TECHNISCHE UNIVERSITÄT MÜNCHEN

Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität

# Occurrence, Detection, Characterization and Description of Selected Beer-Spoilage Lactic Acid Bacteria

Jennifer Schneiderbanger

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Prof. Dr. rer. nat. Horst-Christian Langowski

Prüfer der Dissertation: 1. Hon.-Prof. Dr.-Ing. Friedrich Jacob
2. Prof. Dr. rer. nat. Siegfried Scherer

Die Dissertation wurde am 17.04.2019 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 16.08.2019 angenommen.

„In der Wissenschaft gleichen wir alle nur den Kindern,

die am Rande des Wissens

hie und da einen Kiesel aufheben,

während sich der weite Ozean des

Unbekannten vor unseren Augen erstreckt."

- Sir Isaac Newton -

# PREFACE

## Peer reviewed publications

The following peer reviewed papers (shown in chronological order) were generated in the period of this work.

1. Koob, J.; Jacob, F.; Methner, F.-J.; Hutzler, M. (2016): *Lactobacillus* sp. brewery isolate: A new threat to the brewing industry?
   Brewing Science, Vol. 69, pp. 42 – 49
   DOI: 10.23763/BRSC16-09KOOB

2. Koob, J.; Jacob, F.; Wenning, M.; Hutzler, M. (2017): *Lactobacillus cerevisiae* sp. nov., isolated from a spoiled brewery sample.
   International Journal of Systematic and Evolutionary Microbiology, Vol. 67, pp. 3452 – 3457
   DOI: 10.1099/ijsem.0.002139

3. Schneiderbanger, J.; Schneiderbanger, H.; Jacob, F.; Hutzler, M. (2017): Enhanced cultivation of beer spoilage bacteria in propagation by enforced yeast suppression.
   Brewing Science, Vol. 70, pp. 142 – 147
   DOI: 10.23763/BRSC17-14SCHNEIDERBANGER

4. Schneiderbanger, J.; Grammer, M.; Jacob, F.; Hutzler, M. (2018): Statistical evaluation of beer spoilage bacteria by real-time PCR analyses from 2010 – 2016.
   Journal of the Institute of Brewing, Vol. 124, pp. 173 - 181
   DOI: 10.1002/jib.486

5. Schneiderbanger, J.; Jacob, F.; Hutzler, M. (2019): Genotypic and phenotypic diversity of *Lactobacillus rossiae* isolated from beer.
   Journal of Applied Microbiology, Vol. 126, pp. 1187-1197.
   DOI: 10.1111/jam.14202

## ACKNOWLEDGEMENTS

This work was prepared under the supervision of Prof. Fritz Jacob who granted me this interesting topic and supported me whenever needed. Thank you very much!

Special thanks to Prof. Siegfried Scherer who volunteered to verify this thesis and to Prof. Horst-Christian Langowski for acting as the examination chairperson.

In particular, I would like to thank Dr. Mathias Hutzler who was always there for me to help, discuss, proofread, review, motivate and much more. Without him, my life would have taken an entirely different course.

I also want to thank my colleagues Prof. Dr. Mehmet Coelhan, Dr. Martin Zarnkow, Dr. Tim Meier-Dörnberg, Dr. Maximilian Michel, Dario Cotterchio, Korbinian Haslbeck, Robert Riedl, Susan Illing, Florian Mallok, Dominique Stretz, Friedl Ampenberger, Sebastian Hans, Hubert Walter, Klaus Litzenburger and Josef Englmann for their many years of help, interesting discussions and motivation whenever I needed it.

Many of the analyses included in this work were outsourced to experienced laboratory staff. In particular, I would like to say thanks to Margit Grammer, Anna Kleucker, Veronika Kaindl, Siglinde Huber, Monika Mayerhofer, Irmgard Frey, Karin Würzinger, Tanja Pohl, Sabine Dawidowitsch, Markus Schmidt, Sabine Mageth, Karl-Heinz Bromig and everyone else including Silvia Hubensteiner, Natalie Böck, Georgine Widmann, Jutta Vilser or Josef Pellmeier who positively influenced this work or the working environment it was created in.

Many students supported me during my research with their Diplom, Bachelor’s or Master’s theses. I would like to offer thanks to Barbara Segerer, Julian Großhauser, Harald Reindl, Julian Thünnessen, Lisa Iwo, Lukas Schappals, Michael Hemmerle, Oliver Hammermeister and Tim Papenberg.

I also want to express my thanks to Sarah Silva who has spent many hours carefully proofreading this thesis.

Countless thanks go to my family Ingrid, Werner, Eduard and Marcel for their support and patience in the past few turbulent years.

And finally, I would like to thank my husband, my best friend and obligate beer spoiler Hubertus who never lost sight of the important goals – both with respect to this work and to our lives.

# CONTENTS

**PREFACE** ........ I

**ACKNOWLEDGEMENTS** ........ II

**CONTENTS** ........ III

**ABBREVIATIONS** ........ V

**SUMMARY** ........ - 1 -

**ZUSAMMENFASSUNG** ........ - 3 -

**1. INTRODUCTION** ........ - 5 -

**1.1 THE PROPERTIES OF BEER** ........ - 6 -
1.1.1 ETHANOL ........ - 6 -
1.1.2 LOW pH VALUE ........ - 7 -
1.1.3 DISSOLVED GASES ........ - 7 -
1.1.4 LACK OF NUTRIENTS ........ - 7 -
1.1.5 HOPS ........ - 8 -
**1.2 STRESS TOLERANCE MECHANISMS** ........ - 8 -
1.2.1 ACTIVE HOP RESISTANCE MECHANISMS ........ - 8 -
1.2.2 PASSIVE HOP RESISTANCE MECHANISMS ........ - 10 -
1.2.3 FURTHER STRESS TOLERANCE MECHANISMS ........ - 10 -
**1.3 LACTIC ACID BACTERIA** ........ - 11 -
1.3.1 THE *LACTOBACILLUS* GENUS ........ - 12 -
1.3.2 THE *PEDIOCOCCUS* GENUS ........ - 12 -
1.3.3 THE ROLE OF LACTIC ACID BACTERIA IN BEER ........ - 13 -
1.3.2.1 POSITIVE ROLE OF LACTIC ACID BACTERIA ........ - 13 -
1.3.2.2 NEGATIVE ROLE OF LACTIC ACID BACTERIA ........ - 13 -
**1.4 BEER-SPOILING SPECIES** ........ - 15 -
1.4.1 THE HISTORY OF BEER-SPOILING BACTERIA ........ - 15 -
1.4.2 BEER-SPOILING LACTIC ACID BACTERIA ........ - 15 -
1.4.2.1 FREQUENTLY OCCURRING BEER-SPOILING LACTIC ACID BACTERIA ........ - 15 -
1.4.2.2 LESS FREQUENTLY OCCURRING BEER-SPOILING LACTIC ACID BACTERIA - 18 -
1.4.3 FURTHER BEER-SPOILING MICROORGANISMS ........ - 19 -
1.4.3.1 THE PECTINATUS AND MEGASPHAERA GENERA ........ - 19 -
1.4.3.2 FURTHER GRAM-NEGATIVE BEER-SPOILING BACTERIA ........ - 21 -
1.4.3.3 WILD YEASTS ........ - 21 -
1.4.4 BACTERIAL CLASSIFICATION ACCORDING TO BREWERY-SPECIFIC ASPECTS ........ - 22 -

**1.5 DETECTION AND IDENTIFICATION OF BEER-SPOILING BACTERIA ................................- 24 -**
1.5.1 DETECTION ..........................................................................................- 24 -
1.5.2 IDENTIFICATION.....................................................................................- 25 -
1.5.2.1 PHENOTYPIC ANALYSIS METHODS ......................................................- 26 -
1.5.2.2 CHEMOTAXONOMIC ANALYSIS METHODS.............................................- 26 -
1.5.2.3 GENOTYPIC ANALYSIS METHODS.......................................................- 27 -
1.5.3 DETERMINATION OF BEER-SPOILAGE POTENTIAL ..........................................- 30 -

**2. AIMS ............................................................................................................. - 32 -**

**3. RESULTS (THESIS PUBLICATIONS)........................................................................ - 34 -**

**3.1 PAPER SUMMARIES..........................................................................................- 34 -**
**3.2 CHAPTER A – STATISTICAL EVALUATION OF BEER SPOILAGE BACTERIA BY REAL-TIME PCR ANALYSES FROM 2010 – 2016.......................................................... .- 39 -**
**3.3 CHAPTER B – ENHANCED CULTIVATION OF BEER SPOILAGE BACTERIA BY ENFORCED YEAST SUPPRESSION ..........................................................................- 54 -**
**3.4 CHAPTER C – DETECTION OF A NEW BACTERIAL SPECIES WITH BEER-SPOILAGE POTENTIAL ...........................................................................................................- 60 -**
SECTION 1 *LACTOBACILLUS* SP. BREWERY ISOLATE: A NEW THREAT TO THE BREWING INDUSTRY?...........................................................................................................- 60 -
SECTION 2 *LACTOBACILLUS CEREVISIAE* SP. NOV., ISOLATED FROM A SPOILED BREWERY SAMPLE..........................................................................................................- 68 -
**3.5 CHAPTER D – GENOTYPIC AND PHENOTYPIC DIVERSITY OF *LACTOBACILLUS ROSSIAE* FROM BEER ...........................................................................................................- 78 -**

**4. DISCUSSION ................................................................................................... - 95 -**

**5. REFERENCES................................................................................................. - 106 -**

**6. APPENDIX...................................................................................................... - 127 -**

**6.1 PEER-REVIEWED PUBLICATIONS.......................................................................- 127 -**
**6.2 NON-REVIEWED PUBLICATIONS ........................................................................- 127 -**
**6.3 POSTERS AND ORAL PRESENTATIONS ................................................................- 128 -**
**6.4 BOOK CHAPTER AND AWARDS ..........................................................................- 130 -**
**6.5 PUBLISHER PERMISSIONS TO REPRODUCE PUBLICATIONS ....................................- 131 -**

## ABBREVIATIONS

| | |
|---|---|
| AAB | acetic acid bacteria |
| ABC | ATP-binding cassette |
| ADI | arginine deiminase |
| AFLP | amplified fragment length polymorphism |
| ANI | average nucleotide identity |
| ARDRA | amplified rDNA restriction analysis |
| ATP | adenosine triphosphate |
| BCCM | Belgian Coordinated Collection of Microorganisms |
| BS | beer-spoiling |
| BSB | beer-spoiling bacteria |
| BSM | beer-spoiling microorganisms |
| BSP | beer-spoilage potential |
| BU | bitter unit |
| $CO_2$ | carbon dioxide |
| Ct | threshold cycle |
| DDH | DNA-DNA hybridization |
| DGGE | denaturing gradient gel electrophoresis |
| DMG | diagnostic marker gene |
| DNA | deoxyribonucleic acid |
| DSMZ | Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH; German Collection of Microorganisms and Cell Cultures |
| EMP | Embden-Meyerhof-Parnas pathway |
| EPS | exopolysaccharides |
| FAS | fatty acid biosynthesis |
| FISH | fluorescence in-situ hybridization |
| FTIR | fourier transform infrared spectroscopy |
| FZW BLQ | Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität; Research Center Weihenstephan for Brewing and Food Quality |
| *gtf* | gene encoding glycosyltransferase |
| HGT | horizontal gene transfer |
| *hitA* | hop inducible transporter A |
| *horA* | hop resistance gene A |
| *horC* | hop resistance gene C |
| ISR-PCR | intergenic spacer region PCR |
| ITS | internally transcribed spacer |
| *L.* | *Lactobacillus* |
| LAB | lactic acid bacteria |
| *Lac.* | *Lactococcus* |
| *Leuc.* | *Leuconostoc* |
| LPSN | List of prokaryotic names with standing in the nomenclature |
| LTA | lipoteichoic acid |
| *M.* | *Megasphaera* |
| MALDI-TOF MS | matrix assisted laser desorption ionization — time of flight mass spectrometry |
| MIC | minimum inhibitory concentration |
| MLSA/MLST | multilocus sequence analysis / typing |
| MLVA | multiple locus variable number of tandem repeats analysis |
| $NAD^+$ | oxidized nicotinamide adenine dinucleotide |
| NADH | reduced nicotinamide adenine dinucleotide |
| $O_2$ | oxygen |

| | |
|---|---|
| ORF | open reading frame |
| OTU | operational taxonomic unit |
| *P.* | *Pectinatus* |
| PCR | polymerase chain reaction |
| *Pd.* | *Pediococcus* |
| pmf | proton motive force |
| QC | quality control |
| RAPD | randomly amplified polymorphic DNA |
| rep-PCR | repetitive element palindromic PCR |
| RFLP | restriction fragment length polymorphism |
| RNA | ribonucleic acid |
| *S.* | *Saccharomyces* |
| sp. | species |
| spp. | species pluralis |
| TRFLP | terminal restriction fragment length polymorphism |
| var. | variety |
| VBNC | viable-but-not-culturable |
| v/v | volume per volume |
| WGS | whole-genome sequencing |
| w/v | weight per volume |

## SUMMARY

The driving force of the cost-intensive and elaborate quality control (QC) in breweries is the maintenance of beer quality to fulfill steadily increasing customer demands. The majority of consumers are unaware of beer-spoiling (BS) bacteria and yeasts, which are one of the most frequent causes of complaints. Possible consequences of microorganism contamination include changes of the beer's taste, smell, pH value, texture, and appearance, which in serious cases, cause the consumer to reject a certain product. In addition to the loss of reputation, these factors may lead to considerable monetary damage if spoiled batches cannot be sold and need to be destroyed. Breweries need to take great care and suitable preventive measures are required to ensure beer quality.

The microbiological QC in breweries aims to examine the single process steps for the occurrence of beer-spoiling microorganisms (BSM). The diverse problems microbiological QC need to specifically address include the detection of a small number of spoilage organisms within a large volume, the processing of a variety of sample types that arise during beer production and the slightly variable spectrum of BSM that requires the used methods to be adapted. Thus, the BSM spectrum is limited to a few species as there are hurdles specific to beer that render the growth of numerous microorganisms impossible. The greatest proportion of microorganisms that are able to grow in beer, spoil it with their metabolic products, and form turbidity is the gram-positive bacteria of the *Lactobacillus* and *Pediococcus* genera. In addition to the specified lactic acid bacteria, a few gram-negative bacteria of the *Pectinatus* and *Megasphaera* genera are characterized as beer spoilers.

Even though the group of beer-spoiling bacteria (BSB) is limited to few species, it is not a closed one. Over time, some species were added to this group after being newly described or having gained beer-spoilage ability. Other species were deleted, for example, after losing the ability to grow in beer due to technological changes in beer production or after being taxonomically re-classified as another species. For successful brewing-microbiological QC it is essential to stay up to date with BSM and to adapt all used methods to this spectrum of bacteria and yeasts.

It is worth mentioning that large-scale studies on the occurrence of the individual beer-spoiling species are rare. Insights into BSM frequency are often based on low sample volumes from few breweries or on empirical values. One target of the present work was to provide a current overview on the percentage distribution of the individual bacterial species that do actually occur in beer. For this purpose, more than 13,000 samples were examined for the presence of BSB and their incidences were tracked over seven consecutive years. These samples were also evaluated statistically using the $\chi^2$ test (chi-squared test) for two issues related to the brewing industry: Whether single species are likely to grow in bottom-fermented or in top-fermented beer types and whether they are likely to grow in early or late stages of the production process (= primary or secondary area).

A brewing-microbiological problem derives from the fact that BSB from product samples containing large quantities of yeast (example: propagated yeast samples) are very difficult to detect; in most cases there is a delay and in some cases they are not detected at all. Bacterial cells are morphologically smaller than yeast cells and usually present in considerably smaller concentrations. Thus, they can hide within the yeast cells and the active yeast also restricts

their reproduction. The current routine analysis in the brewing-microbiological laboratory involves repeated incubation in concentrated nutrient media designed to suppress yeast cells and promote bacteria, which often leads to enrichment periods of several weeks. Based on those difficulties, a method was developed that rapidly kills 100 % of yeast cells by adding the antimycoticum Natamax®, which contains natamycin and leaves bacterial cells unimpaired. This increases the bacterial cell number to be detected and reduces the time taken to detect bacterial contaminations.

If a contaminant is isolated that demonstrably causes damages to beer, but has not occurred previously, it needs to be identified as part of microbiological QC. This guarantees that QC stays current and can react to all possible occurring species. In 2013 and 2014, a total of three isolates were obtained from different process steps in a brewery that could not be identified using routine molecular biological methods. Phenotypic, chemotaxonomic and genotypic characteristics of the isolates were determined and compared with known beer spoilers. These comparisons ruled out classification as a known beer-spoiling species.

In a further multivariate analysis, a possible classification of those isolates into closely related, but non-BS species was investigated. The differences established in this study did not conform to the requirements for inclusion into one of the investigated species resulting in the new species description of *L. cerevisiae*. In addition to determining the essential characteristics necessary for new descriptions of a species of the *Lactobacillus* genus, beer-related properties such as the presence of certain hop-resistance genes were determined.

