seven peak classes occurred in spectra of other LAB reference strains present in the database. These results demonstrated that the inclusion of multiple *Lb. malefermentans* reference strains increased the number of species- and strain-specific peak classes present for numerical analysis, and consequently decreased the number of uncorrelated peak classes present in the mass spectrum generated from the enrichment culture of sample A.

Hence, when numerical analysis of the spectrum of an enrichment culture assigns it to a certain species, a comparison of peak classes may or may not suggest the presence of additional species taking into account peak suppression effects, identity and ratios of contaminating strains as described above. For example, peak class analysis of mass spectra generated from the mixtures containing cells of *P. claussenii* LMG 21948^T and *Lb. brevis* LMG 16322 in a ratio of 9:1 and 1:9 (see above) revealed ten and six peak classes, respectively, that were not present in the mass spectra generated from reference strains of respectively *P. claussenii* or *Lb. brevis*. A comparison of peak classes in the spectrum of the 9:1 mixture with those generated from all reference strains correlated eight out of ten peak classes with *Lb. brevis*. In contrast, analysis of the mass spectrum of the 1:9 mixture with those of the reference strains associated the six peak classes with numerous *Pediococcus* reference strains. Yet, the mass spectrum generated of the mixture containing cells of both strains in a 5:5 ratio clustered together with spectra obtained from *Lb. brevis* reference strains. A comparison of peaks in the spectrum of this mixture with those of *Lb. brevis* reference strains revealed 8 peak classes that were not present in more than 540 mass spectra generated for 60 *Lb. brevis* reference strains. Interestingly, five of these eight peak classes were present in mass spectra of *P. claussenii* reference strains, which was indicative of a co-contamination with *P. claussenii*. Remarkably, not a single mass spectrum generated from the mixtures of *Lb. lindneri* LMG 11404 with either *Lb. brevis* LMG 16322 or *P. claussenii* LMG 21948^T grouped together with mass spectra of *Lb. lindneri* reference strains in a numerical analysis. For example, mass spectra generated from most of the mixtures containing cells of *P. claussenii* LMG 21948^T and *Lb. lindneri* LMG 11404 clustered together with those of *P. claussenii* reference strains; the one exception was the mass spectrum obtained from the 1:9 mixture. Numerical analysis of the latter spectrum revealed ten peak classes that were not present in the mass spectra of *P. claussenii* reference strains; however nine out of ten peak classes could be correlated unambiguously with *Lb. lindneri* reference strains.

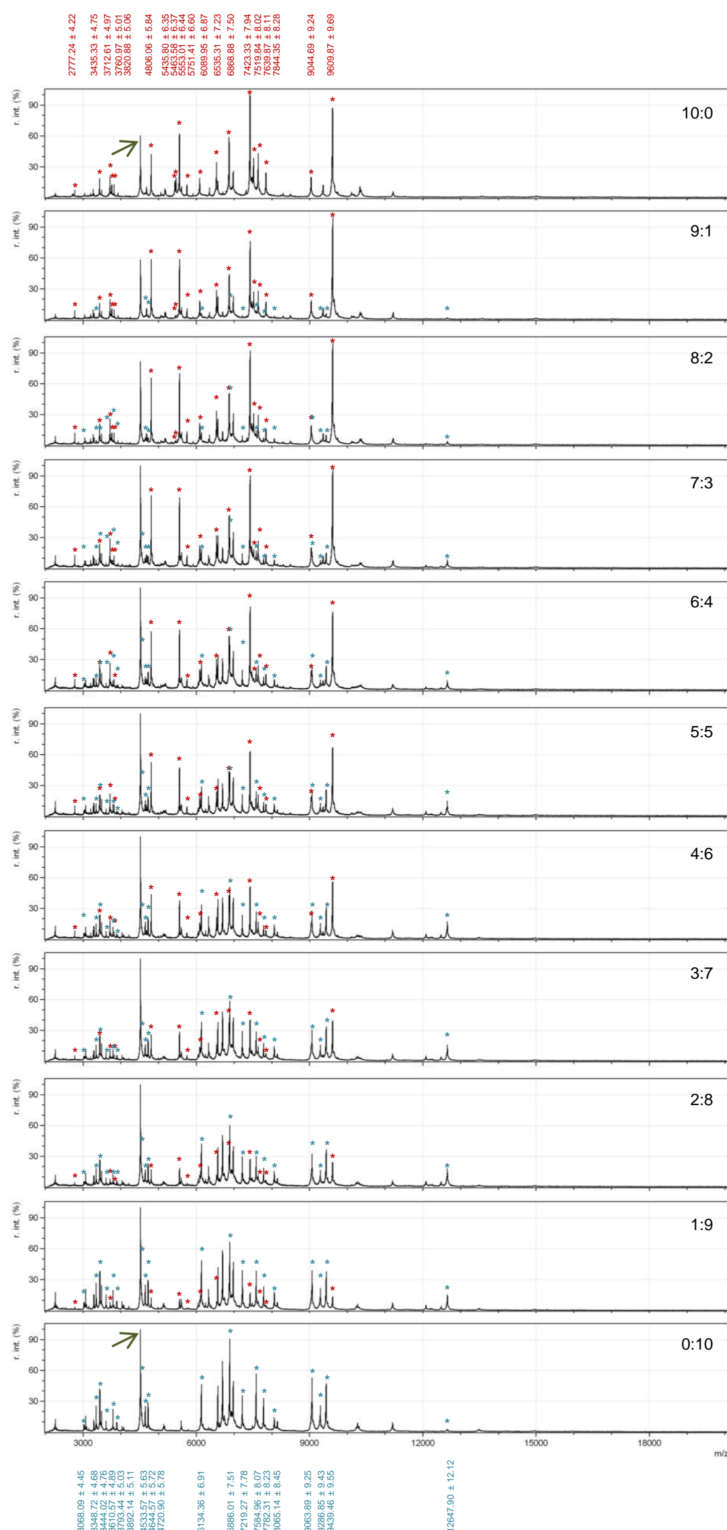

FIG 6.15. Mass spectra generated from mixed suspensions of *P. claussenii* strain LMG 21948[T] and *Lb. brevis* strain LMG 16322 (the ratio of cells of both strains is shown at the right hand side of each mass spectrum). A selection of the most abundant peak classes present in the mass spectra is highlighted and annotated in red (LMG 21948[T]) and in blue (LMG 16322) coloured asterisks. The abundant peak class *m/z* 4520.62 ± 5.62 which is present in each of the mass spectra is highlighted with a green arrow in both mass spectra generated from the axenic cultures. r. int., relative intensity.

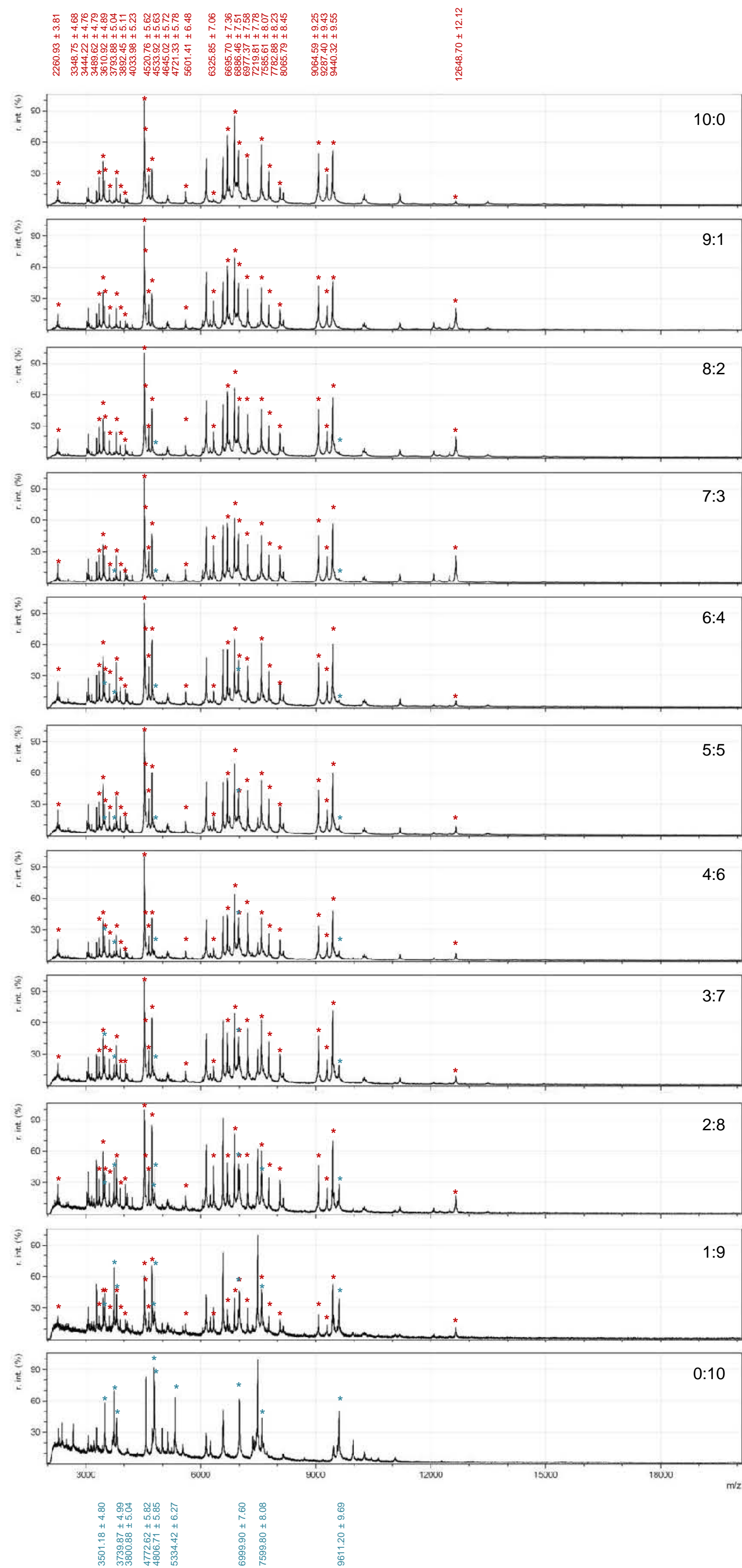

FIG 6.16. Mass spectra generated from mixed suspensions from *Lb. brevis* strain LMG 16322 and *Lb. lindneri* strain LMG 11404 (the ratio of cells of both strains is shown at the right hand side of each mass spectrum). A selection of the most abundant peak classes present in the mass spectra is highlighted and annotated in red (LMG 16322) and in blue (LMG 11404) coloured asterisks. r. int., relative intensity.

6.4 Conclusions

Species level identification of spoilage microorganisms is of utmost importance for the brewing industry even though not all strains of the same species share similar beer spoiling capabilities [1, 3]. Nevertheless, species level identification enables the estimation of the impact of bacterial spoilage and subsequently the prevention of further product deterioration and future spoilage. The present study demonstrated that the beer matrix has a strong impact on the mass spectra generated. A simple modification to the sample preparation protocol by incorporating two additional Milli-Q water washing steps prior to cell extraction facilitated the removal of the matrix compounds. Moreover, an optimised sample preparation based on a filtration procedure coupled to an enrichment step enabled detection and identification of bacteria directly from enrichment cultures of spoiled beer and brewery samples, even in the presence of other yeast or mould cells (**FIG 6.17**). Although this preliminary identification result may provide the brewery's quality manager with pivotal information to prevent further product deterioration or future spoilage, our data demonstrate that it is highly recommended to plate these enrichment cultures and use MALDI-TOF MS as a superior tool to dereplicate and identity the isolates obtained [15, 16] (**CHAPTER 5.1**) to confirm or complement the preliminary identification result.

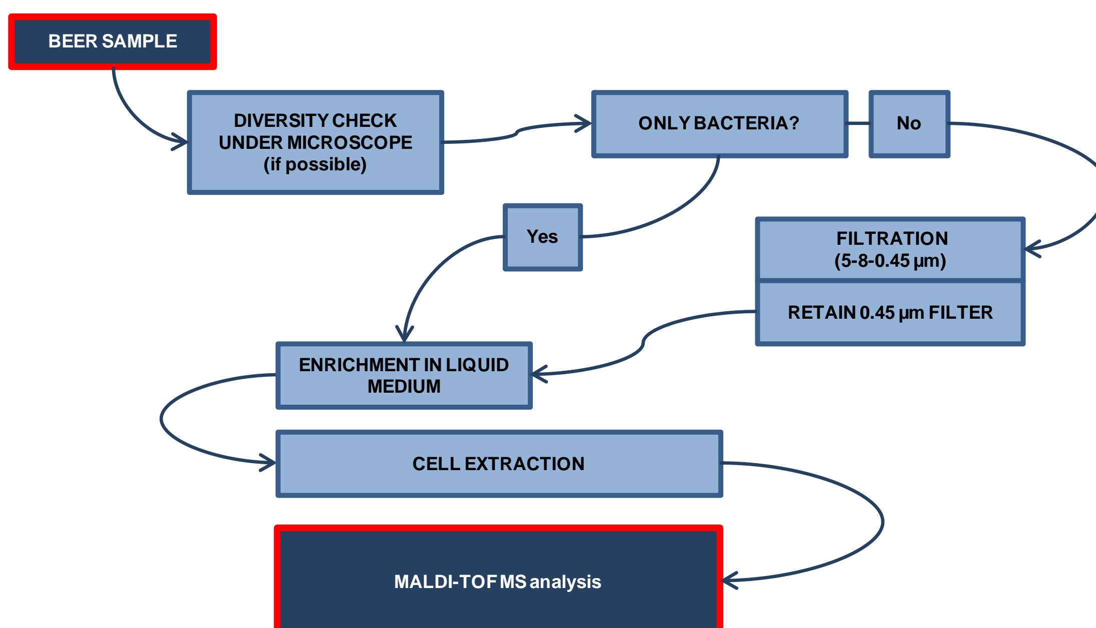

FIG 6.17. Scheme of the general workflow for the detection and identification of beer spoilage bacteria directly from enrichment cultures of beer and brewery samples using MALDI-TOF MS.

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7 Application of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry as monitoring tool for in-house brewer's yeast contamination: a proof of concept

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Author contributions: conceived and designed the experiments: ADW and AVL; performed the experiments and data analyses: ADW; contributed data analyses tools: FS; wrote the manuscript: AW; critically reviewed the manuscript: FS, AVL and PV.

SUMMARY

Contamination of brewer's pitching yeast cultures with wild-type yeasts or bacteria is unwanted as it can corrupt the fermentation outcome and causes huge economic losses for the brewing industry. The applicability of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) as a fast tool to monitor the purity of brewer's yeast cultures was investigated. This proof of concept was examined for a brewer's yeast strain contaminated with wild-type yeast and for bottled beer produced by fermentation with that particular contaminated brewer's yeast strain. The data demonstrated that MALDI-TOF MS is very suitable to discriminate between brewing and non-brewing yeast strains.

7.1 Introduction

A starter culture consists of one or more microorganisms that are added to the raw material (*e.g.*, milk, minced meat, wort, vegetable) to produce a fermented food product (*e.g.*, yoghurt, cheese, sausage, beer, sourdough, pickles). During fermentation, starter cultures metabolise several compounds to speed up and steer this process in the desired direction [1, 2]. This way, added starter cultures contribute to the microbial safety, the improvement of the organoleptic properties, extending shelf life of the fermented product and sometimes even providing health beneficial compounds [3]. It is extremely important for the fermentation industry that these starter cultures are strictly monitored in order to conserve the process, to guarantee safety of the final product and to limit economical losses. Indeed, contamination of starter cultures with foreign, wild-type microorganisms often is detrimental for the end product.

In the brewery, the starter culture used to ferment wort comprises mostly one axenic yeast strain which is well-chosen and characterised for the production of specific beer types. Spoilage by wild-type yeast strains can lead to severe economic losses in processing, preservation and storage. These wild-type yeasts are generally defined as foreign yeasts that are not deliberately used and not under full control, including accidental contamination with in-house production brewer's yeasts [4, 5]. Contamination of the brewer's pitching yeast with either bacteria or wild-type yeasts must be avoided, as these contaminants will cause undesirable flavours and effects in the beer [6]. Acid washing of brewer's yeast cultures between serial repitching is usually effective to eliminate bacterial contaminations, yet has no influence on wild yeast strains [7, 8].

Wild-type yeasts are classified as *Saccharomyces* and non-*Saccharomyces* strains [9]. The *Saccharomyces* wild-type strains pose the greatest threat in the brewing industry because of their high similarity with the in-house production brewer's yeast culture and their frequent occurrence [10, 11]. Certain wild-type yeasts are strictly aerobic and cannot ferment sugars under anaerobic conditions, *i.e.*, *Debaryomyces* spp., *Pichia* spp. and *Williopsis* spp. [4]. The acetic acid-forming *Dekkera/Brettanomyces* spp., although fermentative, usually do not represent a serious threat in the brewing process because they do not flourish under anaerobic conditions [12, 13]. The fermentative yeasts such as *Kluyveromyces*¸ *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces*, on the other hand, can cause serious problems during fermentation [13].

Wild-type yeasts mostly neither flocculate well nor interact with finings [5, 13]. Generally, they pass into conditioning where they can have deleterious organoleptic effects on post-fermentation beers, as well as causing bitter off-flavours, haze and turbidity [4]. Many strains are able to decarboxylate substituted cinnamic acids derived from the barley cell wall. For example, *p*-coumaric and ferulic

acids are decarboxylated into 4-vinylphenol and 4-vinylguaiacol, respectively [14]. These phenolic compounds, which contribute to the characteristic fruity flavour of Belgian white beers, should be avoided in ale and lager beer types [13, 15]. Other wild-type yeasts are able to utilise maltooligosaccharides and dextrins which subsequently lead to super-attenuation of beer [9]. Furthermore, the contamination of bottled beers with diastatic wild-type yeast (*e.g.*, *S. cerevisiae* var. *diastaticus*) is potentially hazardous as abnormally high concentrations of CO_2 can develop and consequently increase the risk of bottle explosion [5]. Generally, wild-type yeasts are competing with the brewer's pitching yeast for nutrients and some yeast strains can even produce certain killer proteins (*i.e.*, zymocins) that are lethal to sensitive yeast strains [16-18].

Contaminating yeasts may not only represent species other than the brewer's yeast species but also wild-type strains of the latter species. Therefore, the analysis of contaminating microbiota requires both species level identification as well as strain differentiation. Currently, differentiation among yeast strains is based on their morphological and biochemical characteristics (*i.e.*, the ability to ferment different carbohydrates, sensitivity to certain antibiotics, etc.) [4, 9, 10, 19]; yet these techniques are time-consuming, laborious and are neither sensitive nor sufficiently specific. Faster and more reliable molecular methods, *i.e.*, random amplified polymorphic DNA-PCR analysis, amplification of certain genes, pulsed field gel electrophoresis of macro-restriction fragments of DNA, contour clamped homogenous electric field gel electrophoresis of chromosomal DNA, restriction fragment length polymorphism analysis of ribosomal, mitochondrial or chromosomal DNA, and sodium dodecyl sulphate polyacrylamide gel electrophoresis of whole cell protein extracts have been used for strain level differentiation of yeast cultures [11, 20-26]. However, these techniques are again time-consuming, laborious and do not facilitate real-time interventions.

Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) has been applied as a rapid, inexpensive and easy-to-use method for the identification [27-35] and typing [33, 36] of clinically relevant yeast strains. Recently, Moothoo-Padayachie *et al*. [37] described the potential of MALDI-TOF MS for the differentiation between important industrial *S. cerevisiae* strains in comparison with molecular-based methods. The present study investigated a proof of concept that MALDI-TOF MS enables to easily differentiate between brewing and non-brewing yeast strains and that this tool is well-suited to monitor the purity of brewer's pitching yeasts throughout the brewing process.

7.2 Materials & Methods

7.2.1 *Yeast isolates*

Two contaminated samples were collected from a Belgian brewery, *i.e.*, a brewer's pitching yeast culture and bottled top fermented beer with refermentation in the bottle produced with that particular brewer's pitching yeast culture. Both contaminated samples were first analysed microscopically. The samples were then serially diluted in saline and plated onto DYPA medium (2% (w/v) D-glucose, 0.5% (w/v) yeast extract, 1% (w/v) peptone and 1.5% (w/v) agar). The agar plates were subsequently incubated aerobically at 30°C for 48h. After growth, six isolates representing two different colony types were picked and purified using the same conditions as described above. An overview of the different yeast isolates is given in **TABLE 7.1**. An axenic culture of the brewer's yeast strain (strain no. T9, see Table 1) was also obtained from the brewery and was grown onto DYPA medium as described above. All yeast strains were stored at -20°C in MicroBank™ vials (Pro-Lab Diagnostic) until further analysis.

7.2.2 *Sample preparation and MALDI-TOF MS data acquisition*

After resuscitation, the six yeast isolates and the brewer's yeast axenic culture were subcultivated twice on the isolation medium. About five mg of wet cells taken from single colonies were suspended into 300 µL Milli-Q water after which 900 µL pure ethanol was added. After centrifugation (3 min, 20817 × *g*, 4°C), 50 µL 70% formic acid and 50 µL acetonitrile were added to the bacterial cell pellet. After shaking vigorously and centrifugation (3 min, 20817 × *g*, 4°C), 1 µL of the supernatant (= the cell extract) was spotted in duplicate onto a MALDI-TOF MS stainless steel target plate to obtain two technical replicates. Immediately after drying, the spots were overlaid with 1 µL matrix solution, which consisted of 5 mg α-cyano-4-hydroxycinnamic acid dissolved in 1 mL acetonitrile/trifluoroacetic acid/Milli-Q water solvent (50:2:48). Prior to analysis the mass spectrometer was externally calibrated using a peptide mixture of adrenocorticotropic hormone (fragment 18-39; Sigma-Aldrich), insulin (Sigma-Aldrich), ubiquitin (Sigma-Aldrich), cytochrome c (Sigma-Aldrich) and myoglobin (Sigma-Aldrich). A 4800 Plus MALDI TOF/TOF™ Analyzer (AB Sciex) was used in the linear mode and covered a mass range from 2-20 kDa. The mass spectrometer used a 200 Hz frequency tripled Nd:YAG laser, operating at a wavelength of 355 nm. Generated ions were accelerated at 20 kV through a grid at 19.2 kV into a 1.5 m, linear, field-free drift region towards the detector. For each spot, 40 subspectra resulting from 50 laser shots from randomized positions within the spot were collected and presented as one spectrum (2000 laser shots in total). The laser intensity was set at 4800 procedure defined units. The Bacterial Test Standard (Bruker Daltonics) was included as positive control.

7.2.3 *MALDI-TOF MS data analysis*

The mass spectra were retrieved as t2d files from the 4800 Plus MALDI TOF/TOF™ Analyzer via the 4000 Series Explorer software. Data Explorer 4.0-software (AB Sciex) was used to convert the t2d files into text files that were subsequently used as input files for the BioNumerics 5.1 and 7.1 software packages (Applied Maths). A mass spectrum was considered of acceptable quality if the absolute signal intensity of the highest peak was > 500 counts, if more than 5 peaks with a signal-to-noise ratio (S/N) > 20 were detected in the 3-20 kDa range and if there were no repetitive signals in the 2.1-3 kDa range. The mass spectral data comprised in the text files were imported into BioNumerics 5.1 software package and converted into fingerprints for further analysis. The curve-based Pearson

product-moment correlation coefficient and UPGMA cluster analysis allowed for a simple and rapid comparison of the obtained yeast isolates [38].

The purchase of a more recent version of this software package allowed biomarker-based analysis of the same spectra. To this end, the spectral data were imported in the BioNumerics version 7.1 software using an optimised preprocessing template including consecutive continuous wavelet transform (CWT) noise estimation, a Savitsky-Golay filter smoothing and a baseline subtraction using the rolling disk algorithm [39]. Each peak with an S/N ratio ≥ 5 and an absolute intensity of at least 6 counts was annotated. Mass spectra of two technical replicates per isolate were combined into a summary spectral profile (SSP). To obtain this SSP, a peak matching analysis was conducted with constant and linearly varying tolerance values of 1 *m/z* and 800 ppm, respectively [39]. The minimum peak detection rate was set at 100%, meaning that each summary peak occurred in both profiles of the technical replicates, thus minimizing the impact of technically induced variations [39]. Subsequently, the signal intensity for each data point in the SSP was calculated by averaging the respective signal intensities from the technical replicates. Afterwards, the peak-based data analysis matches all peaks in the mass spectra to a peak class using constant and linearly varying tolerance values of 2 *m/z* and 800 ppm, respectively [39]. The obtained dataset was converted into a binary character set and SSPs were compared using the binary Dice coefficient to detect strain-specific peaks [40].

7.2.4 *Sequence analysis of D1/D2 region of 26S rRNA genes*

Yeast DNA was obtained using the protocol of Harju *et al.* [41]. Yeast isolates were identified through sequence analysis of the D1/D2 region of the 26S rRNA gene [42]. Amplification and sequencing was performed as described by Snauwaert *et al*. [43] using the primers LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR6 (5'-CGCCAGTTCTGCTTACC-3') (http://biology.duke.edu/fungi/mycolab/primers.htm). The obtained sequences were blasted against several sequence databases via MycoID (http://www.mycobank.org/).

7.3 Results and Discussion

In the brewery, yeast cells are not fully aseptically harvested from previous fermentation batches and repitched in multiple novel fermentation batches [8, 13]. Hence, it is important that the pitching yeast is pure and not contaminated with bacteria, wild-type yeasts or even other in-house production brewer's yeast strains. The applicability of MALDI-TOF MS as a fast technique to verify the purity of brewer's pitching yeast cultures was investigated based on a practical case study. The quality control manager of a Belgian brewery noted deviations in fermentation outcome for several production batches of a top fermented beer with refermentation in the bottle. These deviations were suspected to be caused by contamination of the brewer's pitching yeast with either bacteria or wild-type yeasts.

TABLE 7.1. Overview of yeast isolates used in this study and their morphological and final typing.

Yeast isolate	Source	Morphological type	Final type
T1	Contaminated brewer's pitching yeast culture	1	Wild
T2	Contaminated brewer's pitching yeast culture	1	Wild
T3	Contaminated brewer's pitching yeast culture	2	Original
T5a	Contaminated bottled top fermented beer	1	Wild
T6	Contaminated bottled top fermented beer	1	Wild
T7	Contaminated bottled top fermented beer	2	Original
T9	Axenic brewer's pitching yeast strain	2	Original

The microbial diversity of the obtained samples was microscopically checked for the presence of bacteria after growth in different specific media for beer spoilage bacteria; yet only yeast cells were observed. Two different yeast cell morphologies were observed in both samples suggesting the presence of a contaminating wild-type yeast strain. After plating the samples on DYPA medium, two different colony types were found for each sample. They were referred to as yeast types 1 and 2 and their cell morphologies corresponded with the two microscopically observed cell morphologies (**TABLE 7.1**). The colony and cell morphology of yeast type 1 was characterised by pinpoint, whitish and round colonies and small and round cells; while yeast type 2 was characterised by big, round, whitish colonies and big oval-shaped cells that contained more vacuoles compared to yeast type 1 (**TABLE 7.1**).

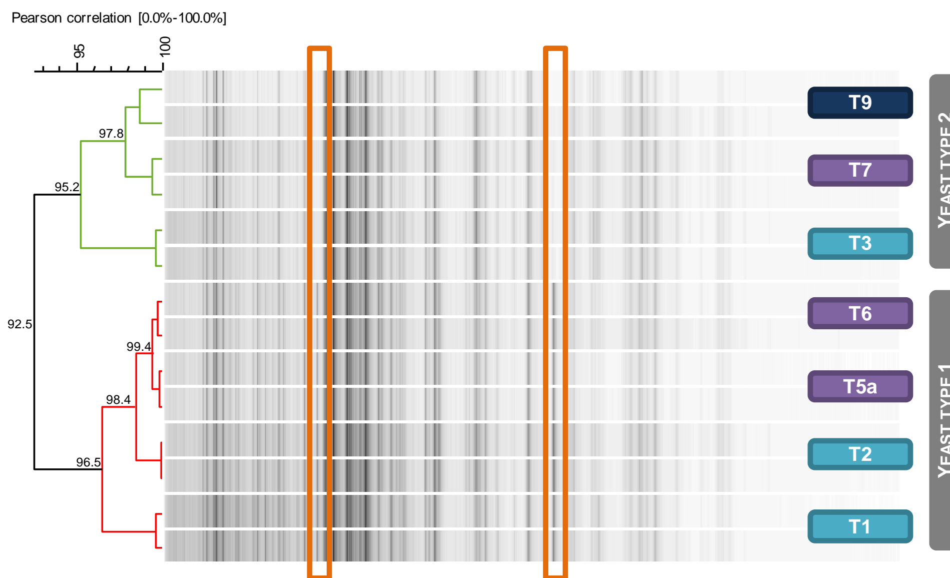

FIG 7.1. Mass spectra and corresponding curve-based cluster analysis using the Pearson product-moment correlation coefficient and UPGMA cluster algorithm of the six isolates and the axenic brewer's pitching yeast strain T9. Rectangles mark two peak classes (*i.e.*, m/z 5806.33 ± 6.65 and 11608.30 ± 11.29) which were only present in the mass spectra generated from the wild-type yeast isolates (T1, T2, T5a and T6, red cluster). The isolates T3 and T7 grouped with the axenic brewer's yeast strain (green cluster), yet separated from the wild-type yeast isolates. Isolates recovered from the contaminated brewer's yeast culture are shown in blue boxes; isolates collected from the bottled top fermented beer with refermentation in the bottle are shown in purple boxes on the right.

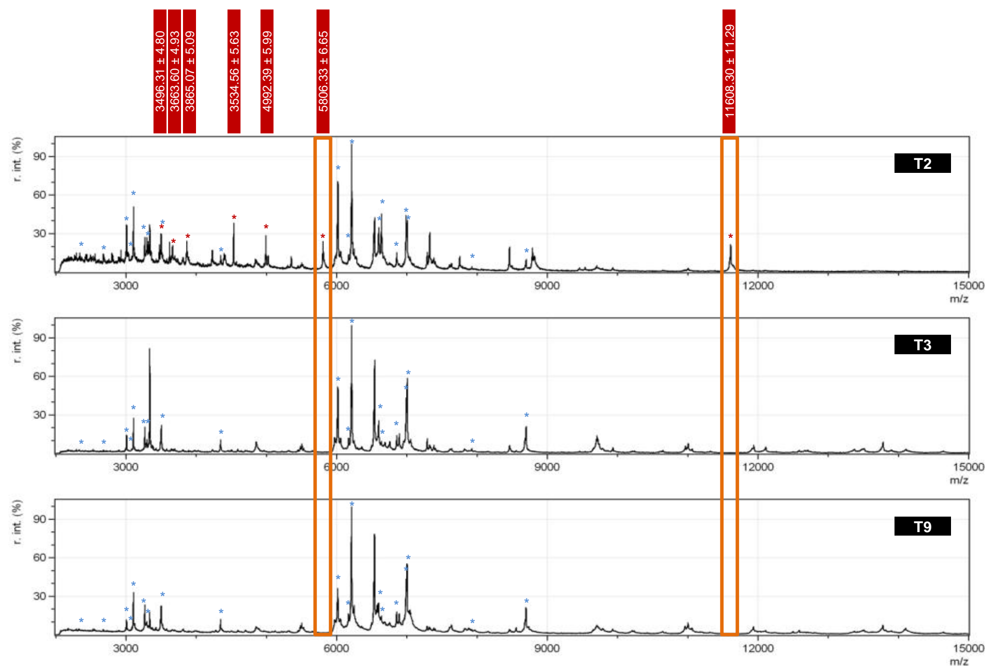

FIG 7.2. Mass spectra derived from wild-type yeast strain T2, brewer's yeast T3 isolated from contaminated yeast culture and axenic brewer's pitching yeast strain T9. The seven wild-type yeast-specific peak classes are indicated by red asterisks and corresponding *m/z* values are shown in red boxes at the top. Rectangles mark the two wild-type yeast-specific peak classes which were visually absent in the mass spectra derived from brewer's yeast strains (**FIG 7.1**). The 19 common peak classes are indicated by blue asterisks and corresponding *m/z* values are shown in. r.int., relative intensity.

MALDI-TOF MS profiles of the 6 isolates (four were yeast type 1 and two were yeast type 2) and of the axenic brewer's yeast strain T9 were subsequently generated and compared using curve-based cluster analysis (**FIG 7.1**). The brewer's yeast strain T9 and isolates T3 and T7 had indistinguishable MALDI-TOF MS profiles, while the remaining isolates (*i.e.*, T1, T2, T5a and T6) also had indistinguishable profiles which clustered separately (**FIG 7.1**). This high similarity in MALDI-TOF mass spectra of strain T9 and isolates T3 and T7 on the one hand, and of isolates T1, T2, T5a and T6 on the other hand clearly demonstrates that isolates T3 and T7 represent subcultures of strain T9, while isolates T1, T2, T5a and T6 represent a contaminating wild-type yeast strain. Moreover, cell and colony morphology of the brewer's yeast strain T9 resembled the morphology observed for subcultures T3 and T7. In addition, peak-based numerical analysis of the SSPs generated from all isolates revealed that seven peak classes occurred solely in the mass spectra generated from each of the wild-type yeast isolates (**FIG 7.2**).

TABLE 7.2. Nineteen peak classes common to SSPs derived from all of the isolates grown on DYPA medium.

m/z values of shared peak classes
2295.54 ± 3.84
2679.10 ± 4.14
3009.56 ± 4.41
3086.15 ± 4.47
3107.80 ± 4.49
3270.15 ± 4.62
3300.08 ± 4.64
3503.90 ± 4.80
4351.26 ± 5.48
6017.83 ± 6.81
6171.47 ± 6.94
6214.08 ± 6.97
6599.30 ± 7.28
6639.75 ± 7.31
6856.35 ± 7.49
6991.56 ± 7.59
7005.40 ± 7.60
7924.18 ± 8.34
8462.77 ± 8.77

These results demonstrated that a wild-type strain contaminated the brewer's pitching yeast and that this contamination persisted until packaging of the top fermented beer with refermentation in the bottle. Despite the presence of multiple differentiating peak classes, the mass spectra generated from all isolates were highly similar and included 19 shared peak classes (**TABLE 7.2**; **FIG 7.2**), suggesting that the contaminating isolates belong to *S. cerevisiae* as well [27, 28, 34, 35]. This was confirmed by sequence analysis of the D1/D2 region of 26S rRNA gene of yeast isolates T1 and T3 which revealed very high sequence similarities towards the *S. cerevisiae* taxonomic reference strain NRRL Y-12632^T (AY048154) (99.9% for both isolates T1 and T3).

In conclusion, this study demonstrated as a proof of concept that MALDI-TOF MS allows discrimination of brewing and non-brewing *S. cerevisiae* isolates by comparing their mass spectra with that of the in-house production brewer's yeast strain. Discrepancies in peak patterns between the mass spectra can confirm or reject possible contamination with non-brewing wild yeasts. Hence, MALDI-TOF MS functions as a rapid and easy authenticity check which suggests that this technique can be a useful monitoring tool in the entire fermentation and starter culture industry.

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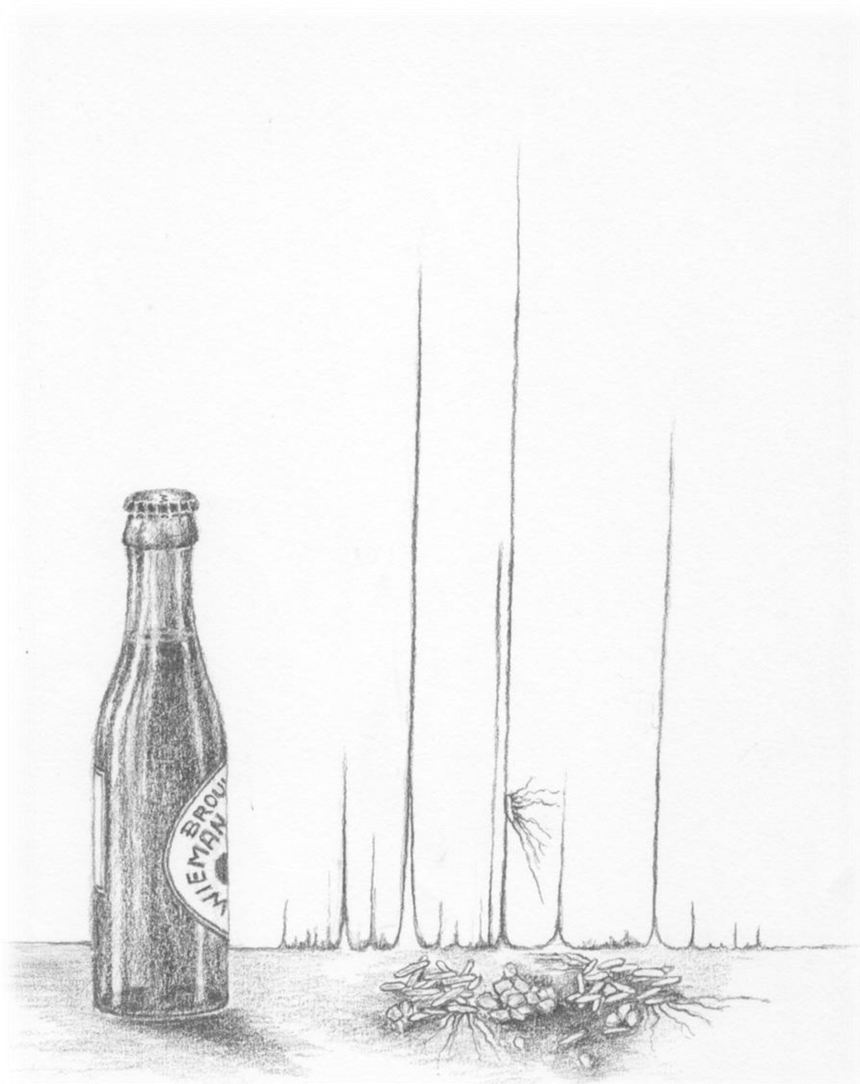

Part IV | General Conclusions & Future Perspectives

8 General reflections and future perspectives

The main objective of the present study was to investigate the potential of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) as a rapid, low cost and high-throughput identification tool for bacteria isolated from spoiled beer and brewery samples. Therefore, an in-house MALDI-TOF MS identification database was constructed containing mainly well-characterised reference strains originating from spoiled beer and other brewery samples, but also from other niches where strains of the same species occur. Moreover, the reference strains were cultivated on to multiple different selective and non-selective growth media to anticipate cultivation condition induced variability in an organism's MALDI-TOF MS profile. The intraspecies diversity and the growth medium induced variability towards the mass spectra generated were determined. State-of-the-art taxonomic standard methods were applied to validate the reassignment of reference strains indicated by MALDI-TOF MS. The MALDI-TOF MS identification database was subsequently evaluated, and errors in the MALDI-TOF MS based classification of reference strains were mostly related to underlying taxonomical errors. The validated MALDI-TOF MS identification database was subsequently used for the identification of bacteria isolated from spoiled beer and brewery samples. Mass spectra generated of the isolates were compared in order to assess the obtained diversity and to select representative isolates. Subsequently, mass spectra of isolates were analysed, and MALDI-TOF MS identification results were confirmed using state-of-the-art taxonomic standard methods. Furthermore, the potential of MALDI-TOF MS was investigated for the detection and identification of bacteria directly from enrichment cultures of spoiled beer and brewery samples with or without the concomitant presence of yeast cells. Finally, MALDI-TOF MS was also explored as a tool to differentiate among brewing and unwanted yeasts. The present chapter comprises a general discussion and the final conclusions of this study, and provides perspectives for future studies.