*L. rossiae* is known as a species that occurs in sourdough and is regarded as phenotypically and genotypically versatile. As this species has also played a role as a beer spoiler in the past decade and because individual *L. rossiae* strains exhibit exopolysaccharide formation, 11 different strains obtained from routine analyses were examined. The specified strains were used to determine if the described versatility could be confirmed, the characteristics that demarcate beer isolates from sourdough isolates, and the extent of the beer-spoilage potential of *L. rossiae*. The great differences between the analyzed strains, which in some cases expand the limits of species description, and the serious possible effects on the beer medium (increased viscosity to verging on becoming slimy) mean that *L. rossiae* must be considered to be an exceptional species within the *Lactobacillus* genus and within the group of BSM.

## ZUSAMMENFASSUNG

Triebfeder der kostenintensiven und aufwändigen Qualitätssicherung (QS) in Brauereien ist der Erhalt der Bierqualität, um die stetig steigenden Verbrauchererwartungen erfüllen zu können. Ein Großteil der Konsumenten ist sich nicht darüber im Klaren, dass bierschädliche (BS) Bakterien oder Hefen eine der häufigsten Ursachen für Reklamationen sind. Auswirkungen einer Kontamination mit diesen Mikroorganismen können Veränderungen der Biere in Geschmack, Geruch, Säuregrad, Textur oder Aussehen sein, die im Ernstfall dafür sorgen können, dass der Verbraucher sich gegen ein Produkt entscheidet. Neben dem Imageverlust kann es zu erheblichen monetären Schäden kommen, wenn verdorbene Chargen nicht in den Handel entlassen werden können und der Vernichtung zugeführt werden müssen. Es bedarf daher großer Sorgfalt und geeigneter Präventionsmaßnahmen seitens der Brauereien, um die Qualität ihrer Biere zu sichern.

Die mikrobiologische QS in Brauereien ist dafür zuständig, die einzelnen Prozessschritte auf das Auftreten von bierschädlichen Mikroorganismen hin zu untersuchen. Diverse Probleme, mit denen die mikrobiologische QS im Speziellen umzugehen hat, sind die Detektion einer geringen Anzahl von Schadorganismen in einem großen zu untersuchenden Volumen, die Verarbeitung verschiedener Probentypen, die während der Bierproduktion anfallen, und die Veränderung des Spektrums bierschädlicher Mikroorganismen (BSM), an die die verwendeten Methoden angepasst werden müssen. Dabei ist das Spektrum an BSM aufgrund spezifischer biereigener „Hürden“, die ein Wachstum vieler Mikroorganismen unmöglich machen, auf wenige Spezies beschränkt. Der größte Teil der Mikroorganismen, die in Bier wachsen und es durch Stoffwechselprodukte und Trübungsbildung schädigen können, sind gram-positive Bakterien der Genera *Lactobacillus* und *Pediococcus*. Zusätzlich zu den genannten Milchsäurebakterien zählen einige wenige gram-negative Bakterien der Genera *Pectinatus* und *Megasphaera* zu den BSM.

Die Gruppe der bierschädlichen Bakterien (BSB) ist zwar auf wenige Spezies limitiert, aber keine geschlossene Gruppe. Im Laufe der Zeit wurden Spezies in die Gruppe aufgenommen, die entweder neu beschrieben wurden oder die Fähigkeit zum Bierverderb erwarben, und andere aus der Gruppe der BSB eliminiert, die z.B. aufgrund von technologischen Veränderungen in Bier nicht mehr wachsen konnten oder taxonomisch einer anderen Spezies zugeordnet wurden. Für eine erfolgreiche brauerei-mikrobiologische QS ist es unumgänglich, im Hinblick auf die Gruppe der BSM auf dem Laufenden zu sein und alle verwendeten Methoden auf dieses Panel an Bakterien und Hefen anzupassen.

Dabei sind großangelegte Studien über das Vorkommen der einzelnen bierschädlichen Spezies selten. Oftmals ergeben sich mikrobiologische Erkenntnisse aus dem Probenaufkommen einiger weniger Brauereien oder aus Erfahrungswerten. Ein Ziel der vorliegenden Arbeit war es, einen aktuellen Überblick über die prozentuale Verteilung der einzelnen bierschädlichen Bakterienspezies zu erstellen. Dabei wurden insgesamt über 13.000 Proben auf die Anwesenheit von bierschädlichen Bakterien hin untersucht und ihr Vorkommen über sieben zusammenhängende Jahre verfolgt. Zusätzlich wurden diese Proben mit Hilfe des $\chi^2$-Tests (Chi-Quadrat-Test) statistisch auf zwei brauereispezifische Fragestellungen hin untersucht: Zum einen auf das bevorzugte Wachstum einzelner Spezies im untergärigen oder obergärigen Sektor und zum anderen auf das bevorzugte Wachstum in frühen oder späten Phasen des Produktionsprozesses (= Primär- oder Sekundärbereich).

Eine weitere mikrobiologische Problemstellung ergibt sich aus der Tatsache, dass BSB in Produktionsproben, die stark hefehaltig sind (Beispiel: Propagationshefe), verspätet oder überhaupt nicht detektiert werden können. Die morphologisch kleineren und meist in bedeutend geringeren Zellzahlen vorkommenden Bakterienzellen können sich zwischen den Hefezellen versteckt halten und werden durch aktive Hefe zusätzlich in ihrer Vermehrung eingeschränkt. Die bisher verwendete Routinemethode im brauerei-mikrobiologischen Labor sieht die wiederholte Inkubation in konzentrierten Nährmedien vor, die Hefezellen unterdrücken und Bakterien fördern sollen, was allerdings in vielen Fällen zu Anreicherungszeiten von mehreren Wochen führt. Aus dieser Problemstellung heraus wurde eine Methode entwickelt, die durch den Zusatz des Natamycin-enthaltenden Antimykotikums Natamax® in kürzester Zeit 100 % der Hefezellen abtötet und gleichzeitig bakterielle Zellen unbeeinträchtigt lässt. Auf diese Weise wird die nachzuweisende Bakterienzellzahl erhöht und die Zeit bis zur Nachweisbarkeit bakterieller Kontaminationen verkürzt.

Im Fall, dass ein Keim isoliert wird, der nachweislich Schäden in Bier hervorruft und bisher noch nicht in Erscheinung getreten ist, muss die mikrobiologische QS die Identifizierung einleiten. Nur so kann gesichert werden, dass sie auf dem Laufenden bleibt und auf alle eventuell vorkommenden Spezies reagieren kann. In den Jahren 2013 und 2014 wurden insgesamt drei Isolate aus verschiedenen Prozessstufen einer Brauerei gewonnen, die mit den routinemäßig eingesetzten molekularbiologischen Methoden nicht identifiziert werden konnten. Nachfolgend wurden phänotypische, chemotaxonomische und genotypische Charakteristika der Isolate bestimmt und mit bekannten Bierschädlingen verglichen. Basierend auf diesem Vergleich wurde die Zuordnung zu einer bekannten bierschädlichen Art ausgeschlossen.

In einer weiteren multivariaten Analyse wurde die Zuordnung dieser Isolate zu nah verwandten, nicht-bierschädlichen Arten überprüft. Die Unterschiede, die in dieser Studie herausgearbeitet wurden, entsprachen nicht den Anforderungen zur Inklusion in eine der untersuchten Spezies, was folglich in der Neubeschreibung einer Spezies, *L. cerevisiae*, resultierte. Zusätzlich zu den für die Neubeschreibung einer Spezies innerhalb des Genus *Lactobacillus* unerlässlichen Merkmalen wurden bierspezifische Charakteristika wie die Präsenz bestimmter Hopfenresistenzgene untersucht.

*L. rossiae* ist bekannt als in Sauerteig vorkommende Spezies und gilt als phäno- und genotypisch besonders versatil. Da diese Art im letzten Jahrzehnt zusätzlich eine Rolle als Bierschädling eingenommen hat, die sich insbesondere durch Exopolysaccharid-Bildung einzelner Stämme auszeichnet, wurden elf aus der Routineanalytik gewonnene *L. rossiae*-Stämme dahingehend untersucht, ob sich die beschriebene Versatilität bestätigen ließ, in welchen Merkmalen sich Bier- und Sauerteig-Stämme unterscheiden und wie hoch der Grad der Bierschädlichkeit von *L. rossiae* ist. Aufgrund der großen Differenzen zwischen den begutachteten Stämmen, die teilweise die Grenzen der bestehenden Speziesbeschreibung erweitern, und den gravierenden möglichen Auswirkungen auf das Biermedium (Viskositätserhöhung bis hin zum Schleimigwerden der Biere), ist *L. rossiae* als Ausnahme innerhalb des Genus *Lactobacillus* und innerhalb der Gruppe der BSM anzusehen.

# 1. INTRODUCTION

The term 'microorganism' designates a microscopically small living organism consisting of a single cell that is independent of other cells and is (occurring individually) invisible to the naked eye. Microbiology deals with the study of those microorganisms that comprise a heterogeneous group of organisms and can have both negative and positive effects on human life (Brock and Madigan, 1991). With regard to the food industry, microorganisms play a positive role if deployed purposefully in food production, such as yeast in the brewing or baking industry or lactic acid bacteria (LAB) in the dairy industry. They can also play a negative role as food spoilage organisms by adversely altering the sensory properties of food and subsequently reducing the generated products' quality.

Beer is regarded as a stable food from a microbiological point of view. But distinct yeast and bacteria species can negatively affect the sensory and organoleptic beer properties to render it inedible to the consumer. To counteract these issues, which can directly affect a brewery's sales, profit and reputation, it is useful to establish microbiological quality control (QC). Whether QC is executed within the brewery itself or by external laboratories often depends on the brewery size, the employees' qualification and the management's sensibility towards the microbiological status of the brewery.

The aims of microbiological QC are, among other things, the detection of beer-spoiling microorganisms (BSM), the identification of harmful germs and the subsequent initiation of appropriate countermeasures such as the retention of batches prior to distribution. To achieve those goals it is sufficient to focus on a small number of microorganisms since only a few yeast and bacteria species can grow in beer and alter its sensory properties, i.e. spoil it. It is particularly important for brewing microbiological QC to detect contaminating microorganisms as quickly and reliably as possible and in small numbers. Once spoilage germs have been detected using appropriate microbiological methods it can be necessary to identify the type of contaminant present, in other words, the genus and species of the contaminating yeast and bacteria. Identification can help to determine the 'in-house flora' and to trace the pathways of contamination. Executed on a large scale and evaluated statistically, species identification can also help to reveal changes within the BSM group and to draw relevant conclusions. It should be noted that the BSM group is not closed but open to the addition of new microorganism species at any time. Those included species are either newly described or have newly acquired the potential for beer spoilage. The spectrum of detrimental germs can also be altered by developments in brewing technologies such as beer production with nearly complete oxygen exclusion. To keep microbiological QC up to date it is essential to be aware of all microorganism species that are able to spoil beer and beer-like beverages and to achieve deeper insights into their beer-spoilage potential and beer-specific characteristics. In comparison with beer-spoiling yeast species, the frequency and degree of spoilage potential are more pronounced with bacteria.

This study focuses on beer-spoiling bacteria (BSB), with a special focus on LAB. In subsections,

- the statistical frequency of individual beer-spoiling (BS) species is determined from brewery samples from Germany and neighboring countries

- a method is proposed for the fast detection of beer-spoiling LAB in culture yeast

- one lactic acid bacterium isolated from spoiled beer is differentiated from known beer-spoiling species

- this BS species is consequently newly described as *Lactobacillus cerevisiae* sp. nov.

- another LAB species, *Lactobacillus rossiae*, primarily isolated from sourdough and recently included in the group of BSB is characterized by multivariate analysis and differentiated from sourdough isolates.

## 1.1 The properties of beer

Beer is protected from microbially triggered spoilage by different intrinsic and extrinsic factors (Menz and Vriesekoop, 2009). Intrinsic factors, called ‘hurdles’ according to LEISTNER (2000), include ethanol content, hops addition, low pH value, carbon dioxide ($CO_2$) content, low oxygen ($O_2$) level, and a low amount of fermentable nutrients (Menz and Vriesekoop, 2009, Suzuki et al., 2006b). These intrinsic factors mean that pathogen microorganisms such as members of the *Bacillus* or *Staphylococcus* genera are not able to grow in beer (Menz and Vriesekoop, 2009, Bunker, 1955). Beer wort does not yet possess most of the specified hurdles. Active culture yeast has to be added immediately after wort production, since a small number of the contaminants present can be suppressed by the yeast’s fermentational force (Campbell, 2003a). Beer types with intentionally reduced intrinsic factors like alcohol-free beer or wheat beer are also more susceptible to microbial spoilage (Riedl et al., 2017).

Extrinsic factors are certain steps of the beer production process, e.g. mashing, wort boiling, filtration, flash pasteurization or cold storage, that impede the insertion of contaminating germs or devitalize germs that were already inserted in the beer or one of its primary stages (Vriesekoop et al., 2012). The following is a description of the particular hurdles and their effect on microorganisms, especially on bacteria.

### 1.1.1 Ethanol

As early as 1935, SHIMWELL described the antibacterial effect of ethanol after his assessment of beers with higher ethanol content being less susceptible to *Saccharobacillus pastorianus* (now: *L. brevis*) (Shimwell, 1935). Ethanol affects the bacterial cell in several ways. It inhibits certain membrane functions, triggers cell membrane leakage, causes transcription and translation errors, induces errors in protein synthesis, and increases membrane permeability to small molecules such as protons and organic acids, which leads to the collapse of the microorganisms’ pH homeostasis (Casey and Ingledew, 1986, Eaton et al., 1982, Barker and Park, 2001, Daifas et al., 2003, Wray, 2015, Haft et al., 2014, Ingram, 1990). It is worth noting

that ethanol kills bacteria more effectively if organic acids are present (synergistic effect) or at lower pH values (Barker and Park, 2001). At typical beer ethanol contents (average content approx. 3.5 – 5.0 % (v/v)), it has little antibacterial effect (Vriesekoop et al., 2012, Menz et al., 2010, Wackerbauer and Emeis, 1969, Menz et al., 2011).

### 1.1.2 Low pH value

Due to the yeast's sugar metabolism, ethanol, carbon dioxide and organic acids are released from the cells resulting in the decrease in beer pH value (Menz and Vriesekoop, 2009). The beer-typical pH value (average approx. 3.4 – 4.8) alone is not sufficient to hinder bacterial growth, because it is only slightly below the optimum pH for most lactobacilli (Wackerbauer and Emeis, 1969). But the average low pH value of beer results in the conversion of weak organic acids to their undissociated forms which are able to penetrate the bacterial cell membrane (Beales, 2004). Due to the higher intracellular pH value the organic acids dissociate leading to the decrease in the intracellular pH and to cell acidification (Beales, 2004). In turn this leads to the blocking of enzyme systems, to the cessation of synthesis of cellular components, to the hampering of nutrient uptake, and finally to the inhibition of bacterial cell growth and division (Booth and Kroll, 1989). The microorganism cell tries to maintain the cellular pH gradient by pumping protons from the inside to the periphery at the expense of a lot of energy. The ability for the maintenance of pH homeostasis, however, varies according to the specific strain and species (Beales, 2004, Booth and Kroll, 1989, Booth, 1985). In addition to the direct impact, the low beer pH value intensifies the antibacterial properties of certain hop components (Simpson, 1993b, Simpson, 1993a, Simpson and Fernandez, 1992, Simpson and Hammond, 1991, Wackerbauer and Emeis, 1969) and also results in the inability of pathogenic strains to grow in beer (Wray, 2015).

### 1.1.3 Dissolved gases

The presence of $CO_2$ as well as the absence of $O_2$ (optimum $\leq$ 0.1 ppm, provided that beers without pre-damage were produced on modern brewing equipment) are also beer-specific hurdles that affect the growth of aerobic microorganisms, especially the growth of pathogens (Vriesekoop et al., 2012). $CO_2$ is formed by the yeast during the first (or second, if executed) fermentation or is directly applied, if permitted by law, up to a final average content of 0.5 % (w/v). $CO_2$ reduces the pH value (Wray, 2015), but does not affect growth of the facultatively anaerobic or microaerophilic lactobacilli in any way (Wackerbauer and Emeis, 1969, Holzapfel and Wood, 2014). The presence of oxygen is generally associated with negative effects in LAB (van de Guchte et al., 2002).

### 1.1.4 Lack of nutrients

The metabolism of the brewing yeast consumes nutrients such as fermentable carbohydrates, amino acids and vitamins during fermentation resulting in a nutrient-poor milieu. As early as 1969, researchers questioned whether a beer‘s nutrient shortage had a great impact on lactobacilli as most beers that do not reach final gravity contain enough fermentable sugar

components to allow bacterial growth. Even beers reaching final gravity contain dextrins which can be utilized by many bacteria for energy generation (Wackerbauer and Emeis, 1969). DOLEZIL AND KIRSOP (1980) figured out that the metabolically most versatile LAB grew best in beer. FERNANDEZ AND SIMPSON (1995) determined a positive correlation between the risk potential of a certain beer for spoilage and its contents of free amino nitrogen, total soluble nitrogen, some individual amino acids and maltotriose. As proposed by SUZUKI ET AL., the ADI system, malolactic fermentation and citrate utilization play an important role for BS LAB (Suzuki et al., 2005b).