8.1 MALDI-TOF MS as a low cost, rapid and high-throughput detection and identification tool for bacteria present in spoiled beer and brewery samples

Beer is a beverage with usually good microbiological stability because it contains almost no oxygen and nutrients for bacterial growth. In addition, low pH, high CO_2-content and the presence of ethanol and antibacterial hop compounds ensure microbiological stability [1-4]. Nevertheless, beer spoilage due to bacteria is a common problem in the brewing industry and causes important economic losses worldwide [5-8]. Nowadays, the hop-resistant lactic acid bacteria (LAB) *Lactobacillus backii*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus lindneri*, *Lactobacillus*

coryniformis, *Lactobacillus plantarum*, *Lactobacillus malefermentans*, *Lactobacillus parabuchneri*, *Pediococcus damnosus*, *Pediococcus inopinatus* and *Pediococcus claussenii* are generally regarded as the most problematic beer spoilage microorganisms [9, 10] (**CHAPTER 1**). The prevalence of strictly aerobic acetic acid bacteria (AAB) has decreased because of improved process filling technology [6]. However, in aerobic environments, AAB can prevail and form biofilms in which other spoilage bacteria are protected and survive [11, 12]. During the last two decades, anaerobic Gram-stain negative bacteria like *Pectinatus*, *Megasphaera* and *Zymophilus* species have gained importance as spoilage bacteria in the brewing industry as well [6, 13, 14] (**CHAPTER 1**).

The taxonomically diverse group of beer spoilage bacteria is currently detected and/or identified using a range of culture-dependent or culture-independent methods (**CHAPTER 2**). These methods are time-consuming and often lack high-throughput capacity and do not facilitate real-time interventions. Furthermore, these methods mostly target only a narrow range of beer spoilage bacteria. Therefore, MALDI-TOF MS was investigated as an alternative identification tool for the most prevalent beer spoilage AAB and LAB by constructing an identification database and evaluating its applicability for the identification of novel spoilage bacteria. The method was validated by state-of-the-art taxonomic standards. Multiple research groups demonstrated the application of MALDI-TOF MS as a high-throughput, accurate and low-cost identification tool for isolates obtained in medical, environmental and food-related studies, enabling the simultaneous identification of a diverse group of microorganisms [15-22] (**CHAPTER 3**).

8.1.1 *Construction of the MALDI-TOF MS identification database*

Currently, there are different commercially available and user-friendly MALDI-TOF MS identification systems for which instrumentation is mostly coupled to a mass spectral database and appropriate data analysis software packages [23-27] (**CHAPTER 3**). In the present study, the 4800 MALDI TOF/TOF^{TM} Analyzer of AB Sciex (Framingham, MA, USA) was used. The instrumentation is not equipped with an identification database, nor with appropriate tools to analyse mass spectra generated from crude protein extracts prepared from the intact cells of microorganisms as it is mainly applied for high-throughput proteomics research. Therefore, efforts were made to construct an extensive mass spectral database in the BioNumerics 5.1 software package (Applied Maths, Belgium). This version of the software supported only curve- or band-based analysis of less complex DNA fingerprinting patterns (*e.g.*, DGGE, AFLP or $(GTG)_5$ fingerprinting patterns). The recent availability (june 2013) of BioNumerics version 7.1 allows for a more comprehensive analysis as the incorporated algorithms enable users to perform an in-depth preprocessing of raw, more complex

MALDI-TOF mass spectra (*e.g.*, by the removal of baseline, estimation of noise, profound peak picking) and to analyse data using either a curve-based (*i.e.*, using the complete mass spectrum) or peak-based (*i.e.*, presence-or-absence analysis of peaks only) approach [28].

The MALDI-TOF MS technique had already previously been successfully explored at the Laboratory of Microbiology at Ghent University for the identification of isolates belonging to the genera *Fructobacillus*, *Lactococcus* and *Leuconostoc* [29]. During this study, both the intact cell method (ICM) and cell extraction method (CEM) were applied for the construction of a robust and reproducible database. Within the course of the present study, both sample treatment procedures were evaluated using a selection of AAB and LAB reference strains. Mass spectra generated using the ICM often did not comply with the established mass spectral quality parameters (see below). Especially mass spectra generated from Gram-stain positive LAB contained an increased slope or a limited number of peaks detected; which substantiated previously reported results [30]. The CEM based on the procedure recommended by Bruker Daltonics (Germany) was preferred for the construction of the present MALDI-TOF MS identification database as the mass spectra generated were more reproducible and of superior quality (**FIG 8.1**).

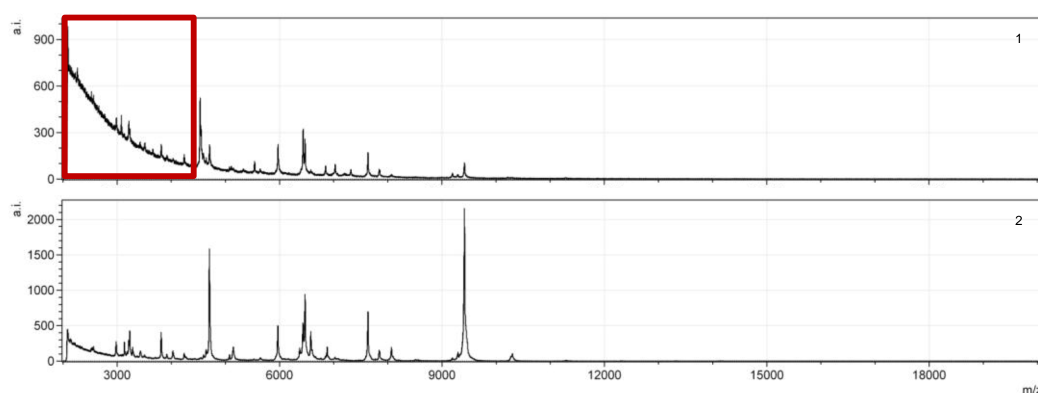

FIG 8.1. Mass spectra of *Lactobacillus paracollinoides* strain LMG 22473^T obtained using the intact cell method (ICM) (**1**) and the cell extraction method (CEM) (**2**). The slope is highlighted in the rectangle. a.i., absolute intensity.

Visual inspection of the mass spectral quality and evaluation of the identification results obtained when using poor quality spectra enabled us to establish cut-off values for three parameters: presence of a slope, the peak signal intensity and the number of peaks detected. For instance, mass spectra characterised by an increased slope tended to group together during curve-based cluster analysis even if mass spectra were generated from strains belonging to different species. Ultimately, the mass spectral quality was considered to be acceptable if the absolute intensity of the highest

peak was more than 500 counts, if more than 5 peaks with a signal-to-noise ratio of more than 20 were detected in the range of 3-20 kDa and if there were no repetitive peaks in the range of 2.1-3 kDa (**FIG 8.2**).

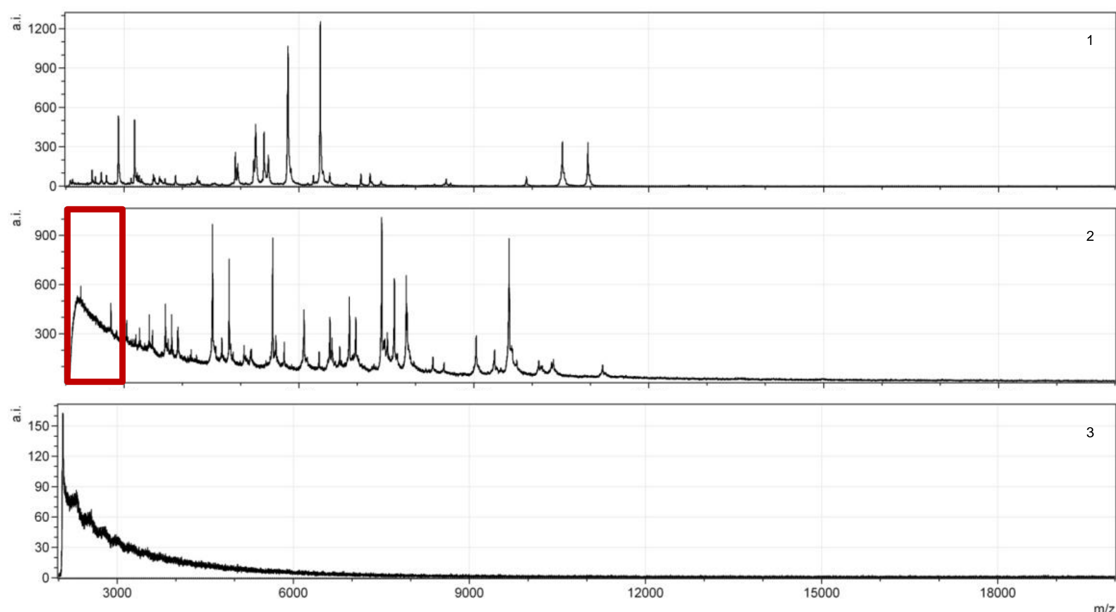

FIG 8.2. Mass spectra of different quality. Mass spectrum **1** is of good quality (*i.e.*, no slope, high signal intensity and data richness). In contrast, a slope (highlighted in the rectangle) is observed in spectrum **2**; yet this mass spectrum still showed a high data richness and signal intensity. Mass spectrum **3** is not-suited for further analysis due to its high slope, low signal intensity and the absence of peaks. a.i., absolute intensity.

At the start of our study, the identification database present in the Laboratory of Microbiology at Ghent University did not contain organisms regarded as potential beer spoilage bacteria (**CHAPTER 1**). Therefore, the identification database was expanded by incorporating 273 (mostly) well-characterised reference strains representing 18 AAB and 34 LAB species for which 4200 good quality mass spectra were generated. These reference strains originated from spoiled beer and brewery samples but also from other niches where these same species occur in order to encompass the intraspecies phenotypic diversity wherever possible [31]. The VITEK MS *in vitro* diagnostic automated microbial identification system (bioMérieux) contains mass spectra generated from ten isolates per species that were obtained from different isolation sources, year and origin [32]. The most effective performance for species level identification of bacteria according to Lartigue *et al*. [33] was obtained by using a MALDI-TOF MS database designed not by selecting one or more strains randomly, but by choosing the strains based on phylogenetic diversity that would represent the species studied. The strain selection for the present identification database was based on their

relevance as beer spoilage bacteria, their isolation source and their availability in international culture collections. For these reasons, *Lb. brevis* was well-represented in the identification database whereas *Lb. malefermentans,* for which few strains have been deposited in international culture collections, was represented by only two strains. All reference strains were cultivated on multiple selective and non-selective growth media to incorporate and anticipate any growth medium induced variation to the mass spectra generated. The extraction procedure was performed from cells obtained from the subsequent 3rd, 4th and 5th generation cultures of each strain per growth medium. The extensive subcultivation of the strains was time-consuming; however these biological replicates, together with the inclusion of technical replicates (duplicate spotting of the cell extract on to the MALDI-TOF MS target plate) enabled us to construct an extensive and robust MALDI-TOF MS identification database.

The present study investigated the effect of the growth medium used on the mass spectra generated and its consequence for species level identification using a set of 25 AAB strains grown on five different growth media (**CHAPTER 4**). To this end, summary spectral profiles (SSPs) were created per strain and growth medium using a peak detection rate of 100%, meaning that each peak accounted for during analysis had to be present in each of the member mass spectra. As such, technical and biological induced variations towards the mass spectra were excluded from further analysis. In general, culture media that did not sustain optimal growth influenced the generated mass spectra strongly; and this effect has also been observed for the other reference strains grown on multiple culture media present in our identification database (**FIG 8.3**). Yet, the growth medium induced differences did not disturb the species level differentiation. It has been recommended to include multiple well-selected growth media during the construction of a MALDI-TOF MS identification database [32]. For that reason, the VITEK MS *in vitro* diagnostic mass spectral database consists of reference mass spectra generated from strains cultured at different growth conditions (*e.g.*, growth medium, different incubation times) [18, 32]. Different incubations times were not included in the present study as cell extracts were prepared from single colonies obtained from young, freshly grown cultures; moreover all of the other growth conditions (oxygen rate and incubation temperature) were fixed for each reference strain according to the providers' specifications.

The present study also revealed that the number of strain-specific peak classes is culture medium dependent and that the selected culture medium affects the potential for strain level differentiation. The data presented in **CHAPTER 4** also demonstrated that the number of shared peak classes appeared to decrease with an increasing number of strains per species examined; and that a growth medium dependent core set of peak classes and thus peptides seems to exist. A similar analysis was

performed using SSPs generated from different LAB reference strains grown on the same culture medium; and revealed that the core set of peak classes decreased when more strains per species were incorporated in the analysis (**FIG 8.4**). The core set of peak classes examined for *Lb. malefermentans* that was represented in our database by two strains only, revealed that additional strains of the species should be included before a reasonably accurate representation of core peptides will be obtained. The inclusion of SSPs generated from three additional *Lb. malefermentans* strains isolated from two different spoiled samples during the course of the present study indeed visualised a clear decrease in shared peak classes with increasing numbers of SSPs examined (**FIG 8.4**). Therefore, we speculate that the inclusion of more reference strains will probably lead to a stabilizing core set of peak classes that could indicate sufficient coverage of the intraspecies diversity of the MALDI-TOF MS identification database (**FIG 8.4**).

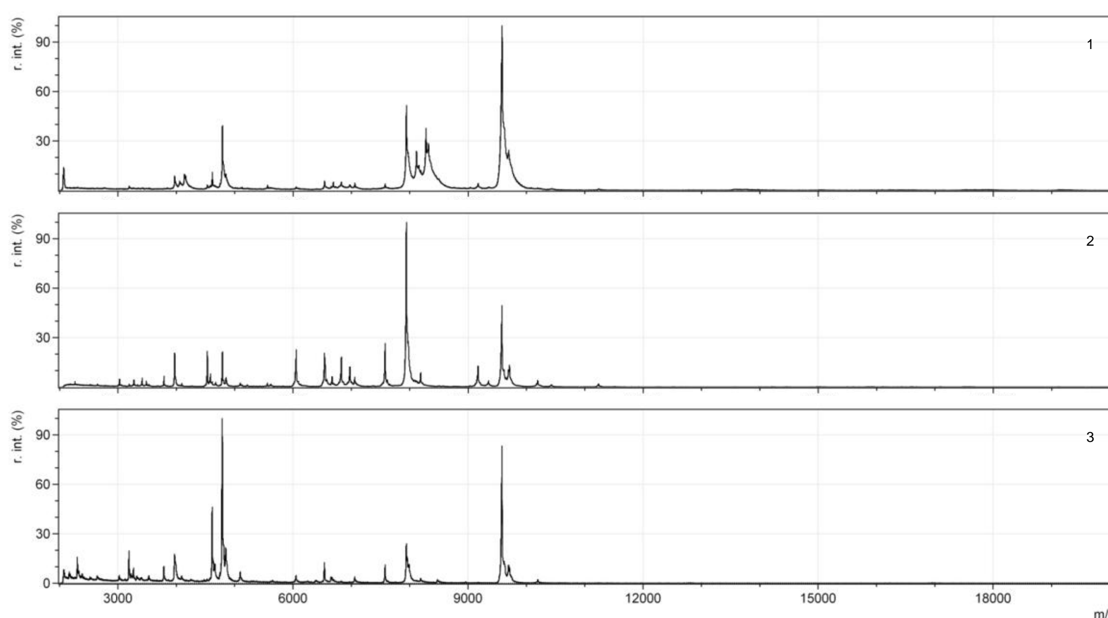

FIG 8.3. The 'Nachweismedium für bierschädliche Bakterien' [NBB; (Conda Pronadisa)] did not support the growth of *P. acidilactici* strain LMG 25667 very well; hence the mass spectrum generated (**1**) differed considerably from those obtained when the strain was grown on either de Man, Rogosa and Sharpe [MRS; (Oxoid)] medium (**2**) or Raka-Ray medium (Oxoid) (**3**). Varying peak signal intensities of the shared peaks were however observed among the latter two mass spectra. r. int., relative intensity.

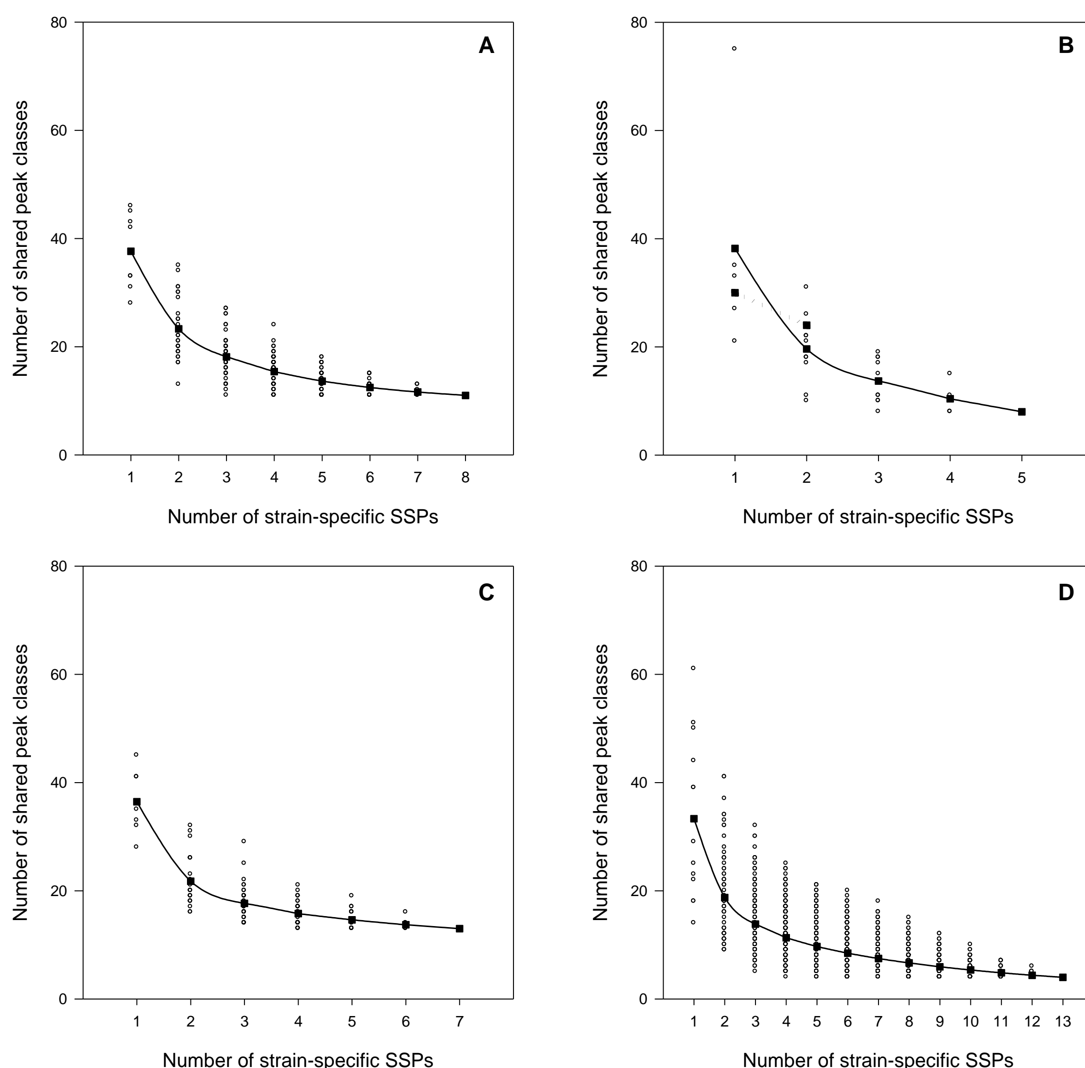

FIG 8.4. The decrease in shared peak classes among SSPs of eight *G. oxydans* strains grown on AAM (**A**), two (dotted curve) and five (full curve) *Lb. malefermentans* strains grown on MRS (**B**), seven *Lb. brevis* strains grown on MRS (**C**) and thirteen *Lb. paracasei* strains grown on MRS (**D**). The number of shared peak classes is plotted as a function of the number of strain-specific SSPs sequentially added. Squares represent the average of shared peak classes per sequential addition of a strain-specific SSP.