### 1.1.5 Hops

In 1945, SHIMWELL discovered that hop bitter acids negatively affect gram-positive, but not gram-negative bacteria. As a result, gram reaction became increasingly important in brewing microbiology (Shimwell, 1945). Further studies showed that hops inhibit the growth of some microorganisms, while they only retard the growth of others (Wackerbauer and Emeis, 1969). It is now known that hop acids have antibacterial properties and, for beer-spoiling LAB, the resistance towards hop acids is the crucial survival criterion (Vriesekoop et al., 2012, Fernandez and Simpson, 1995, Fernandez and Simpson, 1993). Thus, the antibacterial effect not only depends on the amount of bitter compounds (EBC bitter units), but on the composition of the individual hop compounds (Back and Biendl, 2017a, Back and Biendl, 2017b).

Hop acids, especially undissociated iso-α-acids, affect bacterial cells by acting as proton ionophores and consequently destructing their transmembrane pH gradient, which is important for the absorption of vital components (Simpson, 1993a, Simpson, 1993b, Simpson and Fernandez, 1994). Cell leakage induced by hop acids, for example, hampers nutrient uptake as well as RNA and DNA synthesis systems (Vriesekoop et al., 2012). Entering protons lead to internal acidification which reduces the activity of certain enzymes and damages proteins and DNA (van de Guchte et al., 2002). Furthermore, BEHR AND VOGEL (2009) showed that iso-α-acids cause oxidative stress in bacterial cells by participating in transmembrane redox reactions.

## 1.2 Stress tolerance mechanisms

The multitude of stress factors exerted by the beer environment causes bacteria to react with numerous defense mechanisms as illustrated in this chapter. The ability to adapt to a beer's hop content is considered to be the crucial characteristic for beer-spoiling bacteria. Bacterial hop resistance consists of several active and passive defense mechanisms (Behr et al., 2006, Vogel, 2010, Suzuki, 2015).

### 1.2.1 Active hop resistance mechanisms

Proton pumps such as HorA, a multidrug transporter of the ATP binding cassette (ABC) family, and HorC, a proton-motive-force (pmf) -driven multidrug transporter, are active hop defense mechanisms that extrude hop acids from the cell (Iijima et al., 2009, Iijima et al., 2006,

Sakamoto et al., 2001). The corresponding *horA* and *horC* genes were found, irrespective of the species, in 94 % (*horA*) and 96 % (*horC*) of beer-spoiling bacteria genomes (Suzuki, 2011) and are rated as the most important species-independent marker genes related to beer-spoilage potential (BSP) (Haakensen, 2009, Suzuki et al., 2006b). PREISSLER postulated that the presence of the *horA* gene alone did not result in higher minimum inhibitory concentrations (MIC), while the presence of either *horC* or *hitA* yielded significantly higher MICs (Preissler, 2011). Interestingly, TEICHERT discovered that half of BS strains contain only a defective *horA* gene (Teichert, 2009), which reduces the significance of *horA*. However, it is important to note that some bacteria harbor genes related to beer spoilage (such as the *horA* gene) and yet do not demonstrate an ability to spoil beer (Sakamoto et al., 2001, Suzuki et al., 2004b). Further hop resistance genes, *bsrA* and *bsrB*, coding for multidrug ABC transporters, were found in *Pediococcus* strains (Haakensen et al., 2009b).

HitA is a potential divalent cation transporter counteracting the noxious effect of isohumulones by binding $Mn^{2+}$ ions (Hayashi et al., 2001, Yasui et al., 1997). The intracellular content of divalent cations, especially of $Mn^{2+}$ ions, plays a significant role for LAB in hop defense (Behr et al., 2007).

It is assumed that those species-independent genetic markers are taken up by horizontal gene transfer (HGT). This hypothesis is based on the fact that the nucleic acid sequences of those markers are approx. 99 % homologous between different species and genera (Claisse and Lonvaud-Funel, 2001b, Suzuki et al., 2005a, Suzuki et al., 2006b). In many species, *horA*, *horC* and their flanking open reading frames (ORF) were found in conserved areas of the DNA (Iijima et al., 2007, Suzuki et al., 2005a, Suzuki et al., 2006b). Species-independent genetic markers were proven to be located on mobile DNA units such as plasmids and transposons (Suzuki, 2011b). The markers can spread within a brewery by HGT and can be found in different contaminating species. The uptake of such mobile species-independent marker genes is a survival advantage for bacteria while fighting the hostile beer environment (Suzuki, 2015, Haakensen et al., 2007). The HGT theory was also postulated for the glycosyltransferase (*gtf)* and glycerol dehydratase genes associated with the spoilage of wine and cidre (Claisse and Lonvaud-Funel, 2001a, Dols-Lafargue et al., 2008, Werning et al., 2006). Fast adaptation or fast evolution in a stressful and challenging environment are assumed to be results of HGT events triggered by environmental stress (Dziewit and Bartosik, 2014). It is also worth noting that the association with other bacteria in biofilms increases the possibility of genetic material uptake through HGT (Kubota et al., 2008, Timke et al., 2005).

Active hop resistance mechanisms consume a high amount of energy in addition to the impeding factors of beer containing low amounts of residual nutrients and the hampering of nutrient uptake by the protonophoric activity of hop acids (Simpson, 1993b, Simpson, 1993a). Some beer-spoiling strains were examined for their energy consumption under hop influence and SUZUKI ET AL. established that strains from a beer environment were able to produce a higher amount of ATP and to maintain a greater ATP pool inside the cells than non-spoiling strains (Suzuki et al., 2005b). After inoculation into beer, energy was generated by the consumption in particular of citrate, pyruvate, malate, and arginine. The metabolism of organic acids and amino acids is directly or indirectly used for energy production and pmf generation, particularly if nutrients are scarce (Suzuki et al., 2005b). Pmf is further used by LAB as an energy source for numerous transmembrane processes (van de Guchte et al., 2002). Furthermore, hop-resistant bacteria initially have a higher transmembrane pH gradient (Simpson, 1993b).

### 1.2.2 Passive hop resistance mechanisms

Defense mechanisms are regarded as passive if no energy is consumed after their build-up and/or activation (Suzuki, 2011). One example of a passive hop resistance mechanism is the insertion of saturated fatty acids such as $C_{16:0}$ or lipoteichoic acids (LTA) into the membrane of BSB. This results in the loss of membrane fluidity and consequently in the decrease of membrane permeability for hop bitter acids to subsequently protect themselves from hop and acid intrusion (Behr et al., 2006, Yasui and Yoda, 1997, Schurr et al., 2015). LTAs also serve as a long-term reservoir for $Mn^{2+}$ ions that could otherwise form a complex with hop acids to achieve full antibacterial effectivity (Behr et al., 2006, Vogel, 2010).

BEHR ET AL. reported on the increase of $Mn^{2+}$-dependent enzymes in *L. brevis* which take part in energy generation and redox homeostasis (Behr et al., 2007). The removal of $Mn^{2+}$ ions is a useful tool to prevent hops conveying their full antimicrobial force (Geißler et al., 2017). As proposed by GEIßLER ET AL. (2017), there is a magnesium uptake system *CorA* (Kehres et al., 1998) in many beer-spoiling LAB that simultaneously releases manganese from the cell to decrease its effects on hop acids. The exchange of divalent manganese cations with divalent magnesium ions maintains cation homeostasis (Schurr et al., 2015, Preissler, 2011), but they are not completely exchangeable with regard to their physiological functions (Geißler et al., 2017).

### 1.2.3 Further stress tolerance mechanisms

As hop acids, in addition to their protonophoric activity, exhibit redox-reactive decoupling activity, bacteria react with the upregulation of particular enzymes, such as proton-extruding ATP-synthases, for the maintenance of redox homeostasis (Behr and Vogel, 2010, Vogel, 2010, Suzuki, 2015, de Angelis and Gobbetti, 2011). The arginine deiminase (ADI) pathway that is used by several LAB species alkalizes the environment and the generated ATP enables proton extrusion (van de Guchte et al., 2002, Cunin et al., 1986, Sanders et al., 1995). Furthermore, the reduction in cell surface of the bacteria adapted to the beer environment and, therefore, the area that is potentially endangered was observed (Asano et al., 2007, Zhao et al., 2017). The resistance mechanisms can then be deployed more purposefully and effectively on the reduced cell surface (Suzuki et al., 2006b).

To combat the effects of ethanol, LAB fortify their membrane by integrating long-chain fatty acids (> 20 carbons) (Uchida, 1974) and GroES chaperone, heat-shock proteins and glutathione reductase are upregulated (Fiocco et al., 2007, Silveira et al., 2004). Ethanol tolerance is a species-specific characteristic unlike general beer-spoilage ability (Pittet et al., 2011). The ability to adapt to low pH and ethanol is important for LAB, but it is not correlated with the ability to adapt to hops or to spoil beer (Pittet et al., 2011, Menz et al., 2010, Bergsveinson et al., 2015a, Bergsveinson et al., 2015b).

In a recent study, it was determined that dissolved $CO_2$ and the headspace pressure of packaged beer negatively affects LAB growth in beer. This results in cell wall and membrane modifications and in modifications of the cellular transcriptional regulation (Bergsveinson et al., 2015b).

The lack of nutrients in common beer caused by the yeast's metabolism is counteracted by LAB via the use of ABC transporters (Konings et al., 1997). These transfer nutrients into the cell by means of different passive transport systems along an electrochemical ion gradient and via group translocation mechanisms such as the phosphotransferase system (Pittet et al., 2013) that modify internalized molecules (White et al., 2012).

Upon exposure to one or more stress factors, non-spore-forming bacteria defend themselves by entering a dormancy state that is called the 'viable but not culturable' (VBNC) state (Xu et al., 1982, Suzuki et al., 2006a, Oliver, 2005). Some LAB that are linked to beer spoilage are also known to use this survival strategy, while growth and beer-spoilage ability is restored after resuscination (Deng et al., 2015). BS species that were associated with a VBNC state are *L. lindneri*, *L. paracollinoides*, *L. acetotolerans*, *L. casei*, *L. harbinensis* and *L. plantarum* (Suzuki et al., 2006a, Liu et al., 2018, Liu et al., 2017a, Liu et al., 2017b, Liu et al., 2017c, Deng et al., 2015).

A detailed illustration of important defense mechanisms concerning the antibacterial hurdles of beer is given by FRAUNHOFER (Fraunhofer, 2018).

## 1.3 Lactic acid bacteria

Microorganisms are designated as LAB if they belong to the *Lactobacillaceae* family which comprises only two genera, *Lactobacillus* (Beijerinck, 1901) and *Pediococcus* (Claussen, 1903). LAB constitute a heterogeneous group of bacteria (van de Guchte et al., 2002) whose number of species has doubled in the last 12 years to over 200 species (see LPSN: http://bacterio.net/lactobacillaceae.html) (Felis and Dellaglio, 2007). Cells are gram-staining positive, catalase-negative, and non-mobile, and the primary end product of carbohydrate fermentation is lactic acid (Klaenhammer and de Vos, 2011). Their metabolism is considered to be strictly fermentative (Kandler and Weiss, 1984, Wood and Holzapfel, 1995), though respiration has been reported for some species (Brooijmans et al., 2009). Another genus was proposed within the *Lactobacillaceae* family, *Paralactobacillus* (Leisner et al., 2000), but the single species *Paralactobacillus selangorensis* was reclassified as a member of the *Lactobacillus* genus by HAAKENSEN ET AL. (2011). Most LAB species are susceptible to common antibiotics and some species, such as strains of *L. casei*, have therefore acquired 'generally recognized as safe' (GRAS) status (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&sort=GRN_No&order=DESC&startrow=1&type=basic&search=Lactobacillus) (Katla et al., 2001, Teuber et al., 1999). The most closely related family from a phylogenetic perspective is the *Leuconostocaeae* family (Felis and Dellaglio, 2007). A recent study conducted by ZHENG ET AL. (2015) proposed the softening of the two *Lactobacillus* and *Pediococcus* genera to form the *Lactobacillus sensu lato* complex comprising *Lactobacillus* spp. and *Pediococcus* spp. in addition to strains of other genera such as *Oenococcus* spp. (Salvetti et al., 2018).

### 1.3.1 The *Lactobacillus* genus

Cells of the *Lactobacillus* genus (translated as *small rod from milk*), which is the most hazardous genus for beer spoilage, varies widely from long and slender rods to short and bended, or coryneform, coccoid cells. The lactobacilli's size and shape depends, amongst others, on the culture's age, the composition of the medium and specific stress factors (Kandler and Weiss, 1984). The tendency to form cell chains varies according to the species and, in some cases, this variability is even strain specific. The *Lactobacillus* genus shows the need for complex nutrient requirements in relation to amino acids, peptides, nucleic acid derivates, vitamins, salts, fatty acids, and fatty acid esters (often species dependent).

Species are classified as homofermentative, facultatively heterofermentative or strictly heterofermentative ones. The end product of homofermentative metabolism is almost exclusively lactate (> 85 %). Hexoses are degraded via the Embden-Meyerhof-Parnas (EMP) pathway (glycolysis); pentoses and gluconate are not degraded as they lack the phosphoketolase enzyme (Mattarelli et al., 2014). Facultatively heterofermentative species degrade hexoses via the EMP pathway; pentoses and gluconate are fermented as they possess aldolase and phosphoketolase (Mattarelli et al., 2014). End products of heterofermentative metabolism are lactate (min. 50 %), acetate or ethanol, $CO_2$, formate and succinate via 6-phosphogluconate pathway = pentosephosphate pathway (Hammes and Hertel, 2009). Lactobacilli are, in general, aero-tolerant to facultatively anaerobic since they do not possess a respiratory metabolism, but are not killed by moderate oxygen amounts (Priest, 2003). Cells generate energy by substrate-level phosphorylation and regenerate used NADH by electron transfer on lactate (homofermentative) or via acetaldehyde on ethanol (heterofermentative) to $NAD^+$ (Priest, 2003).

Lactobacilli often contain plasmids which are considered to provide resistance towards drugs (Ishiwa and Iwata, 1980) or to affect lactate metabolism (Chassy et al., 1976) (see also Section 1.2 Hop resistance).

### 1.3.2 The *Pediococcus* genus

Cells of the *Pediococcus* genus (translated as *cocci growing in one plane*) are homofermentative, catalase-negative and of coccoid shape. They grow in pairs or, if growing in two perpendicular directions, tetrads, but never in chains, and can be isolated from wort, yeast or beer (Back, 1994a, Mattarelli et al., 2014). Growth of pediococci can be related to slime production, depending on the composition of the residual sugars (Shimwell and Kirkpatrick, 1939). The *Pediococcus* genus is attributed to CLAUSSEN (Claussen, 1903) with the type species *Pd. damnosus* (Garvie, 1974) and 10 further species (Salvetti et al., 2018). From a phylogenetic perspective, *Pediococcus* species cluster together on the basis of 16S rRNA (Salvetti et al., 2012) and specific ribosomal protein and housekeeping gene sequences using MLSA (Salvetti et al., 2018), but are intermixed with *Lactobacillus* clusters.

1.3.3 The role of lactic acid bacteria in beer

#### 1.3.2.1 Positive role of lactic acid bacteria

In some cases, lactic acid bacteria are purposefully added during beer production (Henneberg, 1903, Lowe et al., 2004, Back, 1994a). *Lactobacillus* strains can be added to wort or mash to achieve certain positive characteristics such as improved biological availability of zinc ions (Donhauser and Wagner, 1986), lauter performance (Lowe et al., 2004), taste stability, protein precipitation (Back and Pittner, 1993, Back, 1994a), and the decrease in pH resulting in antimicrobial effects against spoilage germs (see Section 1.1.2) (Vaughan et al., 2005). Strains with the following properties were selected for this purpose: High hop sensitivity, highly thermophilic nature, homofermentative metabolism, inability to produce diacetyl and biogenic amines as well as a high lactic acid formation rate (Back and Bohak, 2005). One species that is often used for wort acidification is *L. amylolyticus* (Bohak et al., 1998). Another method is to add certain defined *Lactobacillus* species, e.g. *L. brevis* or *L. casei*, as starter cultures for specific beer styles such as Berliner Weisse (Wackerbauer and Methner, 1988). Further information is provided in reviews by LOWE AND ARENDT (2004) and VAUGHAN ET AL. (Vaughan et al., 2005). LAB fermentations could pose an interesting possibility in terms of creating new beer styles, and for the increasing numbers of craft beer breweries that are searching for new, non-standard flavors (Bergsveinson and Ziola, 2017).