In conclusion, it is of utmost importance to have an exhaustive MALDI-TOF MS identification database containing good quality mass spectra generated from sufficient reference strains of the same species grown on multiple media [31, 34]. The inclusion of biological and technical replicates per strain grown at a specific culture condition enables the final elimination of biological or technical induced variations towards the mass spectra generated. In addition, an important advantage of MALDI-TOF MS as an identification tool resides in the minimal adaptations to the sample treatment procedure required to expand the identification database to other (types of) microorganisms. The present study focussed mainly on the beer spoiling LAB and AAB; yet good quality mass spectra

were generated from other beer spoilage microorganisms such as *Pectinatus* and *Zymomonas* strains as well using the same sample treatment procedure (**FIG 8.5**). This is a major advantage, especially compared to other detection and/or identification methods for which thorough modifications towards sample treatment protocols, *e.g.*, novel design of primers or probes, are necessary.

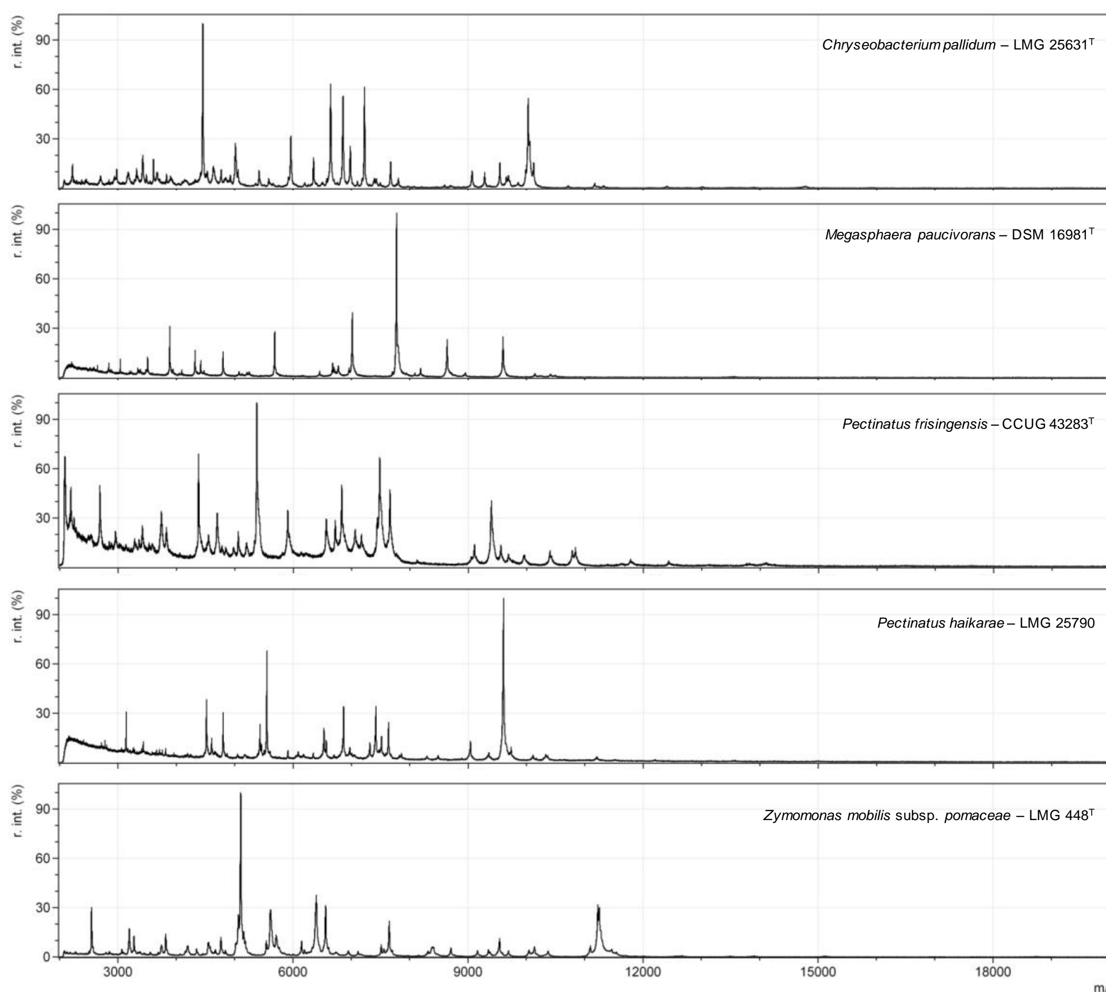

FIG 8.5. Mass spectra of some beer spoilage bacteria or bacteria isolated from the brewery environment that were not included in the present study. r. int., relative intensity.

8.1.2 *Validation of the MALDI-TOF MS identification database*

The performance of the MALDI-TOF MS identification database largely depends on the correct classification of the reference strains examined. Mass spectra generated from each of the reference strains were analysed using either a curve-based or peak-based cluster analysis approach. Mass spectra of 11 species of the 52 included species grouped in multiple clusters and twelve of these

clusters comprised mass spectra of two or more species when the curve-based Pearson product-moment correlation coefficient and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) analysis method was performed. In contrast, the peak-based cluster analysis using the Dice coefficient and UPGMA cluster algorithm enabled species level differentiation far more efficiently. Yet, mass spectra of groups of closely related bacteria like *Lactobacillus plantarum/Lactobacillus paraplantarum* and *Acetobacter cerevisiae/Acetobacter malorum* grouped in a single cluster each [35-37]. The inability to comprehensively discriminate among certain closely related species is not of major concern as long as it is recognised and accounted for during the data interpretations [38-41]. Peak-based numerical analysis of mass spectra of *Lb. plantarum* and *Lb. paraplantarum* revealed only three shared peak classes (*i.e.*, *m/z* 2866.55 ± 4.29, 4560.63 ± 5.65 and 9418.88 ± 9.54) (data not shown); however not a single peak class was found to enable discrimination between mass spectra of both species. In contrast, discrimination among strains of the closely related *Lb. collinoides* and *Lb. paracollinoides* (who had three shared peak classes) was feasible based on the presence of two peak classes characterised by an *m/z* value of 6580.87 ± 7.26 and 6884.81 ± 7.51 (data not shown).

The peak-based cluster analysis revealed that 34 out of 273 (12%) reference strains including some strains isolated from beer or the brewery environment, clustered aberrantly (**CHAPTER 5.1**; **ANNEX 1**). Consequently, sequence analysis of protein coding genes was performed as a taxonomic reference method for accurate species level identification and this consistently confirmed the MALDI-TOF MS cluster result, implying that the previous identification rather than the MALDI-TOF MS identification was wrong. Some of these aberrantly clustering strains represented species that were never reported as potential beer spoilage bacteria such as *Gluconobacter japonicus* and *Acetobacter fabarum.* The potential of these species as spoilage bacteria in the brewing industry therefore merits further attention. Noteworthy in this context was that the mass spectra generated from the type strain of *Pediococcus lolii* grouped together with those of *Pediococcus acidilactici* strains and that an in-depth taxonomical study elaborately discussed in **CHAPTER 5.2** confirmed that the *P. lolii* subcultures deposited in the DSMZ and JCM culture collections belong to *P. acidilactici* [42]. Similarly, mass spectra generated from *Lb. homohiochii* strain LMG 9478^T could not be differentiated from those of *Lb. fructivorans* strain LMG 9201^T, but are clearly distinguished from the closely related *Lb. lindneri* strain R-49605 (**FIG 8.6**). DNA-DNA hybridization values (93%), and 16S rRNA (99.9%) and *pheS* (100%) gene sequence analysis revealed a high degree of similarity among these strains; thereby confirming the MALDI-TOF MS results and suggesting that *Lb. fructivorans* is an earlier heterotypic synonym of *Lb. homohiochii* [43, 44] (unpublished data).

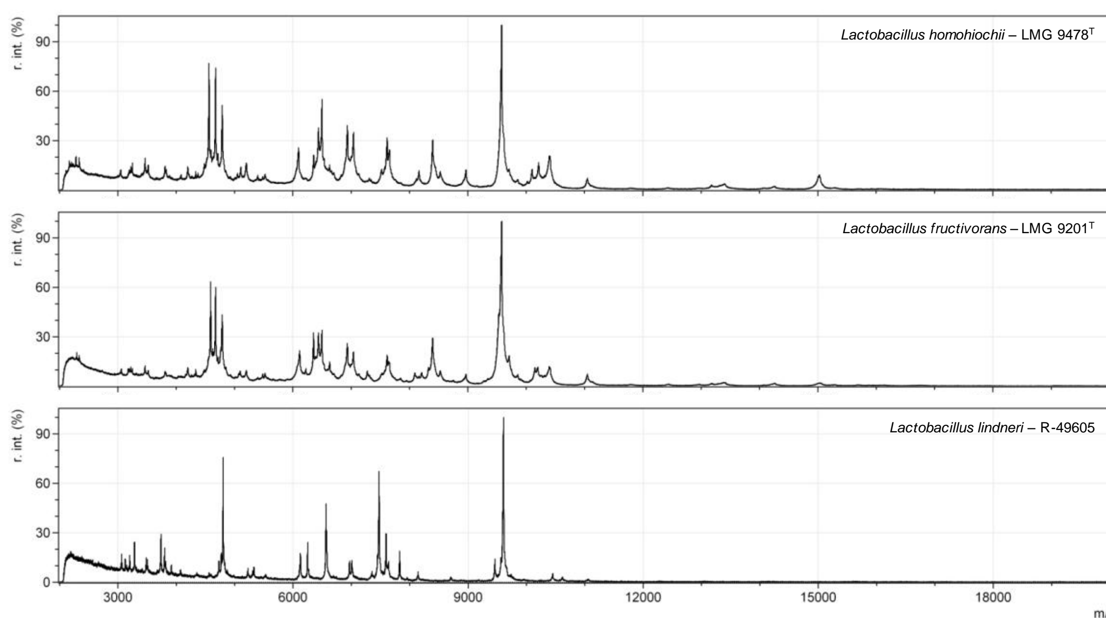

FIG 8.6. Mass spectra obtained from *Lb. homohiochii* strain LMG 9478^{T}, *Lb. fructivorans* strain LMG 9201^{T} and the closely related *Lb. lindneri* strain R-49605. r. int., relative intensity.

8.1.3 *Application of the MALDI-TOF MS identification database to identify unknown bacteria isolated from spoiled beer and brewery samples*

Species level identification of spoilage microorganisms is of utmost importance for the brewing industry even though not all strains share similar beer spoiling capabilities [45, 46]. Nevertheless, species level identification enables the estimation of the impact of bacterial spoilage and the prevention of further product deterioration and future spoilage. In total 29 beer spoilage LAB strains collected from Danish, Czech and Belgian breweries were analysed to test the performance and applicability of our MALDI-TOF MS identification database ([47]; unpublished results). Peak-based cluster analysis of mass spectra generated from these isolates together with those of LAB reference strains enabled rapid and reliable identification of all isolates (number of strains in brackets). The spoilage isolates included strains of the following species: *Lb. brevis* (23), *Lb. plantarum* (3), *Lb. lindneri* (1), *Lb. paracasei* (1) and *P. inopinatus* (1). The MALDI-TOF MS identification results were confirmed using *pheS* gene sequence analysis (data not shown).

Fourteen spoiled beer and brewery samples provided by three Belgian breweries were analysed. Due to the low numbers of bacterial cells present in such samples and the small sample volume that is available for analysis, an enrichment step prior to isolation was necessary. In total 348 isolates were picked from agar plates inoculated with a sample from these enrichment cultures, and were subsequently analysed using MALDI-TOF MS. The diversity observed among this large set of isolates

was rapidly ordered by grouping the mass spectra obtained by means of the curve-based Pearson product-moment correlation coefficient and UPGMA cluster analysis (**CHAPTER 5.1**). In total 15 distinct MALDI-TOF mass spectra were obtained after this dereplication step; and several cluster representatives were chosen depending on the cluster heterogeneity observed. The peak-based cluster analysis correctly identified 94% of the isolates as revealed by subsequent sequence analysis of protein coding genes. The remaining 6% of the isolates were either representing species that were not regarded as potential beer spoilage bacteria (*Acetobacter fabarum*, *Acetobacter indonesiensis*, *Acetobacter persici* and *Gluconobacter japonicus*) and thus not present in our database or represented novel species altogether (*Gluconobacter* sp. and *Gluconobacter cerevisiae* sp. nov. [**Chapter 5.3**]). The expansion of our database by including reference strains of these species will enable a straightforward identification in future experiments. An alternative classifier-based approach allowed for an automated identification analysis; yet yielded a high percentage of inaccurate and unreliable identification results. The underrepresentation of strains of certain reference species (*e.g.*, *Lb. malefermentans*) was responsible for some false identification results (see above, **FIG 8.4**).

In conclusion, the results of the present study demonstrated that MALDI-TOF MS coupled to a well-constructed mass spectral database allowed to identify a large and diverse set of isolates from different spoiled beer and brewery samples. Curve-based cluster analysis was instrumental for the initial isolate dereplication, after which a selection of cluster representative isolates was identified using peak-based cluster analysis.

8.1.4 *The detection and identification of bacteria directly from enrichment cultures obtained from spoiled beer and brewery samples*

The applicability of MALDI-TOF MS as a rapid and high-throughput tool for the identification of beer spoilage bacteria isolated from beer and brewery samples is already demonstrated in this study. Unfortunately, enrichment and isolation of the spoilage bacteria are required prior to MALDI-TOF MS analysis merely because too low numbers of bacterial cells are present in spoilage samples. Therefore, we investigated the potential of MALDI-TOF MS to identify the bacteria directly from the enrichment cultures (**CHAPTER 6**). The minimal cell concentration required to generate good quality MALDI-TOF mass spectra that allowed identification was determined at approximately 5×10^7 CFU/mL enrichment culture which corresponds with previous reports [40, 48]. The (beer) matrix strongly influenced the mass spectra generated; yet two consecutive washing steps using Milli-Q water prior to cell extraction were sufficient to eliminate the matrix effect almost completely.

Moreover, a sample preparation protocol based on a filtration procedure enabled detection and identification of bacteria directly in enrichment cultures of spoiled beer and brewery samples, even in the presence of yeast or mould cells.

Mass spectra generated from the monomicrobially enrichment cultures were of good quality and enabled identification of the potential spoilage bacterium present. However, it is not uncommon that multiple microorganisms simultaneously cause beer spoilage. In the present study, 7 out of 14 spoiled samples were contaminated with cells of more than one species (**CHAPTER 5.1**). The identification of microorganisms present in polymicrobially contaminated samples remained extremely challenging, considering the various types of peak suppression effects when different microorganisms are present in the enrichment culture [38, 49] (**CHAPTER 6**). When peak-based analysis of the mass spectrum of the enrichment culture assigned it to a certain species, a comparison of peak classes may or may not suggest the presence of additional species. Therefore, it is again important that each species is well-represented in the MALDI-TOF MS identification database. For instance, the inclusion of additional *Lb. malefermentans* reference strains would increase the number of peak classes, and consequently decreased the number of uncorrelated peak classes present in the mass spectrum generated from the enrichment culture contaminated with *Lb. malefermentans* (**CHAPTER 6**). Mass spectra of enrichment cultures that group separately from those of reference strains after peak-based cluster analysis could mean that the sample is of polymicrobial nature, that the microorganism is not present in the identification database, or that the sample is contaminated with a novel species altogether (**CHAPTER 5.1**). Although preliminary identification results may provide the brewery's quality manager with pivotal information to start prevention of further product deterioration and future spoilage, our data demonstrated that it is highly recommended to plate these enrichment cultures and use MALDI-TOF MS to dereplicate and identify the isolates obtained in order to confirm the identification results obtained after two to five days [34, 50] (**FIG 8.7**).

It would be interesting to determine which concentration of cells in spoiled beer and brewery samples would allow direct identification of the spoilage bacteria without enrichment step [51]. For instance, the use of affinity-based methods such as coated magnetic nanoparticles requires less time and played already an important role in the MS-based identification of clinically and environmentally relevant microorganisms [52-54]. Fractionation tools such as hollow fiber field flow fractionation or cell sorting by flow cytometry can be applied to separate and concentrate different microorganisms (*i.e.*, different types of bacterial cells and even yeast cells) present in the polymicrobial samples [27, 51, 55].

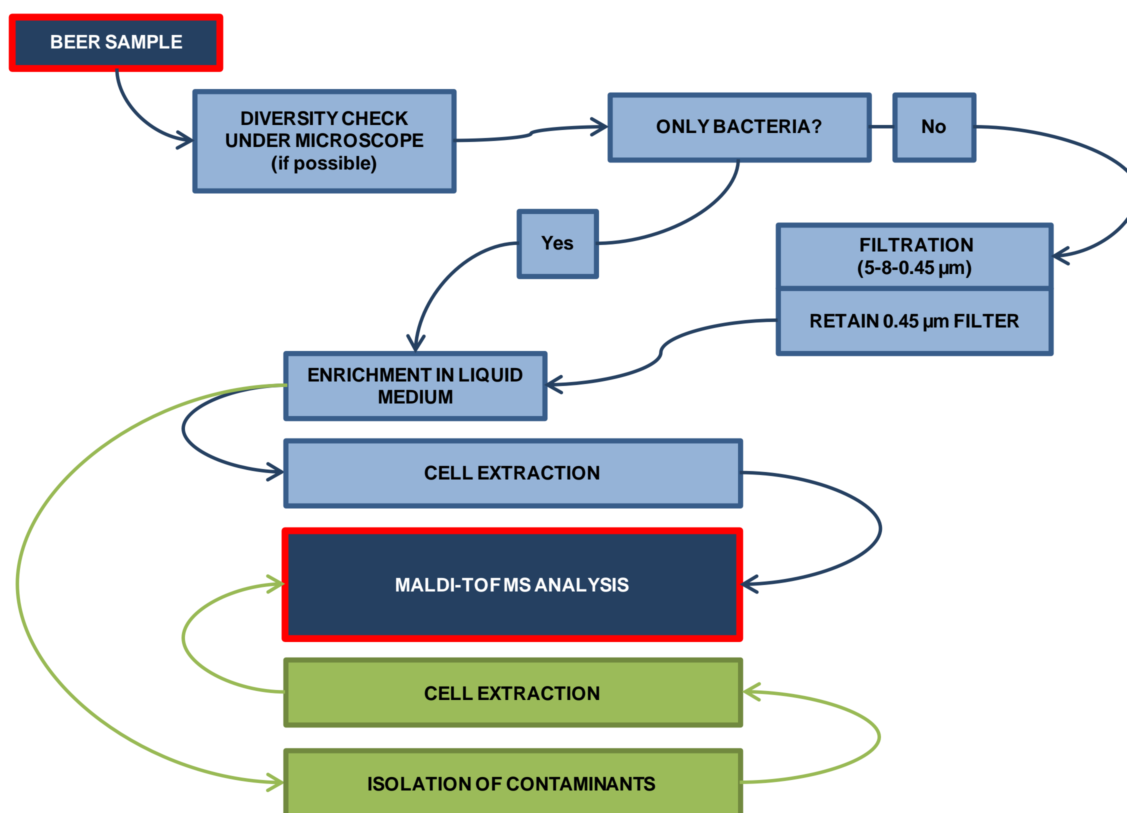

FIG 8.7. Scheme of the general workflow for the detection and identification of beer spoilage bacteria in enrichment cultures of beer and brewery samples using MALDI-TOF MS (blue pathway). Confirmation of these identification results can be obtained by isolating the contaminants present in the enrichment cultures and subsequently identify them via MALDI-TOF MS (green pathway).