Other studies focus on the ability of lactobacilli to produce certain low-molecular weight and heat-stable peptides (Ross et al., 2002). Those so-called bacteriocins can have a bacteriocidal or bacteriostatic effect on other species, especially on closely related ones (Jack et al., 1995, Vaughan et al., 2005). Approaches that involve the addition of nisin, the most widely researched bacteriocin, showed positive results with regard to restricting the growth of beer-spoiling bacteria without affecting the culture yeast or beer flavor (Ogden, 1986, Ogden et al., 1988, Vaughan et al., 2005, Muller-Auffermann et al., 2015b, Muller-Auffermann et al., 2015a). The disadvantages of nisin addition are high costs and the fact that the application of bacteriocins is not permitted in accordance with the German beer purity law (Ogden et al., 1988, Idler and Annemüller, 2001).

#### 1.3.2.2 Negative role of lactic acid bacteria

LAB are ubiquitous in the brewery and can be detected in almost all starting, intermediate and end products, from barley and wheat to the finished beer (Flannigan, 2003, Hollerova and Kubizniakova, 2001, Vaughan et al., 2005). The contamination source is generally characterized as primary or secondary.

The input of spoiling germs by raw or auxiliary materials, brewing water or air into the product is considered to be a primary contamination source (Back, 1994a, Back, 1988). In the production chain, the primary area extends from the brewhouse to the bright beer tanks (after filtration until just before the bottling area). LAB are part of the natural barley flora (< 0.01 % of all present bacteria) and can survive the malting and mashing process steps (O'Sullivan et al., 1999). During steeping, they multiply enormously (Petters et al., 1988). Approximately 0.2–0.4 % of the cell number on green malt survive kilning, after which species such as *L. brevis*, *L. buchneri*, *L. fermentum*, and *L. plantarum* in particular can be found (O'Sullivan et

al., 1999, Flannigan, 2003). Contaminated brewing yeast is also considered to be a primary contamination source (Wackerbauer and Emeis, 1969). If the contamination takes place within the primary area of brewing production (= early production steps), the germs in question are consequently called primary contaminants.

Contamination sources are referred to as secondary sources (and the contaminants therefore as secondary contaminants), if the spoiling microorganisms are introduced in the later beer production steps, during filling and bottling (Back, 1988, Back, 1994a, Back, 1994b). For example, water of the filling process or air that is swirled during filling can act as germ transmitters (Storgards, 2000, Wackerbauer and Emeis, 1969, Henriksson and Haikara, 1991, Dürr, 1984, Paradh et al., 2011). Unsold beer that is brought back to the brewery or contaminated returned empties are regarded as possible sources of secondary contamination.

Biofilm formation plays a major role in LAB contamination (Back, 1994b). Biofilms can establish in hard-to-clean areas in the filling environment in particular. They can be populated with a great number of different microorganisms and can provide a barrier against cleaning and disinfection measures (Storgards et al., 2006b, Flemming and Wingender, 2010). Slime-forming bacteria such as acetic acid bacteria (AAB) and *Enterobacteriaceae* can settle in moist spots that come into contact with the product and build a protective shield against cleaning agents. In the next step, yeasts can accumulate which produce metabolites that serve as nutrients for LAB (Storgards et al., 2006). Those hard-to-clean passages in a brewery can act as a permanent contamination source, if not eliminated as soon as possible.

Thus, lactic acid bacteria occur frequently in breweries and have become increasingly significant as spoiling germs. Species of the *Lactobacillus* and *Pediococcus* genera in particular are known to be beer spoilers (Back, 1994a). Since bacteria are considered to be potential beer-spoilage organisms (end of 19th century) (Pasteur, 1876), brewing microbiology addresses virtually the same microorganism spectrum, besides numerous renamings (Priest, 2003). Possible effects of LAB growth in beer are turbidity, increased viscosity to slime formation based on the synthesis of exopolysaccharides (EPS), increased acidity and the formation of malodorous, atypical substances (Back, 1994a, Rainbow, 1981). The buttery flavor of diacetyl that has a low odor threshold value of 0.15 ppm (Hough et al., 1982) is especially associated with the growth of certain spoiling bacteria in beer. Furthermore, growth of LAB can be accompanied by the production of biogenic amines (Kalac et al., 2002). In the brewing industry, 60–90 % of spoilage incidents are considered to be triggered by beer-spoiling LAB (Back, 1994a, Back, 1994b, Back, 2003). However, the tolerance towards hop components is the crucial characteristic for the degree of spoilage hazard, as mentioned above. It is also worth noting that the ability to spoil beer is not species specific but strain specific, which is the case for LAB from other foods (Sanders et al., 2015).

## 1.4 Beer-spoiling species

1.4.1 The history of beer-spoiling bacteria

In 1871, PASTEUR was the first to detect beer-spoiling bacteria via microscopic analysis (Pasteur, 1876) classifying them at the beginning roughly as rods and cocci. Rods were initially called *Saccharobacillus pastorianus* (van Lear, 1892) and later renamed *Lactobacillus pastorianus* (Bergey et al., 1923). At that time, VAN LEAR determined that those bacteria could not be cultivated on the usual media, but grew only on unhopped beer solidified with gelatine. It is now known that *L. pastorianus* occurs much more often than initially supposed (Iijima et al., 2007). After all beer-spoiling species were originally called *L. pastorianus*, SHIMWELL supposed that there had to be greater species variability within the group of BSB (Shimwell, 1948). This claim was confirmed by detecting *L. malefermentans* and *L. parvus* (Russell and Walker, 1953a, Russell and Walker, 1953b), *L. frigidus* (Bhandari and Walker, 1953) and *L. brevis* (Moore and Rainbow, 1955) as spoilage germs in beer and by determining, for example, that some heterofermentative species share more characteristics with *L. brevis* and some homofermentative strains with *L. plantarum* (Sharpe, 1959, Davis, 1964, Rogosa and Sharpe, 1959, Carriere, 1959). Since the studies of ESCHENBECHER, many beer-spoiling species have been named, described and even classified according to the frequency of appearance (Eschenbecher, 1966, Eschenbecher, 1968a, Eschenbecher, 1968b, Eschenbecher, 1969). Nowadays it is assumed that *L. pastorianus* is a synonym for *L. paracollinoides* since 16S rRNA and protein encoding genes for 6-phosphogluconate dehydrogenase are virtually identical (99.9 %) (Ehrmann and Vogel, 2005b, Suzuki et al., 2008a).

Coccoid bacteria were initially called *Pediococcus cerevisiae* (1844 by BLACKE) (Kitahara, 1974) which corresponds today to *Pediococcus damnosus* (Claussen, 1903). Further beer-spoiling pediococci include *Pd. claussenii* (Dobson, 2002) and *Pd. inopinatus* (Back, 2005, Iijima et al., 2007).

1.4.2 Beer-spoiling lactic acid bacteria

The beer-spoiling lactic acid bacteria consist of the *Lactobacillus*, *Pediococcus, Leuconostoc* and *Lactococcus* genera, which form a 16S rRNA gene-based supercluster within the *Clostridium* branch of gram-positive bacteria (Stackebrandt et al., 1983). In terms of their frequency and spoilage potential, the latter two genera play a minor role in beer spoilage.

#### 1.4.2.1 Frequently occurring beer-spoiling lactic acid bacteria

***Lactobacillus backii* (prior: *L. backi*)** (Bohak et al., 2006, Tohno et al., 2013)

This homofermentative bacterium grows in beers with up to 32 bitter units (BU) resulting in the build-up of turbidity, sediments and acidity. The ability to utilize a narrow carbohydrate spectrum is characteristic of *L. backii* (= named after BACK). The BSP is increased since at least one of the known hop resistance genes *horA* or *horC* can be found within any isolate (Iijima et al., 2007). *L. backii* morphologically resembles *L. coryniformis* showing irregular, club-shaped cells. Even based on their genetics, both species resemble one another which could

be the reason for past misidentifications (Suzuki, 2011). To date, *L. backii* has been exclusively isolated from the brewing environment.

***Lactobacillus brevis*** (Orla-Jensen, 1919)

*Lactobacillus brevis* (= short) is the most frequently occurring beer-spoiling species (Back, 1988, Back, 1994b, Hutzler et al., 2012a, Koob et al., 2014, Back, 1994a). This species consists of strictly heterofermentative rods with rounded ends, growing singly or in short chains (Kandler and Weiss, 1984). *L. brevis* was initially isolated from milk, cheese, sauerkraut, sourdough, silage, cow dung, feces, and the intestinal tracts of humans and rats. The formerly unique species *L. diastaticus* with the ability for super-attenuation and *L. brevisimilis* have been classified as *L. brevis* in the past two decades since the distinguishing features of *L. brevis* were regarded as being too minor (Priest, 2003, Back, 1987, Briggs et al., 2004).

*L. brevis* frequently also occurs outside the brewing environment and is, in general, considered to be an extremely versatile bacterium. This versatility is reflected in the wide temperature range and the ability to grow on many different culture media (Suzuki, 2015). The BSP varies according to the strain and especially between beer isolates and isolates from different areas of the food industry (Kern et al., 2014a, Back, 1994a, Suzuki et al., 2006b, Nakagawa, 1978, Menz and Vriesekoop, 2009). *L. brevis* is considered to be a late biofilm colonizer and some strains are able to produce exopolysaccharides (Back, 2003, Riedl et al., 2019).

***Lactobacillus (para-)buchneri*** (Henneberg, 1903)

*L. (para-)buchneri* (= named after BUCHNER) is a bacterium that shows strictly heterofermentative rods with rounded ends, occurring singly or in pairs, and is therefore morphologically hard to differentiate from *L. brevis.* Differing criteria from *L. brevis* are the ability to ferment melezitose and lactate dehydrogenase migration velocity. *L. buchneri* was isolated from milk, cheese, fermented plant material, and the human oral cavity. The formerly unique species *L. frigidus* and *L. parvus* were classified as *L. (para-)buchneri* (Back, 1981). *L. parabuchneri* was initially isolated from beer and can be differentiated from *L. buchneri* by the ability to ferment certain carbohydrates (Farrow et al., 1988). Both closely related species often appear in different areas of the food industry as spoilage germs.

***Lactobacillus (para-)casei*** (Orla-Jensen, 1916)

The facultatively heterofermentative species *L. (para-)casei* (= cheese) appears as rods, often with angular ends and a strong tendency to form chains (Kandler and Weiss, 1984). The effects of its growth in beer are turbidity, sediment formation and above all the production of diacetyl, a buttery off-odor in German beer styles (Sakamoto and Konings, 2003). The natural habitats of *L. (para-)casei* are milk, cheese, dairy products, sourdough, cow dung, silage, the human intestinal tract, and sewage (Kandler and Weiss, 1984). The differing criterion between *L. casei* and *L. paracasei* is the missing ribose utilization of *L. casei* (Collins et al., 1989). The classification and differentiation of *L. casei*, *L. paracasei* and the closely related species *L. rhamnosus* as well as their subspecies was controversially examined in numerous publications (Collins et al., 1989, Dicks et al., 1996, Ward and Timmins, 1999, Kandler and Weiss, 1986). *L. (para-)casei* grows only weakly in low-hopped beers with increased pH values, resulting in its classification as a potential beer-spoiling bacterium (for classification see Section 1.4.4) (Back, 1994a). In some years, it is the second most frequent beer-spoiling bacterial species after *L. brevis* (e.g. 12.3 % of bacterial incidents in 2012) (Koob et al., 2014).

***Lactobacillus (para-)collinoides*** (Carr and Davies, 1972)

The strictly heterofermentative species *L. collinoides* (= hilly; relating to colony morphology) and *L. paracollinoides* form rods with rounded ends that show the tendency to form filaments and, thus, appear singly, in palisades or in unregular clots. *L. collinoides* was initially isolated from apple juice and cider (Claisse and Lonvaud-Funel, 2000, Funahashi et al., 1998, Carr and Davies, 1972) and so far lacks beer-spoiling potential, in contrast to the species *L. paracollinoides,* which was primarily and exclusively isolated from brewery samples (Suzuki et al., 2004a). Further discriminating characteristics are based on DNA-DNA hybridization values and the ability to ferment D-fructose. *L. paracollinoides* is considered to be specific for breweries and is very closely related to *L. collinoides,* which may have caused misidentifications in the past (Suzuki, 2011).

***Lactobacillus coryniformis*** (Aboelnaga and Kandler, 1965)

The cells of *L. coryniformis* (= club-shaped) are coccoid, short, often pear- or club-shaped rods. *L. coryniformis* was initially isolated from silage, cow dung, the air of dairy plants, and sewage (Kandler and Weiss, 1984). This species is facultatively heterofermentative and causes sediments and diacetyl production during its growth in beer (Back, 1994a). The number of fermentable sugars is limited for *L. coryniformis*.

***Lactobacillus harbinensis*** (Miyamoto et al., 2005)

*L. harbinensis* (= named after Harbin, a Chinese city) is a facultatively heterofermentative, rod-shaped bacterium that was isolated from vegetables, the brewing environment and spoiled soft drinks. At the16S rRNA level, it is closely related to *L. perolens* (Miyamoto et al., 2005). Differentiating criteria to *L. perolens* are the utilization of D- and L-arabinose and the GC content. By-products of its carbohydrate metabolism are lactate, acetate and diacetyl. The ability of *L. harbinensis* to grow in commercial lager beer shows the degree of its BSP (Liu et al., 2018).

***Lactobacillus lindneri*** (Henneberg, 1903, Lindner, 1909, Henneberg, 1926)

The cells of *L. lindneri* (= named after LINDNER) are strictly heterofermentative and morphologically extremely versatile. They are able to change their shape depending on the milieu from long and straight rods in a beer environment to pleomorphic, sharp-edged to coccoid rods in some nutrient media (Back et al., 1996). Usually, growth of *L. lindneri* in beer is associated with increased turbidity, sediment formation and slightly increased acidity, but only with minor changes in taste or smell. It is possible to distinguish this from other beer-spoiling species, for example on the basis of its extremely narrow carbohydrate spectrum indicating *L. lindneri*'s high adaption to the adverse beer environment (Suzuki, 2015). *L. lindneri* is estimated to cause approx. 4–25 % of spoilage incidents, although recent studies indicate a rather small proportion ≤ 10 % of bacterial spoilage incidents (Back, 1988, Back, 1994b, Back, 2003, Back, 1994a, Hutzler et al., 2012a, Koob et al., 2014). Studies conducted by BACK revealed high hop tolerance (Back, 1981) and high tolerance to heat (Back et al., 1992). In 1974, ROGOSA proposed *L. lindneri* to be a synonym of *L. brevis,* which was refuted by BACK based on the sugar spectrum and on certain molecular characteristics (Rogosa, 1974, Back, 1981, Back, 1982). Thus, at the 16S rRNA level, there is a great similarity between *L. lindneri* and *L. brevis* (Yasui et al., 1997). In contrast to *L. brevis*, *L. lindneri* grows poorly on common culture media (Suzuki et al., 2008b) and was exclusively isolated from a brewing environment

(Suzuki, 2015) with a few exceptions from a wine environment (Arevalo-Villena et al., 2010). Additionally, no isolates without BSP were found to date (Storgards et al., 1998). During *L. lindneri*'s growth in beer, very short rods are built that, under certain circumstances, can pass sterile filtration (Asano et al., 2007).

***Lactobacillus (para-)plantarum*** (Orla-Jensen, 1919, Curk et al., 1996)

*L. (para-)plantarum* (= of plants) consists of long and very straight rods with rounded ends that appear singly, in pairs or short chains. The facultatively heterofermentative bacterium *L. plantarum* was isolated from dairy products, silage, sauerkraut, pickled vegetables, sourdough, cow dung, the human intestinal tract, and sewage (Kandler and Weiss, 1984). *L. paraplantarum* was isolated from beer and the human intestinal tract (Curk et al., 1996). The differentiation between *L. plantarum* and *L. paraplantarum* (as well as between the third very closely related species *L. pentosus*) is morphologically or physiologically not possible (Bringel et al., 1996). Thus, DNA-DNA hybridization provides a helpful tool to differentiate the referenced species (Curk et al., 1996, Bringel et al., 2001).

***Pediococcus damnosus*** (Claussen, 1903)

*Pd. damnosus* (= destructive, harmful) is the most frequently occurring coccoid species in the brewery (McCaig, 1983, Priest, 2003). Beers contaminated with *Pd. damnosus* can exhibit high amounts of acetoin and diacetyl (Priest, 2003). Slime formation produced by exopolysaccharides is a strain-specific characteristic (Shimwell, 1948, Priest, 2003). Like *L. lindneri, Pd. damnosus* often adheres to the culture yeast and hides itself this way (Storgards et al., 1997). It grows preferably at low temperatures between 22 and 25 °C. In many cases, *Pd. damnosus*'s growth rate is slower than that of comparable beer-spoiling bacteria. This species is exclusively found in the wine or beer environment (Back, 1994a). *Pd. damnosus* was linked to 1.2–13.0 % of bacterial spoilage incidents in the period 2010 to 2013 (Hutzler et al., 2012a, Koob et al., 2014).

#### 1.4.2.2 Less frequently occurring beer-spoiling lactic acid bacteria

In the following section, further lactic acid bacteria are listed that have less of an impact on the brewing industry because they occur more rarely or have a reduced spoilage potential. In individual cases, contamination with one of these bacteria may lead to serious product damage. For the sake of completeness they are presented in the following in combination with related literature for further information.

***Lactobacillus acetotolerans*** (Deng et al., 2014, Entani et al., 1986, Qian, 2009)

***Lactobacillus cerevisiae*** (Koob et al., 2017, Chapter C, sections 1 and 2, this study)

***Lactobacillus curtus*** (Asakawa et al., 2017)

***Lactobacillus curvatus*** (Klein et al., 1996, Dykes and Vonholy, 1994, Koort et al., 2004, Torriani et al., 1996, Back, 1981)

***Lactobacillus dextrinicus* (prior: *Pediococcus dextrinicus*)** (Back, 1978d, Haakensen et al., 2009a, Coster and White, 1964, Garvie, 1984b)

***Lactobacillus malefermentans*** (Farrow et al., 1988, Jespersen and Jakobsen, 1996, Russell and Walker, 1953a)

***Lactobacillus paucivorans*** (Ehrmann et al., 2010)

***Lactobacillus perolens*** (Back et al., 1999, Miyamoto et al., 2005)

***Lactobacillus rossiae*** (Corsetti et al.,2005, Chapter D, this study)

***Lactococcus lactis*** (Back, 1982, Back, 1994a, Smith et al., 1993, Schleifer et al., 1986)

***Leuconostoc (para-)mesenteroides*** (Garvie, 1984a, Priest, 2003, Garvie, 1983, Farrow et al., 1989, Back, 1994a, Schleifer, 2009b)

***Kocuria kristinae* (prior: *Micrococcus kristinae*)** (Priest, 2003, Back, 1981, Stackebrandt et al., 1995, Suzuki, 2015, Jespersen and Jakobsen, 1996, Kloos et al., 1974, Matoulkova and Kubizniakova, 2018)

***Pediococcus acidilactici*** (Kitahara, 1974, Barney et al., 2001, Lanthoen and Ingledew, 1996, Rouse et al., 2007, Sakaguchi, 1960, Garvie, 1984b, Ahn et al., 2017)

***Pediococcus claussenii*** (Claussen, 1903, Dobson, 2002, Suihko et al., 2003)

***Pediococcus inopinatus*** (Back, 1978a, Back, 1978b, Back, 1978c, McCaig, 1983, Priest, 2003, Lawrence, 1988, Sakamoto and Konings, 2003, Iijima et al., 2007)

***Pediococcus parvulus*** (Barney et al., 2001, Gunther et al., 1962, Werning et al., 2006, Garvie, 1984b)

***Pediococcus pentosaceus*** (Dobrogosz and Stone, 1962a, Dobrogosz and Stone, 1962b, Plengvidhya et al., 2007, Vizoso Pinto et al., 2004, Skytta et al., 1993)

### 1.4.3 Further beer-spoiling microorganisms

#### 1.4.3.1 The *Pectinatus* and *Megasphaera* genera

The beer-spoiling, anaerobic genera *Pectinatus*, *Megasphaera*, *Selenomonas*, and *Propionispira* are of great interest since their cells constitute an intermediate between gram-negative and gram-positive eubacteria (Chaban et al., 2005). Although they possess a cell wall that is typical of gram-negative bacteria, they also exhibit a very thick peptidoglycan layer as well as a plasma membrane, which is characteristic of gram-positive bacteria (Helander et al., 2004). Isolates of the specified genera cluster with gram-positive eubacteria at the 16S rRNA level (Haikara et al., 1981, Schleifer et al., 1990, Paradh, 2015). In recent years, a new class was created and established for this kind of bacteria (*Firmicutes* with gram-negative cell wall), the *Negativicutes* (Marchandin et al., 2010).

The *Pectinatus* genus (= combed bacterium) was initially described by LEE ET AL. (Lee et al., 1978, Lee et al., 1980). Cells are mobile due to peritrichous flagellation (Schleifer et al., 1990). Within this genus there are three species known as beer-spoiling species: *P. cerevisiiphilus* (= beer lover), *P. frisingensis* (= of Freising) and *P. haikarae* (= named after HAIKARA) (Schleifer

et al., 1990, Juvonen and Suihko, 2006). One further species, *P. portalensis*, was described as beer spoiling, but has not yet been isolated from the brewing environment. In addition, the species description was questioned only a few years after its establishment (Gonzalez et al., 2004, Juvonen, 2015, Vereecke and Arahal, 2008). The growth of *Pectinatus* in beer is associated with the formation of pronounced turbidity as well as with the generation of foul-smelling flavor substances such as hydrogen sulfide, propionic acid and methyl mercaptan resulting in a rotten-egg or fecal off-flavor (Back, 1994a, Back, 1979, Membré et al., 1994, Suihko and Haikara, 1990, Paradh et al., 2011, Haikara et al., 1981, Lee et al., 1978, Lee et al., 1980, Haikara, 1980, Haikara, 1985a). The effects caused by the *P. haikarae* species are less severe than the effects of the other two mentioned germ types (Voetz et al., 2010). *Pectinatus* can only grow in beers with pH values above 4.3–4.6 and moderate alcohol contents (Lawrence, 1988, Seidel-Rüfer, 1990). Small to moderate amounts of solved oxygen are tolerated, especially at low temperatures (Soberka et al., 1988, Juvonen, 2015). It is worth mentioning that the oxygen tolerance of *P. frisingensis* exceeds that of *P. cerevisiiphilus* (Haikara, 1985b). It has only recently been established that *Pectinatus* cells can be found in all stages of beer production, although living cells are preferably isolated from the filling area and from the finished beer (Juvonen, 2015, Matoulkova et al., 2012b). *Pectinatus* is particularly found in difficult-to-access areas, e.g. in gaps in the floor area or in the irrigation system (Back, 1994a, Back, 1988, Matoulkova et al., 2012b). Despite its anaerobic nature, *Pectinatus* is often transferred into the product by aerosols (Dürr, 1984) which represents a classic secondary contamination according to BACK (see Section 1.3.2) (Back, 1994a). *Pectinatus* and *Megasphaera* participate in biofilm formation together with other mixed populations (Back, 1994b). It is assumed that yeasts and further aerobic microorganisms exhaust the oxygen present within the forming biofilm in such a way that an anaerobic environment is created. These new conditions enable LAB to grow and produce lactate which can, in turn, be utilized by *Megasphaera* and *Pectinatus* (Lee, 1994, Lee et al., 1981). A slimy sheath shields the biofilm against cleaning agents and dehydration and permits the establishment of a specific environment where strictly anaerobic germs can prosper (Back, 1994b).

In the 1990s, 28 % of bacterial incidents were attributed to contaminations with *Pectinatus* spp. (Back, 1994b), but more recent studies propose a considerably smaller percentage (up to 8 %) (Hutzler et al., 2012a, Koob et al., 2014).

Despite its gram-negative outer envelope, the strictly anaerobic genus *Megasphaera* (= a big sphere) belongs, like *Pectinatus*, to the phylum of gram-positive bacteria (Schleifer et al., 1990, Stackebrandt et al., 1985). Initially, *Megasphaera* was isolated by WEISS ET AL. (Weiß, 1979) and the type species *M. cerevisiae* (= of beer) was first described by ENGELMANN AND WEISS (Engelmann and Weiss, 1985). Two further species, *M. sueciensis* (= of Swedish origin) and *M. paucivorans* (= user of only few substrates) were described in 2006 (Juvonen and Suihko, 2006). *Megasphaera* cells grow primarily in low-alcohol beers (≤ 3.5 % ethanol) and generate extreme off-odors, as is observed for *Pectinatus* (e.g. butyric acid, short-chain fatty acids, hydrogen sulfide) (Back, 1994a, Seidel, 1979, Suihko and Haikara, 2001, Haikara, 1985a, Lee, 1994, Haikara and Lounatmaa, 1987). The cells of this anaerobic bacterium are coccoid and arranged in the majority of cases in pairs and in a few cases like chains (Juvonen and Suihko, 2006, Back, 1994a). Like *Pectinatus*, which shares an ecological niche with *Megasphaera,* it is found primarily in the filling surroundings and in finished beer (Haikara and Helander, 2006). The percentage of spoilage incidents is lower than that of *Pectinatus* (between 0 and 7 %) (Back, 1987, Back, 1994b, Hutzler et al., 2012a, Koob et al., 2014).

#### 1.4.3.2 Further gram-negative beer-spoiling bacteria

Due to their minor importance for beer spoilage, the species and germ groups listed below are indicated only by name, former nomination and related literature.

***Propionispira paucivorans*** **(prior: *Zymophilus paucivorans*)** (Schleifer et al., 1990, Ueki et al., 2014, Juvonen, 2015)

***Propionispira raffinosivorans*** **(prior: *Zymophilus raffinosivorans*)** (Seidel-Rüfer, 1990, Van Vuuren and Priest, 2003, Schleifer et al., 1990, Ueki et al., 2014, Juvonen, 2015)

***Selenomonas lacticifex*** (Seidel-Rüfer, 1990, Van Vuuren and Priest, 2003, Schleifer et al., 1990, Juvonen, 2015)

***Zymomonas mobilis*** (Seidel-Rüfer, 1990, Van Vuuren and Priest, 2003, Sakamoto and Konings, 2003)

**Acetic acid bacteria** including the *Acetobacter*, *Gluconobacter* and, most recently, *Gluconacetobacter* genera (Van Vuuren and Priest, 2003, Yamada et al., 1997, Lawrence, 1988). The aerobic acetic acid bacteria (AAB) can convert ethanol to acetic acid and, therefore, generate an acidic off-taste. As a result of improved hygiene measurements and oxygen-free production technology, AAB lost their significance for beer quality, but can still be isolated from intermediate products of the brewing process. The most important role of AAB in the brewing environment is as a starter culture in biofilm formation (Paradh, 2015).

***Enterobacteriaceae*** (Van Vuuren and Priest, 2003)
This group of bacteria comprises numerous species (e.g. *Obesumbacterium proteus* (previously: *Hafnia protea*) (Priest and Hough, 1974, Paradh, 2015), *Rahnella aquatilis*, *Citrobacter freundii* (Priest et al., 1974, Priest and Hough, 1974), *Enterobacter* spp., *Serratia* spp., *Klebsiella* spp. (Back, 1994a, Van Vuuren and Priest, 2003)). As with AAB, *Enterobacteriaceae* now play a minor role in the beer production process due to improved cleaning and disinfection measurements and oxygen-free technology. *Enterobacteriaceae* can be primarily isolated from wort and yeast and their presence should be monitored, at least sporadically, for quality purposes (Paradh, 2015). AAB and *Enterobacteriaceae* still have a certain impact in the draught beer sector, especially if tap hygiene management is lacking or insufficient, and as starter cultures in biofilm formation (Bokulich and Bamforth, 2013, Quain, 2015, Riedl et al., 2017).

#### 1.4.3.3 Wild yeasts

Yeasts that are not intentionally used in the brewery (bottom or top-fermenting culture yeast), but can cause spoilage if they find their way into beer or intermediates, are called wild yeasts (Gilliland, 1971). They can be isolated from all steps of the beer production process and can trigger problems. Growth of wild yeasts, especially during fermentation, can cause turbidity and slight to severe off-flavors (Lawrence, 1988). Further effects can be problematic fermentations as well as super-attenuation by the production and secretion of glucoamylases in the finished product (Lawrence, 1988, Röcken and Schulte, 1986). Wild yeasts are a heterogeneous group consisting of numerous genera which can be classified roughly as *Saccharomyces* wild yeasts and non-*Saccharomyces* wild yeasts (Kuhle and Jespersen,

1998). Often, wild yeast cells are easily distinguished from culture yeast cells by microscopic analysis (Back, 1994a). Since wild yeasts are not the main target of this thesis, other literature sources are indicated at this point (Bokulich and Bamforth, 2013, Campbell, 2003b, Kuhle and Jespersen, 1998, Vaughan et al., 2005, Ingledew and Casey, 1982).

### 1.4.4 Bacterial classification according to brewery-specific aspects

BACK established a method which is used for the most part to classify beer-spoiling bacteria based on their spoilage potential (Back, 1994a). The spoiling germs that occur in a brewery are therefore categorized into five groups:

- Obligate beer-spoiling bacteria

Bacteria of this category are the most hazardous to beer quality. They can grow in beer without an adaption period and spoil it. In the event of occurrence, immediate counteractive measures must be taken. Example species: *L. brevis*

- Potential beer-spoiling bacteria

They can only grow in beers with reduced selective properties (compare 'hurdle theory' in Section 1.1) such as in beers with increased pH values or reduced hop acid concentrations. Bacteria that grow only after a certain adaption period also count as potential beer-spoiling bacteria. Example species: *L. casei*

- Indirect beer-spoiling bacteria

Those beer-spoilers have, as the name suggests, no direct impact on beer quality. But in case of adverse conditions in the early steps of beer production, they may cause preliminary damage to intermediate products, which cannot be corrected in later steps. Example species: *Enterobacter agglomerans*

- Indicator germs

Those germs indicate that cleaning and disinfection management is incomplete or faulty. They can also point to possible biofilm formation as they belong to the first microorganisms settling in it (= biofilm starter cultures). Within these biofilms, indicator germs can, in later steps, be associated with beer-spoiling bacteria. Example species: *Acetobacter pasteurianus*

- Latent germs

Latent germs are described as those that are only temporarily found in beer and have no impact on beer quality. They survive for a short period of time, but quickly become undetectable. Example: molds

A clear and detailed presentation of these spoilage categories is given by BOHAK (Bohak, 2015). According to HUTZLER ET AL. (2012b, 2013), the individual beer-spoiling species can be characterized on the basis of their beer-spoilage potential, hop tolerance, their tendency towards primary or secondary contamination and their potential to form slime in beer as follows (see Table 1):

Table 1: Overview of beer-spoiling species and their brewery-specific properties (excerpted from Hutzler et al. 2012b and Hutzler et al. 2013)

| Species name | Rods / Cocci | Gram reaction | Beer-spoilage potential | Hop tolerance | Primary / Secondary contamination | Potential for slime formation (brewery isolates) |
|---|---|---|---|---|---|---|
| *L. acetotolerans* | R | + | + | +/- | s>p | − |
| *L. backii* | R | + | ++ | ++ | p>s | − |
| *L. brevis* | R | + | ++ | ++ | s>p | + |
| *L. (para-)buchneri* | R | + | + | + | p>s | + |
| *L. (para-)casei* | R | + | + | +/- | s>p | − |
| *L. coryniformis* | R | + | + | +/- | s>p | − |
| *L. (para-)collinoides* | R | + | ++ | ++ | s>p | − |
| *L. lindneri* | R | + | ++ | ++ | p>s | − |
| *L. perolens* | R | + | + | +/- | s>p | − |
| *L. paucivorans* | R | + | ++ | ++ | p | − |
| *L. plantarum* | R | + | + | +/- | s>p | − |
| *L. rossiae* | R | + | + | +/- | s>p | + |
| *Lac. lactis* | C | + | -/+ | -/+ | s>p | − |
| *Leuc. (para-)mesenteroides* | C | + | -/+ | -/+ | s>p | + |
| *M. cerevisiae* | C | - | ++ | ++ | s | − |
| *M. paucivorans* | C | - | ++ | ++ | s | − |
| *M. sueciensis* | C | - | + | ++ | s | − |
| *Micrococcus kristinae* | C | + | -/+ | +/- | s | − |
| *Pd. damnosus* | C | + | ++ | ++ | p>s | − |
| *Pd. claussenii* | C | + | + | +/- | p>s | v |
| *Pd. inopinatus* | C | + | + | +/- | p>s | − |
| *P. cerevisiiphilus* | R | - | ++ | ++ | s | − |
| *P. frisingensis* | R | - | ++ | ++ | s | − |
| *P. haikarae* | R | - | ++ | ++ | s | − |
| *Zymomonas mobilis* | R | - | -/+ | ++ | p | − |

*L.= Lactobacillus, Lac.= Lactococcus, Leuc.= Leuconostoc, M.= Megasphaera, Pd.= Pediococcus, P.= Pectinatus*

Indirect or potential beer-spoiling bacteria with minor importance: *Bacillus* spp., *Citrobacter freundii*, *Enterobacter* spp., *Klebsiella* spp., *L. curvatus*, *L. malefermentans*, *Obesumbacterium proteus*, *Pantoea agglomerans*, *Pd. acidilactici*, *L. dextrinicus*, *Pd. pentosaceus*, *Rahnella* spp., *Selenomonas lacticifex*, *Serratia* spp., *Zymophilus* spp.

R= rod-shaped, C= coccoid, ++= very high / strong, += high / strong or positive, +/-= positive tendency or majority of strains or adaption necessary, v= variabel, -/+= negative tendency or minority of strains or strong adaption necessary, − = low or negative or no growth, p = primary contamination, s= secondary contamination, s>p= more cases of secondary contamination observed, p>s = more cases of primary contaminations observed

Assessing Table 1, species with a very high BSP ('++') correspond more or less to the category 'obligate beer-spoiling'. Species with a high potential ('+') and those tending towards a high BSP ('+/-') correspond to the 'potential beer-spoiling' category and subsequently, species tending towards a low BSP ('-/+') correspond to the 'indirect beer-spoiling' classification.