8.1.5 *MALDI-TOF MS as infraspecific discrimination tool applied in brewing microbiology*

In the course of our study, the applicability of MALDI-TOF MS as a fast technique to verify the purity of brewer's pitching yeast cultures was investigated based on a practical case study (**CHAPTER 7**). The quality control manager of a Belgian brewery noted deviations in fermentation outcome for several production batches of a top fermented beer with refermentation in the bottle which were potentially related to a contamination of the brewer's pitching yeast culture. Microscopic analysis revealed the presence of two different yeast cell morphologies in both samples. The MALDI-TOF mass spectra generated from isolates obtained from both samples (*i.e.*, the spoiled brewer's pitching yeast culture and a top fermented beer with refermentation in the bottle produced with the spoiled brewer's pitching yeast culture) were compared with those of the axenic culture of the brewer's pitching yeast. The curve-based cluster analysis revealed two distinct types of mass spectra that correlated either with the axenic in-house brewing pitching yeast culture or with a potential wild-type yeast strain. Moreover, sequence analysis of the D1/D2 region of 26S rRNA gene identified both types of yeast strains with 99.9% sequence similarity as *Saccharomyces cerevisiae*. The results

demonstrated as a proof of concept that MALDI-TOF MS allows discrimination of brewing and wild-type *S. cerevisiae* isolates by comparing their mass spectra with that of the in-house production brewer's yeast strain.

Compared to currently available DNA-based strain level differentiation techniques [56], the proteomic-based MALDI-TOF MS approach can be proposed as a more rapid and straightforward tool for infraspecific level discrimination. This approach however demands for high resolution and tends to be more challenging as strains of the same species have substantial genetic overlap, and most of the peak classes observed in the different MALDI-TOF mass spectra are identical [22, 57, 58]. The in-depth peak-based numerical analysis performed in **CHAPTER 4** focussed on the presence of strain-specific peak classes; and revealed that the number of such strain-specific peak classes varied depending on the growth medium used (**CHAPTER 4**). Therefore, it is of utmost importance to cultivate the strains using strict growth conditions when performing comparative infraspecific mass spectral analysis (**CHAPTER 4**; [22]). An in-depth peak-based numerical analysis of the mass spectra of the brewing and wild-type yeast strains grown at the same culture conditions revealed that seven peak classes occurred solely in the mass spectra generated from the wild-type yeast isolates. More advanced MS-based methods could enable the identification of proteins of which these strain-specific peaks originated, thereby providing more insights in the physiological characteristics of the wild-type strain and their contribution in deviating fermentation outcome. Recently, MALDI-TOF MS has been explored to discriminate among isolates of *Lb. brevis* with respect to their beer spoilage potential [*i.e.*, their tolerance towards iso-α-acids and their ability to grow in four different types of beer (wheat beer, lager beer and two different pilsner beers)] [59]. However, of the 17 strains examined, none of the peaks detected could be related exclusively to either strong or weak beer spoilage capability [59]. Remarkably, during the latter study a minimum peak detection rate of 40% only was applied. This parameter is in our opinion crucial when performing infraspecific discrimination studies; and was set at 100% in our study so that each strain-specific peak occurred in each of the mass spectra generated from that particular strain and thereby eliminating biological or technical induced variations (**CHAPTER 4**).

Infraspecific level discrimination allows for a more comprehensive analysis of spoilage microorganisms found in the brewery as it can assist in the determination of the source of contamination [10, 56, 60, 61]. In the present case study, the results demonstrated that a wild-type yeast strain contaminated the brewer's pitching yeast culture and that this contamination persisted until packaging of the top fermented beer with refermentation in the bottle. In earlier studies we isolated *Lb. malefermentans* from two different batches of contaminated bottled dinner beer obtained from the same brewery with a one year interval. Mass spectra generated from all of the

isolates obtained from both samples were very similar and differed from those of the two *Lb. malefermentans* reference strains present in the identification database. Moreover, isolates from both samples had indistinguishable RAPD fingerprints suggesting that *Lb. malefermentans* is a resident spoilage bacterium of this brewery (data not shown). Sampling of critical control points in the brewery (*e.g.*, brewer's yeast cultures, biofilm sensitive dead-ends in piping systems, conveyors in the filling hall) and subsequent MALDI-TOF MS analysis coupled to RAPD fingerprinting analysis of the obtained isolates could rapidly elucidate the real origin of this *Lb. malefermentans* strain and enables the brewery's quality manager to counteract against future spoilage.

8.2 The bacterial diversity observed in the spoiled beer and brewery samples examined

The main goal of the present study was to evaluate the application of MALDI-TOF MS as high-throughput and rapid identification tool for beer spoilage bacteria belonging to AAB and LAB; and not to perform an extensive diversity study of beer spoilage bacteria observed in the brewery. The samples investigated represented a small, but heterogeneous selection of brewery intermediates or end products that are prone to bacterial spoilage (*e.g.*, sweet wort, brewer's pitching yeast cultures, low-alcoholic or non-pasteurised beers) (**CHAPTER 1**; **CHAPTER 5.1**). In total 348 potentially beer spoiling isolates belonging to fifteen AAB and LAB species were obtained from only fourteen samples retrieved from three Belgian breweries (**CHAPTER 5.1**).

The large bacterial diversity observed in these fourteen samples was remarkable; moreover seven out of fourteen samples were contaminated with more than one species. For instance, one sample contained *Lb. malefermentans*, *P. inopinatus*, and four AAB species (*i.e.*, *Acetobacter indonesiensis*, *A. fabarum*, *G. japonicus* and a novel unnamed *Gluconobacter* species). Slime forming AAB are considered to be primary brewery colonizers. Through their growth in biofilms they contribute to the favourable conditions for other beer spoilage bacteria by reducing oxygen levels and providing an acidic environment [5, 11, 12]. The diverse range of such 'indirectly spoiling' AAB species isolated from this sample may be correlated with the presence of biofilms in the piping systems of the filling installation in which *Lb. malefermentans* and *P. inopinatus* can be harboured. Sampling of biofilm-sensitive dead-ends in the piping systems and on conveyors belts in the filling hall could reveal the origin of these contaminants. In total seven out of fourteen samples were contaminated with the obligate beer spoiling bacterium *Lb. brevis*; and four of these samples were of polymicrobial nature. For example, a bottled top fermented beer with an alcohol content of 6.6% v/v was not only contaminated with *Lb. brevis*, but also with the 'indirectly spoiling' AAB species *A. persici* and *A.*

orleanensis. A final kegged pilsner beer was contaminated with two obligate beer spoiling bacteria, *Lb. brevis* and *Lb. backii*.

Microbiological contamination of the brewer's pitching yeast culture is a major concern, as these cultures are often not fully aseptically cropped and could successively spoil other fermentation batches. In our study, four contaminated yeast samples were retrieved from two different breweries; and all of them were contaminated with the indirectly beer spoiling bacterium *A. cerevisiae/A. malorum*. One yeast sample was also contaminated with *Gluconobacter cerinus* and a novel species belonging to the genus *Gluconobacter*. The latter species was also isolated from lambic beer samples in both an industrial and a traditional type of lambic brewery located 74 km apart in Belgium and was described in the present study as the novel species *Gluconobacter cerevisiae* (**CHAPTER 5.3**) [62]. *Acetobacter cerevisiae/A. malorum* was also collected from two bottled dinner beers characterised by different alcohol contents, *i.e.*, 1.8% v/v and 2.25% v/v. Besides *A. cerevisiae/A. malorum*, *Gluconobacter oxydans* was also isolated from the latter dinner beer sample (with an alcohol content of 2.25% v/v) which was artificially sweetened whereas the former was not. The production of non-pasteurised, low- and non-alcoholic beers demands for strict hygienic standards to prevent bacterial spoilage. In the present study, the potential spoilage bacterium *P. claussenii* was isolated from a fruity wheat beer characterised by an alcohol content of 4.3% v/v and low hop content (IBU of 5).

Previous reports stated that the prevalence of AAB species as beer spoilage organisms decreased [9, 63]; however this is in contrast with the results obtained in the present study, *i.e.*, ten out of fifteen species recovered from eight out of fourteen samples belong to the AAB (**CHAPTER 5.1**). Therefore, they should not be neglected as potential spoilage bacteria as they may cause off-flavours, turbidity and ropiness in the presence of oxygen [64]. Moreover, such bacteria will be missed by detection and identification kits currently used in the brewery industry which focus primarily on beer spoiling LAB and some strict anaerobic genera (*e.g.*, *Pectinatus* and *Megasphaera*).

8.3 The risks and benefits of MALDI-TOF MS, some concluding remarks and perspectives

The established culture-dependent and culture-independent methods for the detection and/or identification of beer spoilage microorganisms are summarised in **CHAPTER 2**. In general, these methods are rather time-consuming, often lack throughput capacity and do not facilitate real-time interventions. Furthermore, these methods mostly target only a narrow range of beer spoilage bacteria. **TABLE 8.1** visualises the comparative analysis of these currently established methods with MALDI-TOF MS. The present study demonstrated that MALDI-TOF MS enabled the rapid and accurate identification of a diverse group of beer spoilage bacteria isolated from different samples. Hence, one of the major benefits of MALDI-TOF MS as a microbial detection and identification tool is its flexibility. The mass spectral database constructed in **CHAPTER 5.1** can be easily complemented with (novel) species without the need for modifying the sample treatment procedure. Other detection and identification methods will require modifications of the primers, oligonucleotide probes and monoclonal antibodies applied. This flexibility is a major asset as novel beer spoilage species continue to emerge [10, 62, 65-67] (**CHAPTER 5**). In contrast, data portability is not straightforward for MALDI-TOF mass spectra, and this due to the influences of different parameters (*e.g.*, growth media, sample preparation procedures and instrumentation) on the mass spectra generated [58, 68]. At present, publicly dedicated databases comprising unprocessed mass spectra of microorganisms (cf. DNA sequence databases) are minimally available. The use of internal and/or external control samples and meticulous annotations of different parameters (*e.g.*, growth conditions, instrumentation and sample treatment used) affecting the mass spectra generated could potentially enable future inter-laboratory comparison.

Another major advantage of the MALDI-TOF MS technique resides in its speed of performance and throughput capacity. The sample preparation procedure is very straightforward and consists of only a brief cell extraction procedure followed by automated MALDI-TOF MS analysis. Approximately 200 samples can easily be analysed in one working day. Hence, identifications are obtained faster using MALDI-TOF MS compared to conventional methods. A thorough analysis of a microbial community requires the identification of large numbers of isolates which is not only time-consuming but also expensive [69]. In the present study, a total of 384 isolates was rapidly reduced to only 15 distinct mass spectra. Other studies also reported the suitability of MALDI-TOF MS as a rapid screening method in order to remove redundancy and to select for novelty [69-75]. For instance, the technique is useful for the characterization of LAB associated with the production of diverse

Vietnamese fermented vegetables [76] and for the grouping of heterotrophic marine bacterial isolates from sea-water surface layers of the Norwegian Trondheimsfjord [69].

Finally, the costs of MALDI-TOF MS consumables, operational manipulations and downstream processing of mass spectral data generated are low compared to conventional detection and identification methods [77]. However the initial investment cost and subsequent maintenance costs of the apparatus are high [71], which render its purchase and use in small and medium-sized brewery companies problematic. It seems therefore more appropriate to adopt MALDI-TOF MS in food-related reference laboratories [*e.g.*, Das Bierlabor (Germany) or Accugenix® Microbial ID & Strain Typing (USA)] and to provide these low-cost identification services to the brewing industry.

As stated previously, MALDI-TOF MS like almost each detection and/or identification method currently available in this industry does not allow direct detection and identification of bacteria in spoiled samples due to the low numbers of microbial cells present and therefore requires an enrichment step and ideally the isolation of axenic cultures prior to analysis (**CHAPTER 2**; **TABLE 8.1**). The major disadvantage of the technique is that the inclusion of cultivation steps often leads to misinterpretations of results obtained, as, for instance, fastidious, slow-growing or uncultivable microorganisms will be easily missed [78, 79]. We demonstrated however that MALDI-TOF MS can be used for the direct identification of bacteria in enrichment cultures of monomicrobially contaminated samples. The identification of microorganisms present in enrichment cultures of polymicrobial samples remains challenging. Advancements in the MALDI-TOF MS technology (*i.e.*, improvement of the dynamic range of the instrumentation) and especially in data analysis tools may help to address these outstanding issues. At present, it is better to invest in improving data analysis instead of novel hardware; and thereby attempt to extract as much information as possible from the mass spectra generated.

TABLE 8.1. Overview of characteristics, major benefits and drawbacks of currently established methods used for the detection and/or identification of beer spoilage bacteria (BSB).

	Method	D / I / T	Target	Sensitivity	Time of analysis	Benefits	Drawbacks
GROWTH	Culturing on selective and non-selective growth media[¥]	D	All BSB	1-5 culturable cells/sample	days - weeks	Sensitive (when appropriate culture condition are applied); detection of culturable cells; easy-to-perform	Time-consuming (days up to weeks); often unreliable results; viable but nonculturable cells are not detected
ATP	Detection of bacterial ATP using the enzyme coupled luciferin/luciferase assay[¥]	D	All BSB	50 cells/sample	<1 h (48 h*)	Rapid tool for hygienic monitoring	Interference with chemicals (false-negative results); expensive read-out system; false-positive results; variable sensitivity; poor reproducibility
IMMUNO	Immuno-based assays using MAbs	D / I	Species- or group-specific	3-40 cells/100 mL sample	<1 h (48 h*)	Rapid and sensitive; quantification possible	Expensive and tedious design of MAbs; cross-reactions & background interferences; expensive read-out system; no distinction between viable and dead cells
PROBE	Hybridization of oligonucleotide probes onto specific target sequences[¥]	D / I	Species- or group-specific	10^2-10^5 CFU/mL sample; 1-5 CFU / membrane (microcolony approach)	3 h (48 h*)	Without DNA extraction; quantification possible; detection of viable cells	Investment costs; enrichment step preferred (microcolonies); design of probes; different probemarkers for multiplex detection
PCR	End-point amplification of target DNA sequences	D / I	Species- or group-specific	10^3-10^8 cells/100 mL sample	3-6 h (48 h*)	Easy-to-use; detection of spoilage capabilities	Enrichment and/or pre-filtration step needed; DNA-extraction; PCR-inhibition from beer matrix (false-negative results); unable to distinguish between viable or dead cells; presence of naked DNA (false-positive results); post-PCR processing (only for end-time PCR); primer development; high investment costs
	Real-time amplification and quantification of target DNA sequences[¥]	D / I	Species- or group-specific	10^4-10^5 cells/100 mL sample	2-3 h; (24-72 h*)	Less post-PCR manipulations; real-time follow-up; better cost/benefit ratio	
FINGERPRINTING	Ribotyping, restriction enzyme-pattern analysis of genomic DNA using Southern blot analysis[¥]	I / T	All BSB	Pure cultures	8 h	Automation possible; easy-to-perform; standardised; objective identification; typing possible	High investment and running costs; time-consuming; pure cultures are required; cumbersome sample preparations; identification database-dependent; selection of restriction enzymes (Ribotyping); no high-throughput analyses
	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) protein-profiling	I / T	All BSB	Pure cultures	1-2 days	Objective identification; gene expression-based protein patterns	
MALDI-TOF MS	Mass spectra generated from microbial intact cells or crude protein extractions thereof are typically unique for a certain microorganism	D / I / T	All BSB	Pure cultures or 10^7-10^8 cells/sample (direct analysis of enrichment cultures)	<1 h (24h-days*)	Rapid and accurate; flexible; straightforward sample preparation; high-throughput analysis; automation possible; dereplication tool; low consumable cost; objective identification; typing possible	High investment and maintenance costs; culture-dependent; data portability (inter- and intra-laboratory reproducibility)

[¥]commercialised;

*time of pre-enrichment of sample;

Abbreviations: D, Detection; I, Identification; T, Strain level differentiation possible; MAbs: Monoclonal antibodies

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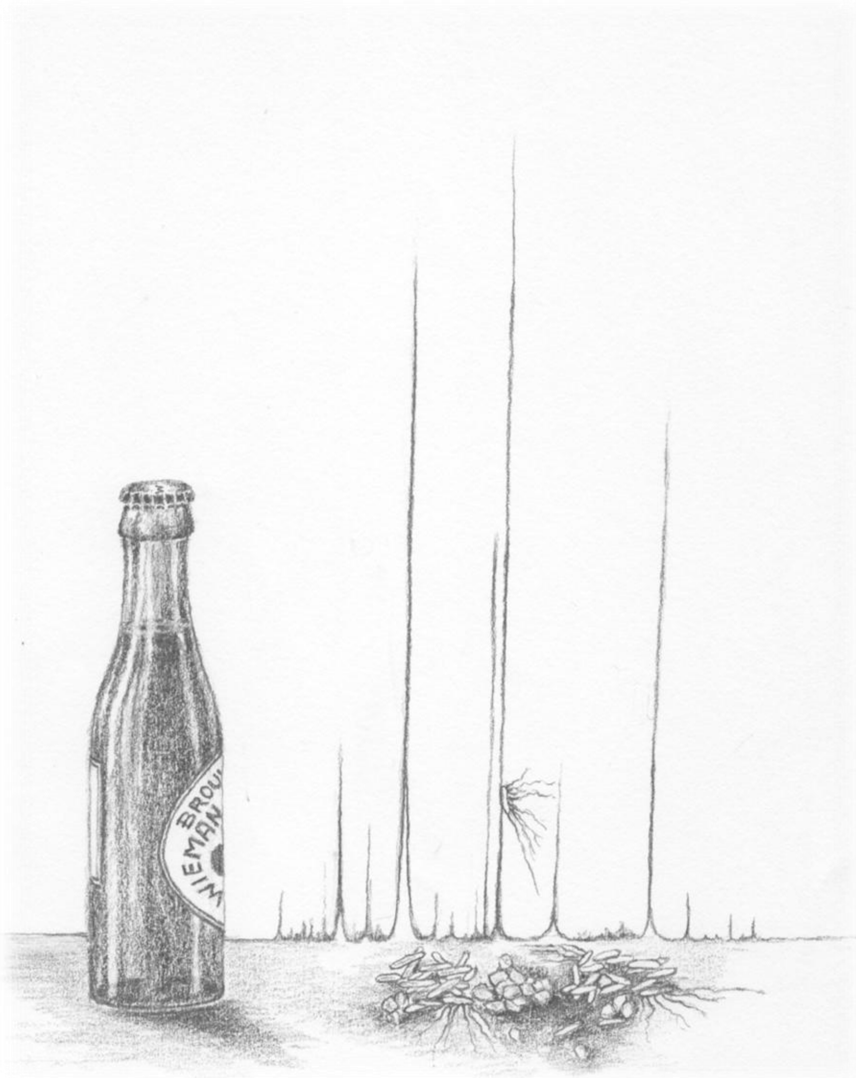

Part V | Summary & Samenvatting

SUMMARY

Beer is a beverage with usually good microbiological stability because it contains almost no oxygen and nutrients for bacterial growth. In addition, low pH, high CO_2-content and the presence of ethanol and antibacterial hop compounds ensure microbiological stability. Nevertheless, beer spoilage due to bacteria is a common problem in the brewing industry worldwide. These bacteria typically cause visible turbidity, acidity and off-flavours. Nowadays, the hop-resistant lactic acid bacteria (LAB) *Lactobacillus backii*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus lindneri*, *Lactobacillus coryniformis*, *Lactobacillus plantarum*, *Lactobacillus malefermentans*, *Lactobacillus parabuchneri*, *Pediococcus damnosus*, *Pediococcus inopinatus* and *Pediococcus claussenii* are generally regarded as the most problematic beer spoilage microorganisms (**CHAPTER 1**). The prevalence of the strictly aerobic acetic acid bacteria (AAB) has decreased because of improved process filling technology. However, in aerobic environments, AAB can prevail and form biofilms in which other spoilage bacteria are protected and survive. During the last two decades, anaerobic Gram-stain negative bacteria species have gained importance as spoilage bacteria in the brewing industry as well (**CHAPTER 1**). The taxonomically diverse group of beer spoilage bacteria is currently detected and/or identified using a range of culture-dependent or culture-independent methods which are time-consuming, often lack high-throughput capacity, do not facilitate real-time interventions, and target only a narrow range of bacteria (**CHAPTER 2**). Multiple research groups demonstrated the usefulness of the soft ionisation technique matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) as a high-throughput, accurate and low-cost identification tool for isolates obtained in medical, environmental and food-related studies, enabling the simultaneous identification of a diverse group of microorganisms (**CHAPTER 3**). Therefore, MALDI-TOF MS was investigated as an alternative identification tool for the most prevalent beer spoiling AAB and LAB.