The above-mentioned brewery-specific classifications as well as the categorization as primary and secondary contaminants are discussed in more detail later on. Additional, recently described beer-spoilers will be inserted into this table and other species will be deleted due to various factors.

## 1.5 Detection and identification of beer-spoiling bacteria

### 1.5.1 Detection

A specific brewing microbiological task is the detection of a small number of contaminating cells within a proportionally large volume. The first step of quality control is typically anaerobic enrichment, with additional physical sample reduction where applicable (e.g. by centrifugation), up to sufficient cell numbers in appropriate nutrient media. Simultaneously, non-spoilage LAB have to be repressed while specific and hard-to-cultivate spoilage strains need to be supported (Bergsveinson and Ziola, 2017). Enrichment should be effected anaerobically, since the most hazardous BSB are of strictly anaerobic or facultatively anaerobic nature, and should take place at temperatures of 25 to 28 °C, which is considered to be optimal for bacterial growth.

The specificity of the nutrient medium is of great importance, since numerous germ types can be found within a brewery sample, even some that do not pose an immediate danger to beer quality such as culture yeast or latent germs. Furthermore, the potential and obligate beer-spoiling microorganisms to be detected are often very adapted to the inhospitable, but unrivalled medium of beer and some even need the beer-specific stress factors for their growth (example: *L. lindneri*). For instance, it was investigated that bacteria adapted to the brewing environment exhibit a considerably lower optimum pH value than other LAB (Suzuki, 2011b). It was also determined that certain gene sequences, enzymes or the preferred sugar spectrum differ between beer-spoiling and non-spoiling strains (Nakakita et al., 2003, Takahashi et al., 1999, Rainbow, 1981). In combination with the fact that some beer-spoiling species, e.g. *L. backii*, were so far exclusively isolated from beer or its intermediates, a close relationship with this very specific medium cannot be denied.

Another requirement in the brewery laboratory is the processing of different sample types which arise in the course of beer production. The later a sample is drawn from the production chain (for example, filling samples), the more reliable the detection has to be and the smaller the microorganism number that has to be detected (Jespersen and Jakobsen, 1996). Additionally, the different sample types of the different brewery sections such as the filtrate and non-filtrate area (wort, highly concentrated yeast samples or finished beer, just to name a few) require different process procedures for the qualitative or quantitative detection, but always a preferably fast and secure detection of the contaminating microorganisms.

Over the decades numerous methods and nutrient media have been developed to detect lactic acid bacteria with or without BSP (Priest, 2003, Casey and Ingledew, 1981, Holzapfel, 1992, Jespersen and Jakobsen, 1996, Riedl et al., 2017). All those methods and media achieve the main criteria of reliability, speed, recovery rate, easy handling, and selectivity more or less satisfactorily. Many methods were developed for the brewing microbiological laboratory to meet the requirements of decreasing time to results and more detection reliability, which could not be established in most cases. Automated turbidimetry (Haikara et al., 1990), microcolony method (Asano et al., 2009) and the application of monoclonal chemiluminescence enzyme immunoassays in combination with a CCD camera (March et al., 2005) may serve as examples.

Despite the comparably long cultivation time, specific nutrient media with subsequent microscopic analysis have asserted themselves in the brewing microbiology laboratory due to their low costs, easy handling and limited need for specialized staff (Novy et al., 2013). A few

media that were developed for the detection of BSB are displayed in the following paragraph in combination with related literature:

MRS (De Man et al., 1960), NBB (Back, 1994a, Bohak et al., 2012), modified NBB (Nishikawa, 1985), VLB-S7 (Emeis, 1969), UBA (Kozulis and Page, 1968), Raka-Ray (Matsuzuwa et al., 1979), KOT (Taguchi et al., 1990), ABD medium (Suzuki et al., 2008b), BMB medium (Barney et al., 1990), SAB medium (Hammes et al., 1992), TJA (Holzapfel, 1992) and wheat beer medium (Riedl et al., 2017).

Pre-evacuated NBB medium (Henriksson and Haikara, 1991), pre-evacuated and/or modified MRS medium (Gares et al., 1993, Watier et al., 1995, Matoulkova et al., 2012a) or SMMP medium (Lee, 1994) are recommended media for the detection of the strictly anaerobic germs *Pectinatus* and *Megasphaera*.

Given the broad variety of beer-spoiling microorganism flora and sample types in a brewery, the method of choice is to combine different nutrient media and their preparations (broth/agar/concentrated medium). No single medium is suitable for detecting all the different kinds of beer-spoilage germs (Taskila et al., 2011, Taskila et al., 2010). It is important to maintain the type and manufacturing method of the selected media to make the results comparable and to be able to statistically analyze the occurrence of a certain germ spectrum, though common isolates that grow fast and easy on routine media skew incident reports. In all of the studies that form the basis of this thesis, MRS agar and broth were used for the non-selective enrichment of LAB, NBB agar and broth for the selective enrichment of beer-spoiling LAB, and micro-inoculum agar and broth (MIB; Difco™, Germany) for the detection of the gram-negative beer-spoiling *Pectinatus* und *Megasphaera* genera.

The search for easy-to-handle, selective, fast, and low-cost methods to detect the limited spectrum of obligate and potential BSB is still ongoing in the brewing microbiology laboratory. A new application that fulfills these specified requirements is the lateral-flow, 16S rRNA PCR-based Milenia Biotec dipstick method (Breitbach et al., 2015). This method also meets the need for low cell count as a detection limit, low microbiological background knowledge and is able to process all brewery samples.

Further brewery-related information about sampling techniques, microorganism enrichment, nutrient media, their application and manufacturing is given elsewhere (Back, 1994a, Back, 2000, Bohak, 2015, Strachotta, 2003, Suzuki, 2011, Koob et al., 2015).

### 1.5.2 Identification

The term ‘identification’ designates the process of deciding whether an unknown organism belongs to a certain, pre-defined group (Holzapfel and Wood, 2014). For a long time it was assumed that every gram-positive, catalase-negative rod that was isolated from yeast, beer or the brewing surrounding was of the *Lactobacillus* genus and further identification at the species level was unnecessary since lactobacilli generally trigger beer spoilage (Priest, 2003). But for some purposes it is useful to identify isolates at a species level, especially with the knowledge now that not all species, or even all strains of one species, exhibit the same BSP (Priest, 2003, Back, 1981). Thus, it is important to consider the difference between the academic researchers’ interest and the brewers’ need for practical information on the spoilage germs (Bergsveinson and Ziola, 2017).

### 1.5.2.1 Phenotypic analysis methods

The differentiation of lactic acid bacteria was, in the past, achieved by a combination of different phenotypic analysis methods (Mohania et al., 2008), some of which are very elaborate and not always reliable. A selection of phenotypic differentiation methods is given here (Priest, 2003, Back, 1994a, Dicks and Endo, 2009):

- Cell and colony morphology

  Due to the small variety of forms and the varying expression of cell and colony morphology depending on culture age and cultivation conditions, those analyses are now of minor importance.

- Determination of sugar spectrum

  A system of fifty sugars and sugar substitutes is needed to identify LAB (for example: API CHL 50 system, bioMérieux). The determination of the ability to form acid from different carbohydrates is still required for the new description of *Lactobacillus* species (Stackebrandt et al., 2002, Back, 1994a, Back, 2000). However, the software (Apiweb$^{TM}$) that belongs to the specified API CHL 50 system is not suited to differentiate BSB species.

- Gas formation from glucose and gluconate to determine fermentation pathway
- Arginine hydrolysis
- Lactate configuration
- Growth tolerances with regard to temperature, alcohol content, NaCl content and pH value
- Voges-Proskauer test

### 1.5.2.2 Chemotaxonomic analysis methods

At present, phenotypic methods are not used exclusively because metabolic groupings are not reliable (Mattarelli et al., 2014). It is also important to note that phenotypic classification does not match rRNA-based phylogeny in the case of lactobacilli (Vandamme et al., 1996). Standard practice is to combine phenotypic, chemotaxonomic and genotypic methods. This enables multivariate or polyphasic analysis for species differentiation, the classification of unknown isolates to an existing species, or the description of a new species or subspecies (Vandamme et al., 1996, Schleifer, 2009a, Holzapfel and Wood, 2014, Mattarelli et al., 2014). Phenotypic analyses become increasingly less important within these investigations, while chemotaxonomic and, in particular, genotypic methods gain in significance.

Possible chemotaxonomic analyses in combination with related literature are listed here:

- MALDI-TOF MS (Kern et al., 2013, Kern et al., 2014b, Wieme et al., 2014, Wenning et al., 2014, Sandrin et al., 2013)
- Whole cell fatty acid analysis (Beverly et al., 1997)
- Fourier transform infrared spectroscopy (FTIR) (Curk et al., 1994, Wenning and Scherer, 2013)
- SDS page (Gancheva et al., 1999)
- Determination of peptidoglycan type (Schleifer and Kandler, 1972)

- Determination of polar lipids (da Costa et al., 2011)
- Electrophoretic mobility of enzymes (Scolari and Vescovo, 2004)

Some disadvantages of the methods listed above are high purchasing and maintenance costs, the requirement of high initial cell concentrations, subsequently long enrichment periods and the need for professional, specially trained staff to perform these procedures. Additionally, some of the mentioned methods are not sufficiently specific to separate the beer-spoiling species and are therefore unsuitable for identification or classification purposes.

#### 1.5.2.3 Genotypic analysis methods

In the modern brewing microbiological laboratory, identifications at the species level are nearly exclusively based on genotypic methods. The determination of evolutionary and phylogenetic relations between single species was revolutionized by the comparison of specific nucleic acid sequences (Woese, 1987) that were previously multiplied using the PCR (polymerase chain reaction) method. The main principles of PCR are illustrated in detail, for instance, by SIEGRIST ET AL. (2015). The sequence comparison of the small ribosomal subunit, called 16S rRNA, which was first established in 1991 by COLLINS ET AL., is an especially useful tool for determining the degree of relation (Collins et al., 1991). It is assumed that the greater the number of different mutations between the specific sequences of two individuals, the more time has elapsed since the separation from the common ancestor. In other words, the smaller the difference between the same gene of two individuals, the closer the individuals are related (Priest, 2003).

Figure 1 shows how LAB species are organized into subgroups according to 16S rRNA sequence comparison and how beer-spoiling LAB species are distributed between these subgroups. An example of genotypic analysis is the 16S rRNA phylogenetic tree conducted by SALVETTI ET AL. (2012), which includes all the LAB species existing in 2012.

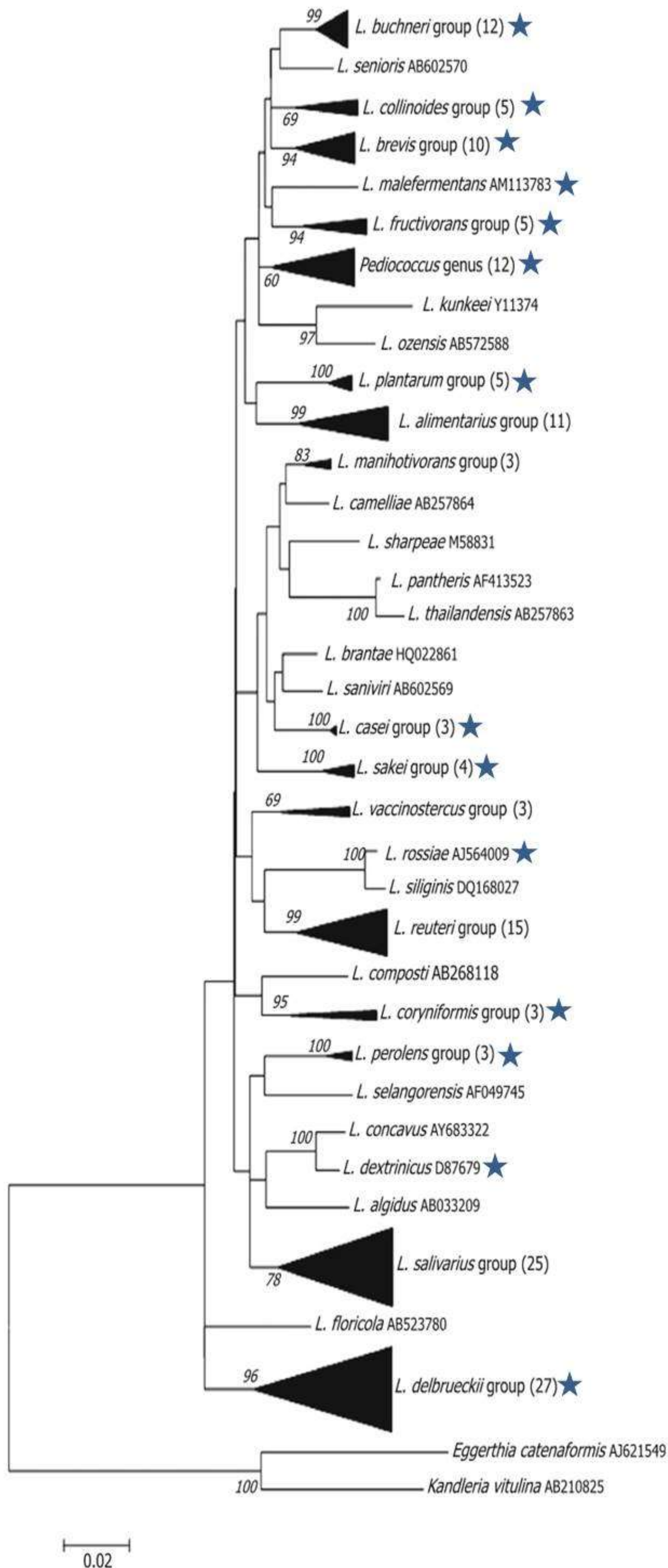

99
L. buchneri group (12)
L. senioris AB602570
69
L. collinoides group (5)
94
L. brevis group (10)
L. malefermentans AM113783
94
L. fructivorans group (5)
60
Pediococcus genus (12)
L. kunkeei Y11374
97
L. ozensis AB572588
100
L. plantarum group (5)
99
L. alimentarius group (11)
83
L. manihotivorans group (3)
L. camelliae AB257864
L. sharpeae M58831
L. pantheris AF413523
100
L. thailandensis AB257863
L. brantae HQ022861
L. saniviri AB602569
100
L. casei group (3)
100
L. sakei group (4)
69
L. vaccinostercus group (3)
100
L. rossiae AJ564009
L. siliginis DQ168027
99
L. reuteri group (15)
L. composti AB268118
95
L. coryniformis group (3)
100
L. perolens group (3)
L. selangorensis AF049745
100
L. concavus AY683322
L. dextrinicus D87679
L. algidus AB033209
78
L. salivarius group (25)
L. floricola AB523780
96
L. delbrueckii group (27)
Eggerthia catenaformis AJ621549
100
Kandleria vitulina AB210825
0.02

Figure 1: Phylogenetic tree showing the relationship between *Lactobacillus* and *Pediococcus* species based on 16S rRNA gene sequences (adopted from (Salvetti et al., 2012) and extended by labelling groups harboring frequently and less frequently occurring beer-spoiling LAB species from Section 1.4.2); bar = number of substitutions per site; subgroups containing more than two species are condensed and marked with the name of the first species described; ★ = subgroup including BS species or single BS species.

Two segments, the 16S rRNA coding for the small ribosomal subunit in prokaryotes and the 18S rRNA coding for the same organelle in eukaryotes, are ideal for identification purposes since they exhibit an advantageous sequence length, mutate at a constant rate, are universally present in cells and do not excessively participate in horizontal gene transfer (Schleifer and Ludwig, 1996, Priest, 2003, Ludwig and Schleifer, 1994). But in some cases, 16S rRNA sequence comparison lacks sufficient discriminative power for closely related species. In those cases, it is necessary to use sequencing of protein-coding genes such as housekeeping genes or fingerprinting techniques (Stackebrandt and Ebers, 2006, Coenye et al., 2005). *Hsp60*, *recA*, *pheS*, *rpoA*, *rpoB*, *gyrB*, *tuf*, and 16S-23S internally transcribed spacer (ITS) region may serve as examples of phylogenetic marker genes that were established to predict a genome relationship (Mattarelli et al., 2014, Tanigawa and Watanabe, 2011, Naser et al., 2007, Coenye et al., 2005).

Further modern genotypic methods for the identification and differentiation of beer-spoiling bacteria are listed below.

- DNA base composition (also known as determination of GC content; important for genus and species description, apart from that not specific enough) (Tamaoka, 1994)
- Randomly amplified polymorphic DNA (RAPD) method (Welsh and McClelland, 1990, Williams et al., 1990)
- Ribotyping (Koivula et al., 2006, Motoyama et al., 1998)
- Fluorescence in-situ hybridization (FISH) (Amann et al., 1995, Bottari et al., 2006)
- Repetitive element palindromic (rep) PCR technique (Gevers et al., 2001, Louws et al., 1998, Versalovic et al., 1998, Versalovic et al., 1991, Versalovic et al., 1994, Lupski and Weinstock, 1992)
- Amplified fragment length polymorphism (AFLP) (Vos et al., 1995)
- Restriction fragment length polymorphism (RFLP) (Gonzalez et al., 2005)
- Terminal restriction fragment length polymorphism (TRFLP) (Liu et al., 1997)
- Denaturing gradient gel electrophoresis (DGGE) (Lopez et al., 2003, Manzano et al., 2005, Tsuchiya et al., 1994, Temmerman et al., 2004)
- Multilocus sequence analysis/typing (MLSA / MLST) (Cooper and Feil, 2004)
- Multiple locus variable number of tandem repeats analysis (MLVA) (van Belkum, 2007)
- Intergenic spacer region (ISR)-PCR (Santos and Ochman, 2004)
- Amplified rDNA restriction analysis (ARDRA) (Ventura et al., 2000)
- Average nucleotide identity (ANI) (Konstantinidis and Tiedje, 2005, Konstantinidis et al., 2006)
- Chromosomal DNA-DNA hybridization (DDH) (Wayne et al., 1987, Stackebrandt and Goebel, 1994, Tindall et al., 2010, Huss et al., 1983)

DDH was considered to be the gold standard of taxonomic analysis for many years as it provides an objective threshold of 70 % DDH for species demarcation (Tindall et al., 2010, Wayne et al., 1987, Rossello-Mora, 2006). 97 % (Stackebrandt and Goebel, 1994) and, more recently, 98.65 % 16S rRNA sequence similarity (Kim et al., 2014a) were determined to

correspond to 70 % DDH. 16S rRNA sequence comparison is widely used due to its simplicity, cost-effectiveness and the availability of innumerable sequences in numerous databases. As the discriminatory power of 16S rRNA comparisons is limited, as stated above, and DDH is labor-intensive and prone to error, researchers are looking for a new genotype-based standard for species delineation (Gevers et al., 2005). DDH is going to be replaced by whole-genome sequencing (WGS) methods with the determination of ANI as a proposed new gold standard (Richter and Rossello-Mora, 2009, Konstantinidis and Tiedje, 2005, Goris et al., 2007). 96 % ANI corresponds to 70 % DDH (Richter and Rossello-Mora, 2009). In some cases, the concatenation of multiple housekeeping gene comparisons is as accurate as whole-genome sequence comparisons (Bohm et al., 2015).

Genotypic analysis methods that are currently used in the brewery microbiological lab for beer-spoiling LAB are displayed by STORGARDS ET AL. (2006), BOKULICH ET AL. (2012) and BOKULICH AND MILLS (2012). Methods for strictly anaerobic beer-spoiling bacteria can be investigated in the thesis of JUVONEN (2009). Overviews of modern PCR-based methods in brewing microbiology are given by BOHAK (2015) and SIEGRIST ET AL. (2015). The basic concept of modern brewing microbiology is the assumption of and search for a genetic variation between spoilage and non-spoilage isolates and between isolates derived from different isolation sources (Bergsveinson and Ziola, 2017).

### 1.5.3 Determination of beer-spoilage potential

Besides the sole detection of germs, the main target of quality control is to quickly give feedback on the beer-spoilage potential of the contaminants. The most reliable test for this purpose is the inoculation of the relevant strains into beer and the subsequent assessment of increasing turbidity which is very time-consuming (Suzuki et al., 2006b). Molecular biological methods are useful in overcoming this disadvantage. In some cases, the beer-spoilage property is species specific and, thus, identification at the species level is sufficient (e.g. *L. backii* or *Pectinatus* spp.). However, if beer-spoiling and non-spoiling strains exist within a single species (e.g. *L. brevis* (Nakagawa, 1978, Suzuki et al., 2006b)), species-independent marker genes have to be applied. The species-independent marker genes are also useful for the detection of beer-spoiling bacteria that have not been described or not yet been described as beer-spoilers. Since hop resistance is suggested to be the key characteristic necessary for growth in beer, the genes related to hop resistance were analyzed to find these marker genes.

The two marker genes that are the most important in terms of the predictability of BSP are *horA* and *horC* (see Section 1.2.1) (Suzuki et al., 2006b, Haakensen, 2009). Further genes associated with BSP are *hitA* (Hayashi et al., 2001), *bsrA* and *bsrB* (Haakensen et al., 2009b), as well as ORF5 (Suzuki et al., 2004c). In search of novel diagnostic marker genes (DMG), it was determined that the *fabZ* gene has shown a significant link to BSP (Behr et al., 2016). It is part of a plasmid encoded complete fatty acid biosynthesis (FAS) cluster and necessary for fatty acid chain elongation. Recent studies show that brewery-specific DNA is mainly found within the plasmidome of the bacterial cell (Behr et al., 2016, Geißler, 2016). In the case of *fabZ*, which plays a major role for *Pediococcus damnosus*, which lacks a complete chromosomal fatty acid biosynthesis, the presence of this gene differentiates beer-spoiling and non-spoiling strains.

The significance of *horA* and *horC* is still undisputed. However, beer-spoiling isolates that exhibit neither of these genes were discovered along with non-spoiling isolates that possess at least one of them, creating the need for further marker genes (or a combination of genes) to reliably differentiate beer-spoiling and non-spoiling strains (Munford et al., 2017, Haakensen et al., 2009b, Suzuki et al., 2006b, Sakamoto et al., 2001). In a novel comprehensive work, nine potential diagnostic marker genes were found for beer-spoiling bacteria, the reliability of which has to be proven in the years ahead (Geißler, 2016).

## 2. AIMS

The group of beer-spoiling bacteria is limited to a few species with the ability to tolerate the adverse conditions of beer milieu, to grow in beer and to spoil it according to their beer-spoilage potential by metabolic products. However, this group is subject to variation as some species lose their significance as beer spoilers from time to time and others are added. Technological changes in beer production can result in bacterial growth being inhibited and sensitive species disappearing from the brewing environment. Some species are excluded from the spectrum of beer-spoiling bacteria by taxonomic re-evaluation and subsequent classification as another species (e.g. *L. frigidus*). The BSB spectrum may be extended if the alteration of technological process parameters positively affects the growth of certain bacteria (e.g. oxygen reduction during beer production leads to the increase of anaerobic species) or if the basic microbiological hurdles change when new beer styles are created or specific aroma-active compounds are added to the beer (ideally outside the scope of the German purity law). Thus, the most considerable factors for BS group expansion are new descriptions of bacterial species with BSP (e.g. *L. paucivorans*) and existing species acquiring BSP over time with subsequent adaption to the beer milieu (e.g. *L. rossiae*). This last part is presumably attributed to the uptake of mobile genetic elements, i.e. plasmids, via HGT whereupon certain resistance genes, in the beer milieu preferred hop resistance genes, are encoded that ensure the bacteria's survival within an inhospitable environment (Bergsveinson et al., 2015a).

To establish microbiological QC and practical detection and identification methods in the brewing microbiological laboratory, it is a basic requirement to be up to date with the spectrum of microorganisms with BSP. It is equally significant to understand the brewery-related characteristics of those germs in combination with their specific spoilage frequency and preferences and to translate the acquired information about spoiling microorganisms into QC detection and identification methods.

There are several overviews on the actual composition of the group of BSB (Back, 1981, Bhandari et al., 1954, Hill, 2009, Lawrence, 1988, Nakagawa, 1978, Rainbow, 1973, Rainbow, 1981, Suzuki, 2011, Hutzler et al., 2012b, Ault, 1965, Campbell, 2003a). However, studies on the frequency of spoilage incidents of the individual species are scarcer. Many researchers in the field of brewing microbiology still refer to early studies conducted by BACK (1980, 1988, 1994b) as there are no large-scale studies. To provide current overviews, thousands of routine samples from the brewing microbiological laboratory of the Research Center Weihenstephan for Brewing and Food Quality (FZW BLQ) were evaluated for spoilage incidents triggered by BSB species (Hutzler et al., 2012a, Koob et al., 2014). A study on the percentage frequency of occurrence of the BS species from 2010 - 2016 is one component of this thesis. 13,000 samples were analyzed for this purpose using PCR-based methods with subsequent identification via melting curve analysis for the presence of BSB. A detailed description of the real-time PCR method and the melting curve analysis is given by HOMANN ET AL. (Homann et al., 2002). Furthermore, the species-related spoilage incidents were evaluated statistically for the production step of the brewing process and for the specific beer type from which the contaminants were isolated. In this way, specific growth tendencies were assumed to be linked to certain BS species.

Another part of this work focuses on an improved LAB-detection method in samples that contain yeast, which pose difficulties in the brewing microbiological laboratory. The challenge

is the required detection of a small number of contaminating bacteria within a considerably higher number of yeast cells without the possibility to physically concentrate the target germs. Additionally, active yeast cells suppress the growth of bacteria, which often results in the contaminants being detected at a later stage, for example, in the culture yeast processing plant or propagation tanks. Thus, the reliable and, simultaneously fast detection of contaminating bacteria in any sample type is one of the main targets of microbiological QC, as stated above. A method was developed that uses the yeast-inhibiting property of the natural antibiotic natamycin to reduce the valuable time taken to detect contaminating LAB. It was developed in order to be low cost, easy to handle and easy to integrate into the daily laboratory routine.

In several cases, routine analysis in the brewing microbiological laboratory, which consists of culture-based enrichment of contaminants in suitable media and subsequent microscopic analysis, is completed by species-level identification using molecular biological methods. One often-used identification method is the PCR method for amplifying the 16S rRNA gene followed by identification using melting curve analysis in real-time, in short, real-time PCR method. There are different kinds of real-time PCR kits that cover the most prominent beer-spoiling bacteria and which are more or less sufficient. Since microbiological QC and, consequently, suitable identification methods must cover the whole range of BSM, it is rare if contaminants identified as beer-spoiling bacteria in preliminary steps occur that cannot be identified via the real-time PCR method. In 2013 and 2014, three isolates were obtained from a brewery that could not be identified via the real-time PCR method. In the following steps, the three isolates were phenotypically, chemotaxonomically and genotypically demarcated from closely related beer-spoiling species. Thereafter, they were examined to determine if they could be assigned to any existing LAB species.

On occasion, LAB species derived from other food acquire beer-spoilage ability and can be isolated from spoiled beer or intermediate samples. To achieve one of the aims of brewing microbiological QC to keep up to date with the spectrum of BSM, it is helpful to determine frequency, the degree of BSP and several beer-related characteristics such as the presence of prominent hop-resistance genes. In 2005, *L. rossiae* was isolated from sourdough and described by CORSETTI ET AL. (Corsetti et al., 2005). As early as 2010, one *L. rossiae* beer-spoilage incident was reported by HUTZLER ET AL. (2012a) followed by increasing numbers of beer-spoilage incidents. As *L. rossiae* isolated from sourdough revealed to be an extremely versatile species from a phenotypic and genotypic perspective (Di Cagno et al., 2007, Scheirlinck et al., 2009), it was investigated if *L. rossiae* BS isolates matched this versatility and if BS isolates could be demarcated from sourdough isolates.

# 3. RESULTS (Thesis publications)

## 3.1 Paper Summaries

CHAPTER A Statistical evaluation of beer spoilage bacteria by real-time PCR analyses from 2010 - 2016

Reproduced with permission from Schneiderbanger, J., Grammer, M., Jacob, F. and Hutzler, M. 2018. Statistical evaluation of beer spoilage bacteria by real-time PCR analyses from 2010 – 2016. Journal of the Institute of Brewing 124 (2), 173 – 181.

---

Large-scale studies presenting the frequency of individual beer-spoiling species are rare as few researchers have the opportunity to consult a high number of contaminated samples from different breweries, companies and countries. The brewing microbiological laboratory of the Research Center Weihenstephan for Brewing and Food Quality analyzes thousands of samples annually for the presence of beer-spoiling microorganisms. The evaluation of the analyses of seven consecutive years (2010 – 2016) considering contaminating species, brewery, kind of contamination (primary or secondary), and kind of contaminated beer type (bottom-fermented or top-fermented) provides a detailed overview of the spectrum of BSM and the changes to these over time, which can provide useful information for brewers and microbiological staff. The samples were either screened for obligate and potential beer-spoiling germs or the contaminants were identified by melting curve analysis, depending on the customers' specification and irrespective of the degree of spoilage.

The individual beer-spoiling species and groups were evaluated for their absolute frequency (findings) and for the percentage of spoilage incidents, which was defined as the occurrence of one species in one brewery within a period of six months. Additionally, the samples were analyzed using a $\chi^2$ test (chi-squared test) for the individual BS species' occurrence in early or in late stages of the production process as well as for the occurrence in bottom or top-fermented beer styles. It was thereby determined if the analyzed species and groups were suspected as being primary or secondary contaminants or were more likely found in the top or bottom-fermented sector (significance level of 0.1 and 0.05). The species and groups that differed significantly from the normal distribution (level 0.05) were *L. (para-)casei* (primary contaminant), *L. lindneri*, *L.* group, *M. cerevisiae* and *P.* group (secondary contaminants). At the same level of significance, *L. brevis* (top-fermented sector), *Pd. damnosus*, *P.* group, *M. cerevisiae*, *L. perolens/harbinensis*, and *L. lindneri* (bottom-fermented sector) deviated from the normal distribution. The $\chi^2$ test provides an easy tool to substantiate empirical values with statistical certainty at different significance levels. The results uncovered practical information about the individual BS species' growth preferences.

---

PARTICIPATION Schneiderbanger, J.: Main author, literature research, statistical evaluation; Grammer, M.: Supply of PCR data; Jacob, F.: Project supervision; Hutzler, M.: Corresponding author, critical content review

## CHAPTER B Enhanced cultivation of beer spoilage bacteria by enforced yeast suppression

Reproduced with permission from Schneiderbanger, J., Schneiderbanger, H., Jacob, F. and Hutzler, M. 2017. Enhanced cultivation of beer spoilage bacteria by enforced yeast suppression. Brewing Science 70, 142 - 147.

One of the main problems of brewing microbiology is the requirement to detect a small number of beer-spoiling bacteria in the different sample types that occur during beer production within large volumes and as quickly as possible. Irrespective of the method used (culture based or molecular biological), it is highly difficult to detect BSB in samples that contain yeast as the small number of contaminants 'hides' within the high number of yeast cells (e.g. $10^2$ cells per mL bacteria in $10^8$ cells per mL yeast). The fact that yeast, especially vital yeast, suppresses the growth of bacteria is a further challenge for fast detection. It must also be noted that it is unfeasible to mechanically concentrate spoiling germs as bacteria and yeast cells cannot be separated in this way. The only possibility for microbiological QC is the repeated cultivation and incubation in specific media, which can take weeks.

Natamycin, a molecule produced by *Streptomyces natalensis*, selectively kills yeast cells while bacteria are unaffected. Though the mechanism of action is not fully understood, the efficiency of natamycin, even to very high yeast cell numbers, is remarkable. Natamax®, which is composed of natamycin and lactose, is a natural antimycotic used to kill yeast and molds during the production of unsterile food. This study investigated if the adding of Natamax® killed yeast concentrations typical of pitching yeast and the exposure time that would be needed to entirely eliminate yeast. Furthermore, it was examined if adding 5 g/L Natamax® to samples that contain yeast promotes the growth of six obligate and potential beer-spoiling species and results in higher bacterial cell numbers (cultural approach) or in smaller Ct (threshold cycle) values (molecular biological approach) compared with samples without Natamax®.

Within 24 h, 5 g/L Natamax® was able to kill 100 % yeast cells in a concentrated sample ($10^8$ cells per mL). The cultural approach showed that the growth was promoted of four out of six BSB species whereas one species could not grow under the test conditions and one (the strongest beer spoiler) was not affected by the suppressing force of the yeast. All six BSB species were detected at higher concentrations using the molecular biological method when Natamax® was added compared with samples without Natamax® addition. The developed method is cost-effective, easy-to-handle and easily integrateable into daily laboratory routine, simultaneously providing faster results.

PARTICIPATION Schneiderbanger, J.: Main author, executing scientist, data analysis; Schneiderbanger, H.: Literature research, data evaluation; Jacob, F.: Project supervision; Hutzler, M.: Corresponding author, critical content review

CHAPTER C Detection of a new bacterial species with beer-spoilage potential

Section 1 *Lactobacillus* sp. brewery isolate: A new threat to the brewing industry?

Reproduced with permission from Koob, J., Jacob, F., Methner, F.-J. and Hutzler, M. 2016. *Lactobacillus* sp. brewery isolate: A new threat to the brewing industry? Brewing Science 69, 42 - 49.

In 2013 and 2014, three bacterial isolates were found in spoiled beer samples that were not identifiable with the conventional real-time PCR methods executed at FZW BLQ. 16S rRNA gene sequence comparison revealed the nearest neighbors to be *L. brevis* and four species not related to the brewing industry, one of them being *L. parabrevis* which was reclassified from *L. brevis* in 2005. A multivariate analysis was conducted with two type strains (*L. brevis* and *L. parabrevis*) and one highly beer-spoiling *L. brevis* isolate to determine if the unknown isolates belonged to one of the specified species or if it differed significantly from them.