The MALDI-TOF mass spectra generated can be used to characterise and identify bacteria at species and even at infraspecific levels. To this end, an MALDI-TOF mass spectral database was constructed containing 273 mainly well-characterised reference strains representing 52 AAB and LAB species originating from spoiled beer and other brewery samples but also from other niches where strains of the same species occur. Moreover, all reference strains were cultivated onto multiple selective and non-selective growth media to anticipate cultivation condition induced variability in an organism's MALDI-TOF MS profile (**CHAPTER 4**). Cultivation media that did not sustain optimal growth influenced the mass spectra generated strongly; yet, the growth medium induced variations had no impact on the final species level identification. The present study also demonstrated that the number of strain-specific peaks is culture medium-dependent and that the selected culture medium

affects the potential for strain level differentiation (**CHAPTER 4**). The data finally also demonstrated that the number of shared mass peaks detected appeared to decrease with an increasing number of strains per species examined, and that a growth medium-dependent core set of peaks and thus peptides seems to exist (**CHAPTER 4**). It is assumed that the inclusion of more reference strains per species will eventually lead to a stabilizing core set of peaks that could indicate sufficient coverage of the intraspecies diversity of the MALDI-TOF mass spectral identification database. It is therefore of utmost importance to have an exhaustive MALDI-TOF MS identification database containing good quality mass spectra generated from sufficient reference strains of the same species grown on multiple culture media.

The performance of the identification database largely depends on the correct classification of the reference strains examined; and in total 34 (12%) out of 273 strains were reclassified based on their position in the peak-based cluster analysis (using the Dice coefficient and UPGMA cluster algorithm; **CHAPTER 5.1**). State-of-the-art gene sequence analysis confirmed each of the MALDI-TOF MS reclassifications implying that the previous identification of those reference strains was wrong. Some of these aberrantly clustering strains represented species that were never reported as potential beer spoilage bacteria such as *Gluconobacter japonicus* and *Acetobacter fabarum*. Noteworthy in this context was that the mass spectra generated from the type strain of *Pediococcus lolii* grouped together with spectra of *Pediococcus acidilactici* strains. An in-depth taxonomical study confirmed that the *P. lolii* subcultures deposited in the DSMZ and JCM culture collections belong to *P. acidilactici* (**CHAPTER 5.2**).

Species level identification of spoilage microorganisms is of utmost importance for the brewery industry even though not all strains share similar beer spoiling capabilities. Nevertheless, species level identification enables the estimation of the impact of bacterial spoilage and the prevention of further product deterioration and future spoilage. The fourteen spoiled beer and brewery samples examined during the present study were provided by three distinct Belgian breweries. These samples represented a small, but heterogeneous selection of brewery intermediates or end products that are prone to bacterial spoilage (*e.g.*, sweet wort, brewer's pitching yeast cultures, low-alcoholic or non-pasteurised beers). Due to the low numbers of bacterial cells present in such samples and the small sample volume that is available for analysis, an enrichment step prior to isolation was necessary. In total 348 isolates were retrieved from these samples, and were subsequently analysed using MALDI-TOF MS. The diversity observed among this large set of isolates was rapidly ordered by grouping the mass spectra obtained by means of the curve-based cluster analysis (using the Pearson product-moment correlation coefficient and UPGMA cluster algorithm; **CHAPTER 5.1**). In total 15 distinct MALDI-TOF mass spectra were obtained after this dereplication step; and several cluster

representatives were chosen depending on the cluster heterogeneity observed. The peak-based cluster analysis (using the Dice coefficient and UPGMA cluster algorithm) correctly identified 94% of the isolates as revealed by subsequent sequence analysis of protein coding genes (*Acetobacter cerevisiae*/*Acetobacter malorum*, *Acetobacter orleanensis*, *Gluconobacter cerinus*, *Gluconobacter oxydans*, *Lactobacillus backii*, *Lactobacillus brevis*, *Lactobacillus malefermentans*, *Pediococcus claussenii* and *Pediococcus inopinatus*). The remaining 6% of the isolates were either representing species that were not regarded as potential beer spoilage bacteria (*Acetobacter fabarum*, *Acetobacter indonesiensis*, *Acetobacter persici* and *Gluconobacter japonicus*) and therefore not present in our database or represented novel species altogether (*Gluconobacter* sp. and *Gluconobacter cerevisiae* sp. nov. [**CHAPTER 5.3**]). The main goal of the study was to evaluate the application of MALDI-TOF MS as high-throughput and rapid identification tool for beer spoilage bacteria belonging to AAB and LAB; and not to perform an extensive diversity study of beer spoilage bacteria observed in the brewery. Previous studies stated that the prevalence of AAB species as beer spoilage organisms decreased; and are therefore not accounted for in currently available detection and identification kits applied in the brewing industry. Yet, the relatively high number of AAB species (10 out of 15) recovered from eight samples in the present study indicated that these species should not be ignored as potential beer spoiling bacteria (**CHAPTER 5.1**).

To speed-up analysis, the applicability of MALDI-TOF MS was investigated for the detection and identification of bacteria directly from enrichment cultures of spoiled beer and brewery samples with or without the concomitant presence of yeast cells (**CHAPTER 6**). The minimal cell concentration required to generate good quality MALDI-TOF mass spectra that allowed identification was determined at approximately 5×10^7 CFU/mL enrichment culture. The (beer) matrix strongly influenced the mass spectra generated; yet, two consecutive washing steps using Milli-Q water prior to cell extraction were sufficient to eliminate the matrix effect almost completely. Moreover, a sample preparation protocol based on a filtration procedure enabled detection and identification of bacteria directly in enrichment cultures of spoiled beer and brewery samples, even in the presence of yeast or mould cells. Mass spectra generated from the monomicrobially enrichment cultures were of good quality and enabled identification of the potential spoilage bacterium present. When peak-based cluster analysis of the mass spectrum of the enrichment culture assigned it to a certain species, an in-depth comparison of peaks may or may not suggest the presence of cultures of additional species. The identification of microorganisms present in polymicrobially contaminated samples remained extremely challenging, considering the various types of peak suppression effects when different microorganisms are present after growth in the enrichment culture. Nevertheless, preliminary identification results may provide the brewery's quality manager with pivotal

information to start prevention of further product deterioration and future spoilage. The data demonstrated that it is highly recommended to plate the enrichment cultures and use MALDI-TOF MS to dereplicate and identify the isolates obtained in order to confirm the identification results.

Compared to currently available DNA-based strain level differentiation techniques, the proteomic-based MALDI-TOF MS approach can be proposed as a more rapid and straightforward tool for infraspecific level discrimination. The results presented in this PhD study demonstrated as a proof of concept that MALDI-TOF MS allows discrimination of brewing and wild-type *Saccharomyces cerevisiae* isolates by comparing their mass spectra with that of the in-house production brewer's yeast strain (**CHAPTER 7**). An in-depth peak-based numerical analysis of the mass spectra of the brewing and wild-type yeast strains grown at the same culture conditions revealed multiple strain-specific peaks in the mass spectra generated from the wild-type yeast.

In conclusion, the results of the PhD study demonstrated that MALDI-TOF MS coupled to a well-constructed mass spectral database is a powerful tool for the detection, identification and typing of spoilage microbiota in the brewing industry. Compared to currently established detection and/or identification techniques applied in brewing microbiology, MALDI-TOF MS facilitates high-throughput, accurate and rapid detection and identification of a taxonomically diverse group of bacteria.

SAMENVATTING

Bier is een alcoholische drank met een relatief goede microbiële stabiliteit en dit omwille van het zuurstofarme milieu en de beperkte aanwezigheid van nutriënten voor de groei van bacteriën. Deze stabiliteit wordt nog versterkt door de lage pH, het hoge CO_2-gehalte en de aanwezigheid van alcohol en antibacteriële hopcomponenten. Bacterieel bierbederf is desondanks een veelvoorkomend probleem in de brouwerijindustrie met een zware economische impact tot gevolg. Bierbederf wordt immers vaak gekenmerkt door een troebel, verzuring en de aanwezigheid van onaangename geur- en smaakcomponenten. Over het algemeen worden de hopresistente Grampositieve melkzuurbacteriën (MZB), zoals *Lactobacillus backii*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus lindneri*, *Lactobacillus coryniformis*, *Lactobacillus plantarum*, *Lactobacillus malefermentans*, *Lactobacillus parabuchneri*, *Pediococcus damnosus*, *Pediococcus inopinatus* en *Pediococcus claussenii* beschouwd als de meest problematische bierbedervers (**HOOFDSTUK 1**). Het gebruik van zuurstofarme afvulsystemen in de brouwerij resulteerde in een verlaagd voorkomen van strikt aerobe azijnzuurbacteriën (AZB). Echter, in een zuurstofrijk milieu kunnen deze AZB zich profileren en uitgroeien tot biofilms waarin andere bederfbacteriën worden beschermd en kunnen overleven. In de voorbije twee decennia kenden de strikt anaerobe Gramnegatieve bacteriën een opmars als potentiële bedervers in de brouwerij (**HOOFDSTUK 1**). Momenteel wordt de taxonomisch diverse groep van bacteriële bierbedervers gedetecteerd en/of geïdentificeerd met behulp van tijdrovende en onvoldoende nauwkeurige cultuurafhankelijke en cultuuronafhankelijke technieken. Deze laatste zijn vaak niet breed en gemakkelijk toepasbaar, en slechts gericht op een beperkte groep van bedervers (**HOOFDSTUK 2**). Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) werd door verschillende onderzoeksgroepen succesvol geïmplementeerd als een snelle, accurate en goedkope identificatietechniek voor een diverse groep van micro-organismen van medische, omgeving en voedingsoorsprong (**HOOFDSTUK 3**). In het doctoraatsonderzoek, werd MALDI-TOF MS onderzocht als een alternatieve identificatiemethode voor potentiële bacteriële bierbedervers met de focus op de AZB en MZB.

De gegenereerde MALDI-TOF massaspectra kunnen worden gebruikt om bacteriën te karakteriseren en te identificeren op species- en zelfs op infraspecifiek niveau. Tijdens dit onderzoek werd een identificatiedatabank opgebouwd bestaande uit spectra van 273 voornamelijk goed gekarakteriseerde referentiestammen die in totaal 52 species behorende tot AZB en MZB vertegenwoordigen. Deze stammen werden geïsoleerd uit gecontamineerde bier- en brouwerijproducten, maar ook uit andere niches waarin stammen van hetzelfde species voorkomen.

Elke referentiestam werd opgekweekt op verschillende selectieve en niet-selectieve groeimedia om de groeimedium geïnduceerde variabiliteit ten opzichte van de gegenereerde spectra te onderzoeken (**HOOFDSTUK 4**). Als het cultuurmedium niet gunstig was voor de groei van de bacterie, werden er duidelijke variaties in de spectra opgemerkt. Deze hadden echter geen invloed op de finale speciesdifferentiatie. De studie toonde ook aan dat het aantal gedetecteerde stamspecifieke pieken in de spectra cultuurafhankelijk is, met als gevolg dat er mag geconcludeerd worden dat het gebruikte groeimedium een duidelijke invloed zal hebben op stamdifferentiatie (**HOOFDSTUK 4**). Het aantal gemeenschappelijke pieken daalt naarmate er meer spectra van stammen van hetzelfde species werden geanalyseerd en deze groep van gemeenschappelijke pieken blijkt ook cultuurafhankelijk te zijn. Het insluiten van meerdere referentiestammen van hetzelfde species zal uiteindelijk leiden tot een vaste groep van gemeenschappelijke pieken en kan daarbij een maat zijn voor de intraspecies diversiteit van de MALDI-TOF MS identificatiedatabank. Finaal mag gesteld worden dat een MALDI-TOF MS identificatiedatabank het beste wordt opgebouwd met voldoende referentiestammen die gegroeid werden op verschillende cultuurmedia (**HOOFDSTUK 5.1**).

Het succesvol gebruik van de databank berust grotendeels op de correcte taxonomische classificaties van de stammen ingesloten in het referentiekader. De piekgebaseerde clusteranalyse (m.b.v., Dice coëfficiënt en UPGMA clusteralgoritme) van de gegenereerde spectra van alle referentiestammen toonde aan dat in totaal 34 (12%) van de 273 stammen in eerder onderzoek aan het verkeerde species werden toegewezen (**HOOFDSTUK 5.1**). Sequentieanalyses van huishoudgenen bevestigden elk van deze MALDI-TOF MS hergroeperingen, wat impliceerde dat de eerdere identificaties van deze referentiestammen incorrect waren. Sommige van deze incorrect geclassificeerde stammen vertegenwoordigden species die nooit eerder als potentiële bierbedervers werden vermeld, zoals *Gluconobacter japonicus* en *Acetobacter fabarum*. Opmerkelijk is dat de gegenereerde spectra van de typestam van *Pediococcus lolii* samen groepeerden met spectra gegeneerd van de *Pediococcus acidilactici* referentiestammen. Een grondige polyfasische taxonomische benadering bevestigde dat de *P. lolii* subculturen gedeponeerd in de internationale DSMZ- en JCM-cultuurcollecties behoren tot *P. acidilactici* (**HOOFDSTUK 5.2**).

Hoewel niet alle stammen van hetzelfde species eenzelfde bederfprofiel hebben, is de identificatie van de geïsoleerde bacteriën tot op speciesniveau uitermate belangrijk voor de brouwerijindustrie. Aan de hand van deze resultaten kan de kwaliteitsmanager ingrijpen en het bederf eventueel inperken en zelfs vermijden in de toekomst. In totaal werden veertien gecontamineerde bier- en brouwerijproducten onderzocht tijdens deze studie. De stalen vertegenwoordigden een heterogene groep van brouwerij tussen- en eindproducten die gevoelig zijn aan bacterieel bederf (bv, wort, culturen van de brouwersgist, laag alcoholische en niet gepasteuriseerde bieren). Aanrijken in een

vloeibaar medium was nodig omdat in de stalen lage bacteriële celaantallen aanwezig zijn en het beschikbare staalvolume beperkt was. In totaal werden 348 isolaten verkregen waarvan de massaspectra na MALDI-TOF MS-analyses werden gegroepeerd met behulp van curvegebaseerde clusteranalyse (m.b.v., Pearson product-moment correlatie coëfficiënt en UPGMA cluster algoritme; **HOOFDSTUK 5.1**). In totaal werden vijftien verschillende MALDI-TOF massa spectra weerhouden. Een aantal isolaten werden geselecteerd als vertegenwoordigers van elk van de vijftien clusters en dit gebeurde volgens de geobserveerde heterogeniteit. De piekgebaseerde clusteranalyse (m.b.v., Dice coëfficiënt en UPGMA cluster algoritme) liet vervolgens toe om 94% van de isolaten op een betrouwbare manier te identificeren (*Acetobacter cerevisiae*/*Acetobacter malorum*, *Acetobacter orleanensis*, *Gluconobacter cerinus*, *Gluconobacter oxydans*, *Lactobacillus backii*, *Lactobacillus brevis*, *Lactobacillus malefermentans*, *Pediococcus claussenii* and *Pediococcus inopinatus*). Deze MALDI-TOF MS-identificatieresultaten werden gevalideerd en bevestigd via sequentieanalyses van huishoudgenen (**HOOFDSTUK 5.1**). De resterende 6% van de isolaten waren ofwel species die nooit eerder als bierbedervers werden omschreven (*Acetobacter fabarum*, *Acetobacter indonesiensis*, *Acetobacter persici* en *Gluconobacter japonicus*) en daarom ook niet werden opgenomen in ons MALDI-TOF MS- referentiekader of ze vertegenwoordigden nieuwe species (*Gluconobacter* sp. en *Gluconobacter cerevisiae* sp. nov. [**HOOFDSTUK 5.3**]). Het voornaamste doel van dit onderzoek was om de toepassing van MALDI-TOF MS als een snelle en betrouwbare techniek voor de identificatie van bierbedervende AZB en MZB te evalueren en niet om een uitgebreide diversiteitstudie uit te voeren. Eerder onderzoek toonde aan dat de prevalentie van AZB in de brouwerijen duidelijk was afgenomen en toch werden tijdens dit onderzoek een groot aantal AZB geïsoleerd (10 van de 15 species) uit acht van de veertien onderzochte stalen (**HOOFDSTUK 5.1**). Hieruit blijkt dat deze groep van bierbedervers niet zou mogen worden genegeerd als potentiële bederforganismen, zoals dat nu wel het geval is in de courant gebruikte commerciële detectie- en/of identificatiekits.

Om de tijdrovende isolatiestap te vermijden, werd het potentieel van MALDI-TOF MS onderzocht voor de detectie en identificatie van de bedervers rechtstreeks uit de aanrijkingsculturen van gecontamineerde stalen (**HOOFDSTUK 6**). De minimale celconcentratie die nodig was om spectra met een goede kwaliteit te genereren werd bepaald op ongeveer 5×10^7 KVE / ml aanrijkingscultuur. Zoals verwacht, had de (bier)matrix een sterke invloed op de gegenereerde massaspectra, maar met behulp van twee opeenvolgende wasstappen met Milli-Q water werden storende achtergrondcomponenten verwijderd. De finale staalvoorbereiding bestaat uit een filtratiestap waarbij gisten en schimmels werden verwijderd, vervolgens werd alleen de bacteriële fractie aangerijkt. Spectra gegenereerd uit monomicrobieel gecontamineerde aanrijkingsculturen waren van een goede kwaliteit en maakten de speciesidentificatie van de potentiële bederver mogelijk.

Indien de piekgebaseerde clusteranalyse een spectrum toewijst aan spectra gegenereerd van een bepaald species, kan een verdere grondige vergelijking van pieken in deze spectra al dan niet wijzen op de aanwezigheid van additionele species. De identificatie van micro-organismen in polymicrobiële stalen blijft een uitdaging. De aanwezigheid van meerdere micro-organismen in de aanrijkingscultuur leidt immers tot pieksuppressie-effecten waardoor het zeer moeilijk wordt om de pieken van verschillende micro-organismen te onderscheiden van elkaar. Een initiële identificatie biedt de kwaliteitsmanager echter al cruciale informatie om direct in te grijpen en het bederf in te perken en te vermijden in de toekomst. Het blijft aangewezen om de aanrijkingsculturen uit te platen op verschillende cultuurmedia en de potentiële bedervers te isoleren en vervolgens te identificeren.

In vergelijking met huidige DNA-gebaseerde technieken, kan MALDI-TOF MS sneller en eenvoudiger zijn voor de differentiatie op infraspecifiek niveau. Dit onderzoek toonde als een "proof of concept" aan dat MALDI-TOF MS het mogelijk maakt om *Saccharomyces cerevisiae* brouwers- en wild-type gisten te onderscheiden door de spectra van beiden te vergelijken met de spectra van de originele cultuur van de brouwersgist (**HOOFDSTUK 6**). Meerdere stamspecifieke pieken werden geïdentificeerd bij de piekgebaseerde numerieke analyse en deze maakten het mogelijk om deze stammen te differentiëren.

De MALDI-TOF MS techniek gecombineerd met een uitstekend opgebouwde identificatiedatabank zijn een uitermate geschikte methode voor de detectie, identificatie en infraspecifieke differentiatie van potentieel bierbederf micro-organismen in de brouwerijindustrie. MALDI-TOF MS maakt het dus mogelijk om op een snelle, nauwkeurige wijze een taxonomisch diverse groep aan micro-organismen te detecteren en te identificeren.

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Anneleen Wieme

16 mei 2014

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2009 – 2014 (May) **Ph.D. student in biochemistry and biotechnology**

Laboratory of Microbiology; Faculty of Sciences & Laboratory of Brewing and Biochemistry; Faculty of Bioscience engineering; Ghent University
Dissertation: 'Application of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in the brewery environment'
Promotors: Prof. Dr. Peter Vandamme & Prof. Dr. Anita Van Landschoot

EDUCATIONAL BACKGROUND

2007 – 2009 **Master in industrial sciences: biochemistry**

Faculty of Applied Engineering Sciences, University College Ghent
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2004 – 2007 **Professional bachelor degree: chemistry, biochemistry**

Department of Technology (Biochemistry), University College Ghent
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SCIENTIFIC OUTPUT

a1-publications

1. **Wieme A**, Cleenwerck I, Van Landschoot A, Vandamme P. 2012. *Pediococcus lolii* DSM 19927^{T} and JCM 15055^{T} are strains of *Pediococcus acidilactici*. International Journal of Systematic and Evolutionary Microbiology 62:3105-3108.

2. **Wieme AD**, Spitaels F, Aerts M, De Bruyne K, Van Landschoot A, Vandamme P. 2014. The effects of the growth medium on matrix-assisted laser desorption/ionization time-of-flight mass spectra: a case study of acetic acid bacteria. Applied and Environmental Microbiology 80:1528-1538.

3. Spitaels F, Li L, **Wieme A**, Balzarini T, Cleenwerck I, Van Landschoot A, De Vuyst L, Vandamme P. 2014. *Acetobacter lambici* sp. nov., isolated from fermenting lambic beer. International Journal of Systematic and Evolutionary Microbiology 64, 1083-1089.

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5. Spitaels F, **Wieme AD**, Janssens M, Aerts M, Daniel H-M, Van Landschoot A, De Vuyst L, Vandamme P. 2014. The microbial diversity of traditional spontaneously fermented lambic beer. *PLoS ONE* 9, e95384.

6. **Wieme AD**, Spitaels F, Aerts M, De Bruyne K, Van Landschoot A, Vandamme P. 2014. Identification of beer spoilage bacteria using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. International Journal of Food Microbiology (*accepted*).

7. Li L, **Wieme A**, Spitaels F, Balzarini T, Nunes OC, Manaia CM, Van Landschoot A, De Vuyst L, Cleenwerck I, Vandamme P. 2014. *Acetobacter sicerae* sp. nov., isolated from cider and kefir and identification of *Acetobacter* species by *dnaK*, *groEL* and *rpoB* sequence analysis. International Journal of Systematic and Evolutionary Microbiology (*in press*, doi:10.1099/ijs.0.058354-0).

8. **Wieme AD**, Spitaels F, Vandamme P, Van Landschoot A. 2014. Application of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as monitoring tool for in-house brewer's yeast contamination: a proof of concept. The Journal of The Institute of Brewing (*submitted*).

9. Spitaels F, **Wieme AD**, Janssens M, Aerts M, Van Landschoot A, De Vuyst L, Vandamme P. 2014. The microbial diversity of an industrially produced lambic beer shares members of a traditionally produced fermented lambic beer and reveals a core microbiota for lambic beer fermentation. Applied and Environmental Microbiology (*submitted*).

10. Spitaels F[¥], Van Kerrebroeck S[¥], **Wieme AD**, Snauwaert I, Aerts M, Van Landschoot A, De Vuyst L, Vandamme P. 2014. Microbiota and metabolites of aged bottled gueuze beers converge to the same composition. Food Microbiology (*submitted*).

11. **Wieme AD**, Spitaels F, Van Landschoot A, Vandamme P. 2014. Direct detection and identification of bacteria in enrichment cultures of spoiled beer and brewery samples using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry: a proof of concept. (*in preparation*).

b2-publication

Wieme AD, Spitaels F, Vandamme P, Van Landschoot A. 2014. The microbial stability of beer and the use of MALDI-TOF MS as fast identification tool for beer spoilage bacteria – a current update. 2014. In Beer stability – A Challenge for the 21st Century Brewers, pp. 329-346. Eds. Campbell I, Antkiewicz P, Tuszyński T, Błażewicz J. Ogólnopolskie Stowarzyszenie Wspierania Inicjatyw NAUKA-PRZEMYSŁ, Kraków, Poland. ISBN: 978-83-930745-3-2.

ORAL PRESENTATIONS

2014 **Wieme A.D.**, Microbial stability of beers and MALDI-TOF MS for the identification of microbial contaminants, at the 17th School of Fermentation Technology – Beer stability – a challenge for the 21th century brewers; University of Rolniczy, Kraków (Poland).

2012 **Wieme A.D.**, Use of MALDI-TOF MS for the identification of beer spoilage bacteria, at the "Studiedag van de Brouwerij and Mouterij", Koninklijke Oud-Studentenbond Fermentatio Hogeschool Gent; University College Ghent, Ghent (Belgium).

2011 **Wieme A.D.**, MALDI-TOF MS as a high throughput identification tool for beer spoilage bacteria, at the Mass Spectrometry in Food and Feed conference; Royal Flemish Chemical Society (KVCV), Ghent University, Merelbeke (Belgium).

POSTER PRESENTATIONS

2013 Poster presentation at the Trends in Food Analysis VII seminar; Royal Flemish Chemical Society (KVCV); Ghent University, Ghent (Belgium).

2013 Poster presentation at the 34th European Brewing Convention Congress (EBC); European Brewery Convention; NCCK Luxembourg (Luxembourg).

2012 Poster presentation at the World Brewing Congress (WBC); American Society of Brewing Chemists, Portland (Oregon, United States of America).

2012 Poster presentation at the workshop "Microbial Diagnostic Applications of Mass Spectrometry"; HPA Microbiology Services, Colindale (London, United Kingdom).

2011 Poster presentation at the 10th Symposium on Lactic Acid Bacteria; Federation of European Microbiological Societies and Netherlands Society for Microbiology; Egmont-aan-Zee (The Netherlands).

2010 Poster presentation at the Exchange conference, Open Innovation for Feed, Food & Health; Ghent (Belgium).

WORKSHOPS AND TRAINING

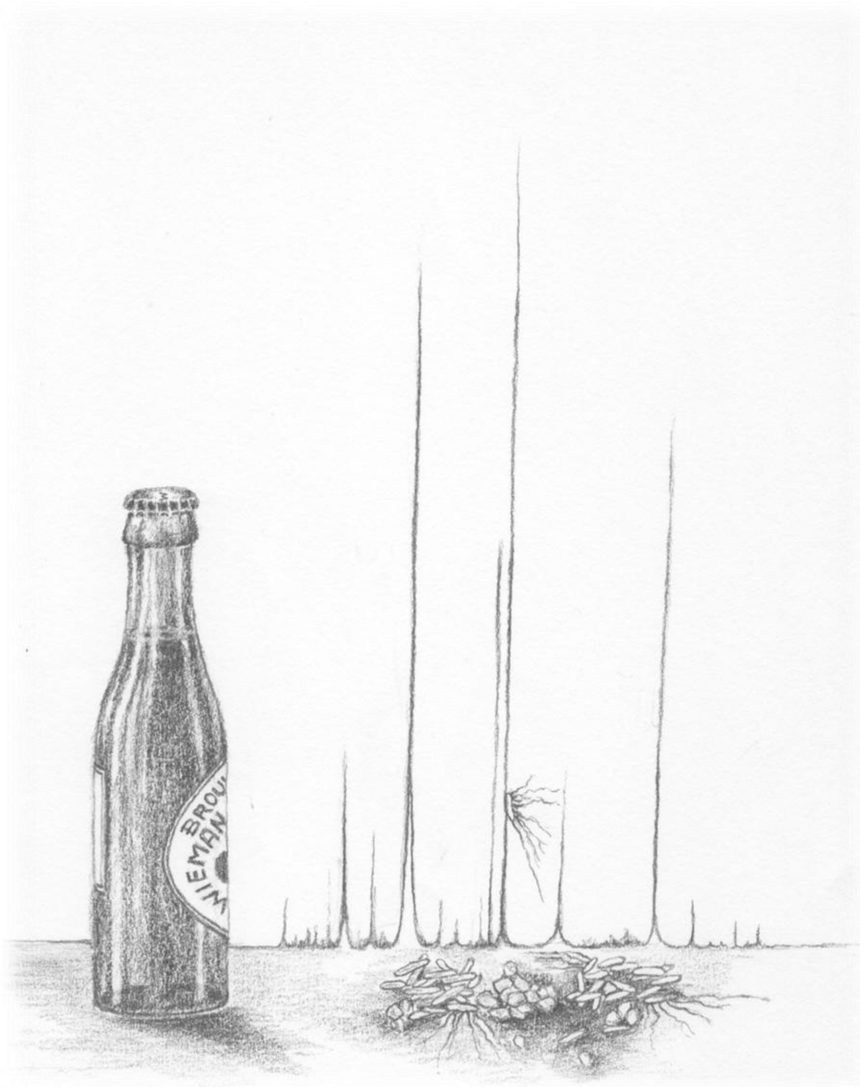
2012 Workshop: "Microbial Diagnostic Applications of Mass Spectrometry" organised by HPA Microbiology Services, Colindale in collaboration with Micototeca da Universidade do Minho, Braga, Portugal (Londen, Verenigd Koninkrijk).

2010 Basic Training Workshop on BioNumerics and GelCompar II organised by Applied Maths (Sint-Martens-Latem, Belgium).

2010 Advanced Academic English: Writing Skills and Conference Skills; organised by Doctoral Schools of Life Sciences and Medicine, Ghent University (Ghent, Belgium).

2010 Project Management; organised by Doctoral Schools of Life Sciences and Medicine, Ghent University (Ghent, Belgium).

2010 Sociomicrobiology and Food Fermentations; Ghent University (Ghent, Belgium).



Annex

ANNEX 1. List of 273 reference strains used for the construction of the MALDI-TOF MS identification database. *Corresponding strains from the mentioned species were reclassified in the present study. LMG, Belgian Co-ordinated Collections of Microorganisms/Laboratory of Microbiology, Ghent University; LAB and R, research collections of the Laboratory of Microbiology, Ghent University.

Species	Strain	Source	Received as*
Acetobacter aceti	LMG 1504^T	Beech-wood shavings of vinegar plant	
	LMG 1505	Quick vinegar (The Netherlands, Zwolle)	
	LMG 5	Beer (Belgium)	
Acetobacter cerevisiae	LMG 1625^T	Ale beer in storage (Canada, Toronto)	
	LMG 1545	Film in fermentor of rice vinegar (Japan, Aichi)	
	LMG 1599	Brewer's pitching yeast (UK)	
	LMG 1608	Beer (The Netherlands)	
	LMG 1682	Beer (Ireland)	
Acetobacter cerevisiae / Acetobacter malorum	LMG 1549	Top fermented beer	*Acetobacter pasteurianus*
	LMG 1587	Ale beer	*Acetobacter pasteurianus*
	LMG 1597	Brewer's pitching yeast and beer (UK)	*Acetobacter pasteurianus*
	LMG 1604	Sour beer (UK)	*Acetobacter pasteurianus*
	LMG 1698	Brewery (UK)	*Acetobacter pasteurianus*
	LMG 1699	Brewery (UK)	
	R-50418	Brewer's pitching yeast (Belgium)	
Acetobacter estunensis	LMG 1626^T	Cider (UK, Bristol)	
	LMG 1572	Cider (UK, Bristol)	
	LMG 1580	Beer (The Netherlands, Leiden)	
Acetobacter fabarum	LMG 1701	Beerwort (South Africa, Alberton)	*Acetobacter pasteurianus*
	LMG 24630	Kefir grains (Belgium, Gembloux)	*Acetobacter lovaniensis*
Acetobacter lovaniensis	LMG 1579^T	Sewage on soil (Belgium, Becquevoort)	
Acetobacter malorum	LMG 1746^T	Rotting apple (Belgium, Ghent)	
Acetobacter orleanensis	LMG 1583^T	Bottled beer (Belgium)	
Acetobacter pasteurianus	LMG 1262^T	Beer (The Netherlands)	
	LMG 1553	Spoiled beer (Japan, Osaka)	
	LMG 1590	Unknown source	
	LMG 1591	Vinegar	
	LMG 1639	Beer (The Netherlands)	
Gluconacetobacter liquefaciens	LMG 1381^T	*Diospyros* sp. dried fruit (Japan)	
Gluconobacter cerinus	LMG 1368^T	*Prunus* sp. (cherry) (Japan, Osaka)	
	LMG 1415	Beer (The Netherlands)	
	LMG 1679	Amstel Beer (The Netherlands, Leiden)	
Gluconobacter frateurii	LMG 1365^T	*Fragaria ananassa* (Japan, Osaka)	
Gluconobacter japonicus	LMG 1678	Amstel Beer (The Netherlands, Delft)	*Gluconobacter cerinus*
Gluconobacter oxydans	LMG 1408^T	Beer	
	LMG 1398	Beer (UK)	
	LMG 1406	Spoiled beer	
	LMG 1519	Spoiled top fermented beer (UK)	
	LMG 1581	Beer (The Netherlands, Delft)	
	LMG 1674	Beer (The Netherlands, Delft)	
	LMG 1676	Amstel Beer (Belgium, Leuven)	

Species	Strain	Source	Received as*
Gluconobacter oxydans	LMG 1683	Beer (Ireland)	
Gluconobacter sphaericus	LMG 1414^T	Grapes (Japan)	
Gluconobacter thailandicus	LMG 1743	Rotting pear (Belgium, Ghent)	*Gluconobacter cerinus*
Komagataeibacter europaeus	LMG 18890^T	Submerged culture vinegar generator (Germany, Esslingen)	
	LMG 1510	Vinegar (Denmark, Copenhagen)	
	LMG 1521	Vinegar (Kenya, Nairobi)	
Komagataeibacter swingsii	LMG 25	Unknown source	*Komagataeibacter xylinus*
Komagataeibacter xylinus	LMG 1515^T	Mountain ash berries	
	LMG 1518	Unknown source	
Lactobacillus acetotolerans	LMG 10751^T	Spoiled rice	
Lactobacillus acidophilus	LMG 13550^T	Human	
Lactobacillus backii	LMG 23555^T	Lager beer (Germany, Bavaria)	
	LMG 23556	Lager beer (Italy)	
Lactobacillus bifermentans	LMG 9845^T	Blown Dutch cheese	
Lactobacillus brevis	LMG 6906^T	Human faeces	
	LAB 286	Cherry mash	
	LAB 288	Plum mash	
	LAB 1003	Unpasteurised Heineken beer (The Netherlands, Zoeterwoude)	
	LAB 1008	Unpasteurised Heineken beer (The Netherlands, Zoeterwoude)	
	LAB 1146	Beer spoiler	
	LAB 1160	Beer spoiler	
	LAB 1161	Beer spoiler	
	LAB 1163	Tank cleaning water	
	LAB 1222	Beer spoiler	
	LAB 1223	Beer spoiler	
	LAB 1224	Beer spoiler	
	LAB 1315	Possible beer spoiler	
	LAB 1316	Possible beer spoiler	
	LAB 1318	Possible beer spoiler	
	LAB 1326	Unknown source	
	LAB 1357	Brewery culture collection	
	LAB 1359	Brewery culture collection	
	LAB 1364	Wort	
	LAB 1373	Spoiled beer	
	LMG 7761	Green fermenting olives	
	LMG 11401	Beer	
	LMG 11437	Silage	
	LMG 11771	Unknown source	*Lactobacillus parabuchneri*
	LMG 11974	Marinated fish	*Lactobacillus buchneri*
	LMG 11988	Dried yeast culture	
	LMG 12023	Human intestine	

Species	Strain	Source	Received as*
Lactobacillus brevis	LMG 14527	Spoiled beer (Germany)	
	LMG 16322	Spoiled beer	
	LMG 18940	Unknown source	*Lactobacillus perolens*
	LMG 22109	Beer (12% v/v alcohol)	
	R-21108	Cellar tank	
	R-21111	Unpasteurised beer	
	R-21115	Final product of beer	
	R-21120	Unpasteurised beer	
	R-21122	Final product of beer	
	R-49143	Final beer (8% v/v alcohol) (Denmark)	
	R-49144	Final beer (The Czech Republic)	
	R-49147	Unknown source	
	R-49148	Final unpasteurised pilsner beer (Denmark)	
	R-49149	Final unpasteurised pilsner beer (Denmark)	
	R-49151	Final unpasteurised pilsner beer (Denmark)	
	R-49152	Final unpasteurised pilsner beer (Denmark)	
	R-49153	Final unpasteurised pilsner beer (Denmark)	
	R-49154	Final unpasteurised pilsner beer (Denmark)	
	R-49155	Final unpasteurised pilsner beer (Denmark)	
	R-49156	Final unpasteurised pilsner beer (Denmark)	
	R-49157	Final unpasteurised pilsner beer (Denmark)	
	R-49158	Final unpasteurised pilsner beer (Denmark)	
	R-49159	Final unpasteurised pilsner beer (Denmark)	
	R-49160	Final unpasteurised pilsner beer (Denmark)	
	R-49161	Final unpasteurised pilsner beer (Denmark)	
	R-49162	Final unpasteurised pilsner beer (Denmark)	
	R-49163	Final unpasteurised pilsner beer (Denmark)	
	R-49164	Final unpasteurised pilsner beer (Denmark)	
	R-49165	Final unpasteurised pilsner beer (Denmark)	
	R-49166	Final unpasteurised pilsner beer (Denmark)	
	R-49603	Top fermenting beer in CCT (Belgium)	
	R-49604	White beer in cask (Belgium)	
	R-50070	Final kegged beer (Belgium)	
Lactobacillus buchneri	LMG 6892^T	Tomato pulp	
	LMG 11439	Unknown source	
	LMG 11985	Oral cavity	
	LMG 12000	Silage	*Lactobacillus* sp.
	LMG 22102	Final product of beer	
Lactobacillus buchneri	R-21109	Final product of beer	
	R-21117	Ketchup	
	R-21118	Ketchup	

Species	Strain	Source	Received as*
Lactobacillus buchneri	R-27972	Sourdough (Belgium)	
Lactobacillus casei subsp. *casei*	LMG 6904^T	Cheese	
Lactobacillus collinoides	LMG 9194^T	Fermenting apple juice (not a beer spoiler)	
	LMG 9195	Cider and apple juice	
	LMG 18850	Distillation cider (France, Pont l'Evèque Normandy)	
	LAB 285	Apple mash	*Lactobacillus brevis*
Lactobacillus coryniformis subsp. *coryniformis*	LMG 9196^T	Silage	
Lactobacillus coryniformis subsp. *torquens*	LMG 9197^T	Air of dairy barn	
Lactobacillus curvatus	LMG 9198^T	Milk	
	LAB 158	Sauerkraut	
	LAB 195	Sausage material	
	LAB 289	Mettwurst	
	LAB 290	Salami (Hungary)	
	LAB 962	Spoiled vacuum sealed sausage	
	LMG 12006	Hard cheese (Italy)	
Lactobacillus delbrueckii subsp. *bulgaricus*	LMG 6901^T	Bulgarian yoghurt	
	LAB 55	Unknown source	
	LAB 464	Unknown source	
	LMG 12168	Homemade yoghurt	
Lactobacillus delbrueckii subsp. *delbrueckii*	LMG 6412^T	Distillery sour grain mash incubated at 45°C	
	LAB 1276	Unknown source	
	LMG 22235	Human urine (Sweden, Göteborg)	
Lactobacillus delbrueckii subsp. *indicus*	LMG 22083^T	Traditional dairy fermented products (India)	
	LMG 22084	Traditional dairy fermented products (India)	
	LMG 22085	Traditional dairy fermented products (India)	
Lactobacillus delbrueckii subsp. *lactis*	LMG 7942^T	Emmental cheese	
	LAB 463	Unknown source	
	LMG 13136	Unknown source	
	LMG 18223	Unknown source	
	LMG 6401	Unknown source	
Lactobacillus dextrinicus	LMG 11485^T	Silage	
Lactobacillus fermentum	LAB 1307	Possible beer spoiler	
Lactobacillus fructivorans	LMG 9201^T	Unknown source	
Lactobacillus harbinensis	LMG 24040^T	Chinese fermented vegetable (China, Harbin)	
	LAB 1446	Unknown source	*Lactobacillus perolens*
	LMG 18938	Orange lemonade (The Netherlands)	*Lactobacillus perolens*
Lactobacillus hilgardii	LMG 7934	Wine	"*Lactobacillus brevis* subsp. *gravesensis*"
	LMG 7935	Wine	"*Lactobacillus brevis* subsp. *otakiensis*"
Lactobacillus lindneri	LMG 14528^T	Spoiled beer (Germany)	
	DSM 20691	Spoiled beer	
	LMG 11404	Spoiled beer	

Species	Strain	Source	Received as*
Lactobacillus lindneri	R-49605	Top fermenting beer in CCT (Belgium)	
Lactobacillus malefermentans	LMG 11455^T	Beer	
	LMG 11416	Beer	
Lactobacillus parabrevis	LMG 11984^T	Wheat	
	LMG 11494	Farmhouse red cheshire cheese	
Lactobacillus parabuchneri	LMG 11457^T	Human saliva (UK)	
	LAB 1170	Soft drink	
	LAB 1172	Beer Spoiler	
	LAB 153	Sauerkraut	
	LMG 11768	Unknown source	
	LMG 11769	Unknown source	
	LMG 11770	Unknown source	
	LMG 11772	Unknown source	
	LMG 11773	Unknown source	
	LMG 11973	Brewery	
	LMG 11987	Unknown source	
	LMG 22038	Malt whisky fermentation (UK)	
	LMG 22103	Beer (The Czech Republic)	
	LMG 22462	Malt whisky fermentation (UK)	
Lactobacillus paracasei subsp. *paracasei*	LMG 13087^T	Unknown source	
	LAB 313	Green olives	
	LAB 460	Unknown source	
	LAB 523	Feta cheese brine	
	LAB 524	Feta cheese brine	
	LMG 11961	Port wine, Portugal	
	LMG 11963	Machinery used in port wine production (Portugal)	
	LMG 13722	Young red table wine (Portugal)	
	LMG 13731	Spoiled port wine (Portugal)	
	LMG 7955	Unknown source	
	LMG 9207	Cheshire cheese	
	R-21110	Unpasteurised beer	*Lactobacillus parabuchneri*
	R-21121	Final product of beer	*Lactobacillus parabuchneri*
	R-49150	Final unpasteurised pilsner beer (Denmark)	
Lactobacillus paracasei subsp. *tolerans*	LMG 9191^T	Pasteurised milk	
Lactobacillus paracollinoides	LMG 22473^T	Brewery environment (Japan)	
	LMG 9204	Beer	
Lactobacillus paraplantarum	LMG 16673^T	Beer (France)	
Lactobacillus paucivorans	LMG 25291^T	Beer storage tank (Germany)	
Lactobacillus pentosus	LMG 10755^T	Unknown source	
	LAB 160	Sauerkraut	

Species	Strain	Source	Received as*
Lactobacillus pentosus	LAB 170	Sausage starter preparation	
	LAB 351	Green olives	*Lactobacillus plantarum*
	LAB 352	Green olives	*Lactobacillus plantarum*
	LMG 17677	Chili bo (Malaysia)	
	LMG 9210	Lactic acid fermentation of waste sulphite liquor	
	R-18410	Unknown source	
Lactobacillus perolens	LMG 18936^T	Orange lemonade (Germany)	
	LAB 1190	Soft drink	
	LAB 1191	Soft drink	
	LAB 1423	Unknown source	
	LMG 18937	Orange lemonade (Germany)	
	LMG 18939	Beer wort (Germany)	
Lactobacillus plantarum subsp. *plantarum*	LMG 6907^T	Pickled cabbage	
	LAB 1018	Starterculture for spent grains acidification	
	LAB 1159	Beer Spoiler	*Lactobacillus buchneri*
	LAB 530	Fresh Feta cheese	
	LAB 541	Fresh Feta cheese	*Lactobacillus paracasei* subsp. *paracasei*
	LAB 547	Feta cheese brine	
	LMG 11405	Silage	
	LMG 22106	Beer (The Czech Republic)	
	LMG 22107	Beer (The Czech Republic)	
	LMG 9209	Ensilage vegetables	
	LMG 22108	Final product of beer	*Lactobacillus brevis*
	R-30196	Sourdough (Belgium)	
	R-49145	Final beer (The Czech Republic)	
	R-49146	Unknown source	
Lactobacillus plantarum subsp. plantarum	R-49262	Unknown source	
Lactobacillus plantarum subsp. *argentoratensis*	LMG 9205^T	Fermented corn product (Ogi) (South Nigeria)	
Lactobacillus rossiae	LMG 22972^T	Wheat sourdough (Italy)	
	LAB 1192	Soft drink	*Lactobacillus perolens*
	LAB 1193	Soft drink	*Lactobacillus perolens*
Lactobacillus sakei subsp. *carnosus*	LMG 17302^T	Raw sausage	
	LMG 18295	Raw sausage (Germany)	
Lactobacillus sakei subsp. *sakei*	LMG 9468^T	Starter of sake (Moto)	
Pediococcus acidilactici	LMG 11384^T	Barley	
	LAB 173	From sausage starter preparation	
	LAB 196	Sausage starter	
	LMG 25667	Ryegrass silage	*Pediococcus lolii*
	LMG 26010	Beer (UK)	
	LMG 26011	Beer (UK)	*Pediococcus damnosus*
	LMG 26012	Beer (UK)	*Pediococcus inopinatus*

Species	Strain	Source	Received as*
Pediococcus claussenii	LMG 21948^T	Spoiled beer (Canada, Toronto)	
	LAB 1004	Unpasteurised beer (The Netherlands, Zoeterwoude)	
	LAB 1232	Unknown source	
	LAB 1329	Unknown source	
	LAB 1330	Unknown source	*Pediococcus damnosus*
	LAB 1331	Unknown source	*Pediococcus damnosus*
	LAB 1453	Unknown source	*Pediococcus damnosus*
	LAB 994	Unknown source	
Pediococcus damnosus	LMG 11484^T	Lager beer yeast	
	LAB 1362	Unknown source	
	LAB 1370	Spoiled beer	
	LAB 1447	Unknown source	
	LAB 1449	Unknown source	
Pediococcus inopinatus	LMG 11409^T	Brewing yeast	
	LAB 1451	Unknown source	
	LAB 1452	Unknown source	
	LAB 1454	Unknown source	
	LMG 11410	Beer	
	LMG 22104	Final product of beer	
	LMG 22105	Beer 10% v/v alcohol	
	R-21106	Beer 10% v/v alcohol	
	R-21112	Final product of beer	
	R-21126	Final product of beer	
	R-49606	Dinner beer during lagering (Belgium)	
Pediococcus pentosaceus	LMG 11488^T	Dried American brewer's yeast	
	LAB 676	Unknown source	
	LMG 11385	Sake Mash	
	LMG 13373	Grass samples	

ANNEX 2. Strainlist

Strain number	Genus	Species	Source	Isolation year	Geographical origin
R-49483	*Lactobacillus*	*backii*	Final kegged pilsner beer	2010	Belgium
R-49484	*Lactobacillus*	*backii*	Final kegged pilsner beer	2010	Belgium
R-49531	*Lactobacillus*	*brevis*	Spoiled pitching yeast culture	2012	Belgium
R-49532	*Lactobacillus*	*brevis*	Spoiled pitching yeast culture	2012	Belgium
R-49533	*Staphylococcus*	sp.	Spoiled pitching yeast culture	2012	Belgium
R-49534	*Corynebacterium*	sp.	Spoiled pitching yeast culture	2012	Belgium
R-49601	*Acetobacter*	*cerevisiae*	Spoiled pitching yeast culture	2012	Belgium
R-49602	*Acetobacter*	*cerevisiae*	Spoiled pitching yeast culture	2012	Belgium
R-49856	*Lactobacillus*	*brevis*	Bottled beer (alc. cont. of 7.5% v/v)	2012	Belgium
R-49857	*Lactobacillus*	*brevis*	Bottled beer (alc. cont. of 7.5% v/v)	2012	Belgium
R-49858	*Staphylococcus*	sp.	Final kegged pilsner beer	2010	Belgium
R-49859	*Staphylococcus*	sp.	Final kegged pilsner beer	2010	Belgium
R-49860	*Gluconobacter*	*oxydans*	Bottled sw eetened dinner beer (alc. cont. of 2.25% v/v)	2010	Belgium
R-49861	*Gluconobacter*	*oxydans*	Bottled sw eetened dinner beer (alc. cont. of 2.25% v/v)	2010	Belgium
R-49862	*Acetobacter*	*orleanensis*	Bottled beer (alc. cont. of 6.6% v/v)	2012	Belgium
R-49863	*Pediococcus*	*claussenii*	Fruity w heat beer (alc. cont. of 4.3% v/v)	2010	Belgium
R-49864	*Lactobacillus*	*brevis*	Spoiled pitching yeast culture	2012	Belgium
R-49868	*Lactobacillus*	*malefermentans*	Bottled dinner beer (alc. cont. of 1.5% v/v)	2012	Belgium
R-49869	*Staphylococcus*	sp.	Fruity w heat beer (alc. cont. of 4.3% v/v)	2012	Belgium
R-49877	*Lactobacillus*	*brevis*	Bottled beer (alc. cont. of 7.5% v/v)	2012	Belgium
R-49878	*Bacillus*	sp.	Bottled dinner beer (alc. cont. of 1.5% v/v)	2012	Belgium
R-49879	*Lactobacillus*	*brevis*	Spoiled w ort	2012	Belgium
R-49880	*Staphylococcus*	sp.	Fruity w heat beer (alc. cont. of 4.3% v/v)	2010	Belgium
R-49881	*Lactobacillus*	*paracollinoides*	Bottled beer (alc. cont. of 8.5% v/v)	2009	Belgium
R-50064	*Acetobacter*	*persici*	Bottled beer (alc. cont. of 6.6% v/v)	2012	Belgium
R-50069	*Lactobacillus*	*backii*	Final kegged pilsner beer	2010	Belgium
R-50070	*Lactobacillus*	*brevis*	Final kegged pilsner beer	2010	Belgium
R-50294	*Staphylococcus*	sp.	Fruity w heat beer (alc. cont. of 4.3% v/v)	2012	Belgium
R-50295	*Staphylococcus*	sp.	Fruity w heat beer (alc. cont. of 4.3% v/v)	2012	Belgium
R-50296	*Staphylococcus*	sp.	Fruity w heat beer (alc. cont. of 4.3% v/v)	2012	Belgium
R-50297	*Staphylococcus*	sp.	Fruity w heat beer (alc. cont. of 4.3% v/v)	2012	Belgium
R-50344	*Acinetobacter*	sp.	Water closure fermentation tank	2013	Belgium
R-50345	*Klebsiella*	sp.	Water closure fermentation tank	2013	Belgium
R-50346	*Leuconostoc*	sp.	Cleaning w ater from lauter tun	2013	Belgium
R-50347	*Enterococcus*	sp.	Cleaning w ater from mashing tun	2013	Belgium
R-50348	*Lactococcus*	sp.	Cleaning w ater from mashing tun	2013	Belgium
R-50349	*Lactococcus*	sp.	Cleaning w ater from mashing tun	2013	Belgium
R-50350	*Lactococcus*	sp.	Cleaning w ater from mashing tun	2013	Belgium
R-50351	*Enterococcus*	sp.	Cleaning w ater from plate cooler	2013	Belgium
R-50352	*Bacillus*	sp.	Sw ab boiling kettle	2013	Belgium
R-50353	*Enterobacter*	sp.	Cleaning w ater boiling kettle	2013	Belgium
R-50354	*Lysinbacillus*	sp.	Cleaning w ater from lauter tun	2013	Belgium
R-50355	*Lysinbacillus*	sp.	Cleaning w ater from lauter tun	2013	Belgium
R-50356	*Lysinbacillus*	sp.	Cleaning w ater from lauter tun	2013	Belgium
R-50357	*Leuconostoc*	sp.	Tab from lauter tun to boiling kettle	2013	Belgium
R-50358	*Weisella*	sp.	Tab from lauter tun to boiling kettle	2013	Belgium
R-50359	*Leuconostoc*	sp.	Tab from lauter tun to boiling kettle	2013	Belgium
R-50360	*Enterococcus*	sp.	Brew ing liquid from plate cooler	2013	Belgium
R-50361	*Gluconobacter*	sp.	Packaged beer	2013	Belgium
R-50362	*Acetobacter*	*indonesiensis*	Packaged beer	2013	Belgium
R-50363	*Gluconobacter*	*japonicus*	Packaged beer	2013	Belgium
R-50364	*Bacillus*	sp.	Outlet Whirlpool	2013	Belgium
R-50365	*Bacillus*	sp.	Oxoniumbath	2013	Belgium
R-50366	*Bacillus*	sp.	Oxoniumbath	2013	Belgium
R-50367	*Bacillus*	sp.	Oxoniumbath	2013	Belgium
R-50368	*Bacillus*	sp.	Oxoniumbath	2013	Belgium
R-50369	*Leuconostoc*	sp.	Milk kefir	2013	Belgium
R-50370	*Acetobacter*	sp.	Kombucha	2013	Belgium
R-50371	*Gluconobacter*	*oxydans*	Kombucha	2013	Belgium
R-50372	*Lactobacillus*	*nagelii*	Kombucha	2013	Belgium

Strain number	Genus	Species	Source	Isolation year	Geographical origin
R-50373	*Acetobacter*	sp.	Kombucha	2013	Belgium
R-50374	*Gluconobacter*	sp.	Kombucha	2013	Belgium
R-50375	*Komagateibacter*	sp.	Kombucha	2013	Belgium
R-50376	*Lactobacillus*	*nagelii*	Kombucha	2013	Belgium
R-50377	*Lactobacillus*	*nagelii*	Kombucha	2013	Belgium
R-50378	*Gluconobacter*	*oxydans*	Kombucha	2013	Belgium
R-50379	*Lactobacillus*	*nagelii/paracasei*	Kombucha	2013	Belgium
R-50380	*Lactobacillus*	*nagelii*	Kombucha	2013	Belgium
R-50381	*Lactobacillus*	*nagelii*	Kombucha	2013	Belgium
R-50382	*Acetobacter*	sp.	Kombucha	2013	Belgium
R-50383	*Lactobacillus*	*paracasei*	Kefir	2013	Belgium
R-50384	*Lactobacillus*	*harbinensis*	Kefir	2013	Belgium
R-50385	*Acetobacter*	sp.	Kefir	2013	Belgium
R-50386	*Acetobacter*	sp.	Kefir	2013	Belgium
R-50387	*Acetobacter*	sp.	Kefir	2013	Belgium
R-50388	*Lactobacillus*	*hilgardii*	Kefir	2013	Belgium
R-50389	*Acetobacter*	sp.	Kefir	2013	Belgium
R-50390	*Lactobacillus*	*parabuchneri*	Kefir	2013	Belgium
R-50391	*Lactobacillus*	*parabuchneri*	Kefir	2013	Belgium
R-50392	*Lactobacillus*	*paracasei*	Kefir	2013	Belgium
R-50393	*Acetobacter*	*sicerae*	Kefir	2013	Belgium
R-50405	*Bacillus*	sp.	Water closure fermentation tank	2013	Belgium
R-50406	*Lactobacillus*	*nagelii*	Kombucha	2013	Belgium
R-50407	*Lactobacillus*	*plantarum*	Kombucha	2013	Belgium
R-50408	*Lactococcus*	sp.	Cleaning w ater from mashing tun	2013	Belgium
R-50409	*Lactobacillus*	*paracasei*	Kefir	2013	Belgium
R-50410	*Lactobacillus*	*paracasei*	Kefir	2013	Belgium
R-50411	*Acetobacter*	*sicerae*	Kefir	2013	Belgium
R-50412	*Acetobacter*	*sicerae*	Kefir	2013	Belgium
R-50413	*Acetobacter*	*sicerae*	Kefir	2013	Belgium
R-50414	*Acetobacter*	*sicerae*	Kefir	2013	Belgium
R-50415	*Sphingobacterium*	sp.	Cleaning w ater from w hirlpool	2013	Belgium
R-50416	*Gluconobacter*	*cerinus*	Spoiled pitching yeast culture	2013	Belgium
R-50417	*Gluconobacter*	*cerinus*	Spoiled pitching yeast culture	2013	Belgium
R-50418	*Acetobacter*	*cerevisiae/malorum*	Spoiled pitching yeast culture	2013	Belgium
R-50419	*Gluconobacter*	*cerevisiae*	Spoiled pitching yeast culture	2013	Belgium
R-50445	*Lactobacillus*	*acetotolerans*	Kefir	2013	Belgium
R-50446	*Lactobacillus*	*acidophilus*	Milk kefir	2013	Belgium
R-50643	*Gluconobacter*	*japonicus*	Bottled dinner beer (alc. cont. of 1.5% v/v)	2013	Belgium
R-50644	*Lactobacillus*	*malefermentans*	Bottled dinner beer (alc. cont. of 1.5% v/v)	2013	Belgium
R-50645	*Acetobacter*	*indonesiensis*	Bottled dinner beer (alc. cont. of 1.5% v/v)	2013	Belgium
R-50646	*Lactobacillus*	*malefermentans*	Bottled dinner beer (alc. cont. of 1.5% v/v)	2013	Belgium
R-50647	*Lactobacillus*	*malefermentans*	Bottled dinner beer (alc. cont. of 1.5% v/v)	2013	Belgium
R-50648	*Pediococcus*	*inopinatus*	Bottled dinner beer (alc. cont. of 1.5% v/v)	2013	Belgium
R-50649	*Lactobacillus*	*malefermentans*	Bottled dinner beer (alc. cont. of 1.5% v/v)	2013	Belgium
R-50650	*Acetobacter*	*fabarum*	Bottled dinner beer (alc. cont. of 1.5% v/v)	2013	Belgium
R-50651	*Pediococcus*	*inopinatus*	Bottled dinner beer (alc. cont. of 1.5% v/v)	2013	Belgium
R-53261	*Acetobacter*	*cerevisiae/malorum*	Bottled dinner beer (alc. cont. 1.5% v/v)	2010	Belgium
R-53262	*Acetobacter*	*cerevisiae/malorum*	Bottled dinner beer (alc. cont. 1.5% v/v)	2010	Belgium
R-53263	*Acetobacter*	*cerevisiae/malorum*	Bottled sw eetened dinner beer (alc. cont. 2.25% v/v)	2010	Belgium
R-53264	*Klebsiella*	sp.	Fruity w heat beer	2012	Belgium
R-53265	*Acetobacter*	*cerevisiae/malorum*	Spoiling pitching yeast culture	2012	Belgium
R-53266	*Acetobacter*	*cerevisiae/malorum*	Spoiling pitching yeast culture	2012	Belgium
R-53267	*Acetobacter*	*cerevisiae/malorum*	Spoiling pitching yeast culture	2012	Belgium
R-53268	*Lactobacillus*	*brevis*	Spoiling pitching yeast culture	2012	Belgium
R-53269	*Acetobacter*	*cerevisiae/malorum*	Spoiling pitching yeast culture	2012	Belgium
R-53270	*Lactobacillus*	*brevis*	Bottled beer (alc. cont. of 6.6% v/v)	2012	Belgium
R-53271	*Lactobacillus*	*brevis*	Bottled beer (alc. cont. of 6.6% v/v)	2012	Belgium
R-53272	*Lactobacillus*	*brevis*	Bottled beer (alc. cont. of 7.5% v/v)	2012	Belgium
R-53273	*Lactobacillus*	*brevis*	Bottled beer (alc. cont. of 7.5% v/v)	2012	Belgium