For this purpose, genotypic (16S rRNA gene sequence, *rpoA* and *pheS* housekeeping gene comparisons, DNA-DNA hybridization) and phenotypic analyses (carbohydrate fermentation pattern, temperature, acid, salt and alcohol tolerance) were carried out. To determine beer-spoilage ability, the isolates were incubated in five different beer types with subsequent physico-chemical analysis of the contaminated beers and the presence of three prominent genes proposed to impart hop resistance (*horA*, *horC*, *hitA*) was examined.

The three unknown isolates could not be distinguished from *L. brevis* or *L. parabrevis* based on 16S rRNA gene similarity, but showed sufficient similarity between each other to consider them to be strains of the same species with one as the working strain (strain 2301). The sequences of the housekeeping genes *rpoA* and *pheS* and DNA-DNA hybridization results showed less similarity than necessary for classification into the same species. The carbohydrate fermentation pattern and further physiological properties did not provide any justification for assignment to or exclusion from the species in question.

The examined isolate grew in four of the five tested beer types degrading alanine, acetaldehyde and the organic acids pyruvate and citric acid in significant amount and increasing the concentrations of acetic acid and lactic acid. Only the lager beer which displayed the highest antimicrobial hurdles of the tested beers was not spoiled by the unknown isolate, though two out of three hop resistance genes were detected using a real-time PCR method specifically established in this study.

PARTICIPATION Koob, J.: Main author, executing scientist, data evaluation; Jacob, F.: Project supervision; Methner, F.-J.: Taxonomy expertise, critical content review; Hutzler, M.: Corresponding author, critical content review

CHAPTER C Detection of a new bacterial species with beer-spoilage potential

Section 2 *Lactobacillus cerevisiae* sp. nov., isolated from a spoiled brewery sample

Reproduced with permission from Koob, J., Jacob, F., Wenning, M. and Hutzler, M. 2017. *Lactobacillus cerevisiae* sp. nov., isolated from a spoiled brewery sample. International Journal of Systematic and Evolutionary Microbiology 67, 3452 - 3457.

After exclusion from the species *L. brevis* and *L. parabrevis*, another multivariate analysis was carried out to investigate the unidentified strain 2301 obtained from a turbid and slightly acidified bright beer tank sample to determine if it could be assigned to two closely related species with no connection to the brewing environment, *L. yonginensis* and *L. koreensis* (99.2 % and 99.5 % 16S rRNA gene sequence similarity, respectively).

The evaluation of the housekeeping gene sequences (*rpoA* and *pheS*) of strain 2301, *L. yonginensis* THK-V8$^T$ and *L. koreensis* DCY50$^T$ had a higher resolution potential than 16S rRNA gene sequence comparisons as was proposed for the *Enterococcus* and *Lactobacillus* genera. Very low DNA-DNA hybridization values (30.5 % and 19.4 %, with ≥ 70 % species threshold postulated by Wayne et al. (1987)) were decisive for the postulation of a new species, *L. cerevisiae,* in terms of its isolation from beer.

Numerous phenotypic, chemotaxonomic and genotypic properties had to be determined to describe a new species of the *Lactobacillus* genus (Mattarelli et al., 2014), which were outlined along with the properties of closely related species. In addition to the genotypic differences outlined above, *L. cerevisiae* can be distinguished from the three nearest neighbors (*L. parabrevis*, *L. yonginensis* and *L. koreensis*) from a phenotypic perspective by the development of two different colony forms (circular with either smooth or fringed edges) and by the fermentation of D-mannitol and the non-fermentation of D-arabitol. From a chemotaxonomic perspective, the fatty acid profile and the difficulty of determining the cell-wall composition are differential features.

The type strain of the newly described species *L. cerevisiae* is deposited at the Research Center Weihenstephan for Brewing and Food Quality as culture collection number TUM BP 140423000-2250$^T$, at the DSMZ as DSM 100836$^T$ and at the Belgian Coordinated Collection of Microorganisms (BCCM) as LMG 29073$^T$.

PARTICIPATION Koob, J.: Main author, executing scientist, data evaluation; Jacob, F.: Project supervision; Wenning, M.: Molecular biological expertise, critical content review; Hutzler, M.: Corresponding author, critical content review

## CHAPTER D Genotypic and phenotypic diversity of *Lactobacillus rossiae* from beer

Reproduced with permission from Schneiderbanger, J., Jacob, F. and Hutzler, M. 2019. Genotypic and phenotypic diversity of *Lactobacillus rossiae* from beer. Journal of Applied Microbiology 126, 1187-1197.

The species *Lactobacillus rossiae* was described in 2005 as a participant in sourdough fermentations and was soon recognized as phenotypically and genotypically diverse (Corsetti et al., 2005, Scheirlinck et al., 2009, Di Cagno et al., 2007). A few years after its description, *L. rossiae* appeared occasionally in beer, which is unsurprising as sourdough and beer resemble one another in their sugar composition. Due to the ability of some *L. rossiae* strains to increase a beer's viscosity by exopolysaccharide production, in extreme cases resulting in an oily consistency, it must be perceived as a dangerous BSB and should be further characterized. In the process it should be noted if the general variability of this species was also apparent with isolates obtained from beer.

From 2010 to 2016, 1.52 % of all samples that were identified as being positive for the presence of BSB, were attributed to contaminations by *L. rossiae* (see Chapter A). Eleven strains of seven breweries from different spoilage incidents were chosen to uncover phenotypic and genotypic diversity. Results and gene sequences from different studies conducted with sourdough isolates were included to compare the beer isolates to sourdough isolates.

In summary, the eleven beer isolates showed great variability in gas production from gluconate and simultaneously in $NH_3$ production from arginine, in temperature and NaCl tolerance. The evaluation of the carbohydrate fermentation pattern confirmed the broad capability attributed to this species. Comparison of the 16S rRNA gene and *rpoA* housekeeping gene sequences showed no discriminatory power, either between the beer isolates or between beer and sourdough isolates. However, $(GTG)_5$ rep fingerprints, *pheS* housekeeping gene sequence comparison and DNA-DNA hybridization executed with three isolates from three different main clusters, revealed large discrepancies between beer isolates. Consequently, it should be considered that certain thresholds relating to the LAB species definition and proposed for the used genotypic methods must be adapted to match this species or the isolates of *L. rossiae* need to be split into several subspecies, which need to be established.

Uniform distinguishing features between beer and sourdough isolates were exclusively determined as the ribose utilization and the non-utilization of lactose. A comparison of further *L. rossiae* isolates from the beer environment with *L. rossiae* strains from different areas of the food industry and using a modern WGS method will be an interesting field of research as the observed versatility reflects the capabilities of lactobacilli in general.

PARTICIPATION Schneiderbanger, J.: Main author, executing scientist, data evaluation; Jacob, F.: Project supervision; Hutzler, M.: Corresponding author, critical content review

## 3.2 CHAPTER A – Statistical evaluation of beer spoilage bacteria by real-time PCR analyses from 2010 - 2016

Research article

Received: 29 October 2017 Revised: 5 January 2018 Accepted: 15 January 2018 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/jib.486

# Statistical evaluation of beer spoilage bacteria by real-time PCR analyses from 2010 to 2016

**Jennifer Schneiderbanger, Margit Grammer, Fritz Jacob and Mathias Hutzler***

**A total of 13,802 samples over seven years were investigated using real-time PCR for the presence of beer spoilage bacteria, providing a rare large-scale overview of the incidence of individual species. At the same time, the isolation site (early stages of the brewing process = type I and late stages = type II) and the type of contaminated beer and intermediate products (bottom-fermented or top-fermented) were evaluated using chi-squared analysis. The most frequently occurring species were *Lactobacillus brevis* (7 year average 41.9%), *Lactobacillus (para-)casei* (10.4%) and *Lactobacillus backii* (9.5%). *L. (para-)casei* was found at significantly higher rates in the early stages of the brewing process, whereas *Lactobacillus lindneri*, the *Lactobacillus* [*L. (para-) plantarum* and *L. coryniformis*] and the *Pectinatus* (*P. cerevisiiphilus*, *P. frisingensis* and *P. haikarae*) groups and *Megasphaera cerevisiae* predominated in the later stages and in package (significance level 95%). On a significance level of 95%, *Pediococcus damnosus*, *M. cerevisiae* and *L. lindneri* together with the *Pectinatus* and *Lactobacillus* groups grew predominantly in bottom fermented samples. *L. brevis*, in contrast, was found most frequently in top fermented beers.** **Copyright © 2018 The Institute of Brewing & Distilling**

Additional Supporting Information may be found online in the supporting information tab for this article.

**Keywords:** beer spoilage bacteria; *Lactobacillus*; real-time PCR; statistics; chi-squared distribution

## Introduction

Much to the regret of brewers, beer contamination with beer spoilage bacteria is not uncommon. The growth of these harmful bacteria causes quality deficiencies that range from impaired flavour and aroma to acidification of the product, haze, sedimentation and, in the most extreme cases, slime formation. Beer as a product is characterized by various properties that restrict the growth of microorganisms, for example the $CO_2$ content of ~5 g/L, alcohol content of ~4.5–5.5 abv or the hop content *(1–3)*. Several bacterial species, especially from the group of lactic acid bacteria, have developed resistance that enable them to grow in this inhospitable medium.

A particular focus in microbiological quality assurance in breweries is on the timely detection of spoilage organisms. This helps breweries to take rapid action, block affected batches before they reach the consumer or, for instance, start thorough cleaning of the affected brewery area. The tools of the trade in quality assurance consist of product, rinse water and swab samples, which are analysed in the microbiology laboratory for the full range of potential contaminants. Not every brewery analyse the samples in house and these investigations are assigned to third parties for identifying beer spoilage bacteria.

In the accredited microbiology laboratory (DIN EN ISO 17025) of the Research Center Weihenstephan for Brewing and Food Quality, thousands of samples are analysed annually for the presence of spoilage bacteria and yeast. The laboratory staff uses culture-based methods as well as molecular biology methods. The polymerase chain reaction (PCR) technique in particular has become increasingly important in brewery laboratories in recent years. A specific section of the bacteria's target DNA is replicated and visualized in real time, using specialist primers and probes. By using the melting curve analysis, it is also possible to identify the bacterial species (commercial kit based on hybridization probe technology; Biotecon, Potsdam, Germany). A PCR run therefore provides information on the quantity of the spoilage organism present and the identity of the microorganisms. The choice of PCR kit determines which beer spoilage bacteria can be identified using the melting curve.

Routine microbiological analysis is focused on the detection and the subsequent identification of spoilage bacteria as the growth of these in beer often has major consequences in terms of quality. Species of the *Lactobacillus* (*L.*) and *Pediococcus* (*Pd.*) genera are found, which belong to the Gram-positive, microaerophilic family of Lactobacillaceae, or the lactic acid bacteria. Other key species are the Gram-negative, strictly anaerobic genera *Megasphaera* (*M.*) and *Pectinatus* (*P.*).

The spoilage potential of beer spoilage bacteria is variable and is categorized according to Back *(3,4)* into several levels. Bacteria are described as obligate beer spoilage organisms if they can grow in normal beer without an adaptation phase. Potential beer spoilers can only grow after an adaptation period or in beers that have reduced selective properties (e.g. non-alcoholic beers). Indirect and latent beer spoilage bacteria as well as hygiene indicators are further categories that have less importance in the brewing microbiology. Routine brewery analysis is often only concerned with

* Correspondence to: Mathias Hutzler, Technische Universität München, Research Center Weihenstephan for Brewing and Food Quality, Alte Akademie 3, 85354 Freising, Germany. E-mail: hutzler@wzw.tum.de

Technische Universität München, Research Center Weihenstephan for Brewing and Food Quality, Alte Akademie 3, 85354, Freising, Germany

 Copyright © 2018 The Institute of Brewing & Distilling

bacteria in the first two categories – the obligate and potential beer spoilage bacteria.

Large scale studies and statistical evaluation of the incidence of individual species of beer spoilage bacteria are rare *(5,6)*. However, there have been numerous reports about beer spoilage bacteria in the last few decades *(7–14)*. Statistical evaluation of the PCR results of the Research Center Weihenstephan for Brewing and Food Quality from 2010/2011, as well as 2012/2013 have already been published *(15,16)*. In this study, the PCR results from 2014 to 2016 were statistically evaluated and combined with the previous results to compare contamination events over several consecutive years.

## Materials and methods

### Samples

The samples came from routine analysis carried out at the Research Center from 2010 to 2016. Various sample types at different stages of the brewing process were analysed including product, rinse water, yeast samples, swab and membrane filter samples and samples for microscopic analysis. In the seven years reported here, a total of 13,802 samples were investigated using PCR technology. All bacteria that were detectable by the real-time PCR method (see below) in one of the analysed samples were recorded, independent of the spoilage degree of the sample.

### Enrichment

The detection limit, for PCR-based methods is between 100 and 1000 cells/mL *(17–19)*. As the loading can be be below this, the contaminants can be enriched under optimum growth conditions. With beer spoilage bacteria, optimum conditions are an oxygen-free incubation at 28 ± 1°C. An acid indicator contained in the culture media displays a colour change to indicate the growth of the microorganisms. The culture media used for the proliferation of present beer spoilage bacteria were NBB® broth (for *Lactobacillus* contaminations; Döhler, Germany) and micro inoculum broth (for *Megasphaera* and *Pectinatus* contaminations; Difco™, Germany). All cultures were incubated anaerobically for 2–14 days, depending on the initial cell count. In some cases, the bacteria were already recovered on culture agar by the sending laboratory, which enabled analysis of individual colonies visually and microscopically confirmed that the same species was contained within the colonies. Colonies with varying colony morphology on an agar plate were analysed individually.

### Preparation, screening, identification

A 100 μL sample of the liquid enrichment media was used for PCR analysis; a colony was used in each case from the agar plates. The foodproof® ShortPrep III Kit (Biotecon Diagnostics) was used according to the manufacturer's specifications for the DNA extraction. The quantitative real-time PCR was performed using the foodproof® beer screening kit (Biotecon Diagnostics) on a Light Cycler® 480 II (Roche) according to the manufacturer's specifications for the most common and hazardous beer spoiling bacteria species. The quantity of bacterial DNA present is determined using the *ct* value (cycle threshold). The ct value describes the cycle in which the exponential growth curve exceeds a specific background value *(20)*. The earlier this value is achieved and the lower the *ct* value, the more target DNA was present in the sample; *ct* values between 15 and 30 were assessed as being positive in the screening (see Fig. 1). It should be noted that the *ct* value and the number of colony forming units (CFU) do not correlate well.

The bacteria species can be identified using the melting curve analysis based on a kit-specific hybridization probe mixture (probe sequences are not published by Biotecon). Melting curves are measured in two fluorescence channels. At increasing temperatures labelled hybridization probes (interaction of two fluorescence dyes) are separated from their DNA target region.

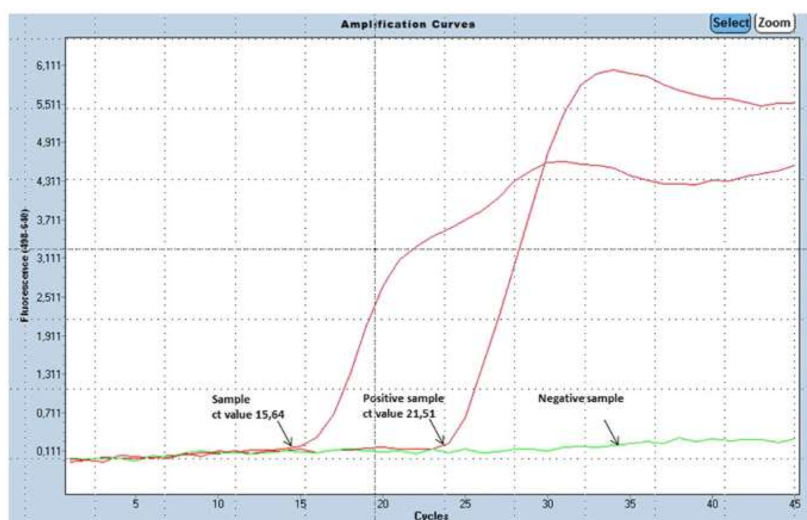



**Figure 1.** Example of a quantitative real-time polymerase chain reaction (PCR) analysis. Figure is reproduced in colour in online version. [Colour figure can be viewed at wileyonlinelibrary.com]


Copyright © 2018 The Institute of Brewing & Distilling

Melting curve and temperature are characteristic for each species (see Fig. 2) *(21)*.

The customer decides whether they want the laboratory to screen for the presence of beer spoilage bacteria (group screening) or identify the bacteria based on the melting curve analysis. If multiple contaminants are present in a sample, it is usually possible to read off multiple identities from the modified melting curve (depending on the mixing ratio of species and their DNA concentrations).

### Beers spoilage bacteria

The bacteria species from Table 1 are recorded by the primers contained in the foodproof® beer screening kit. Several of these are displayed as a group (*Lactobacillus* group, *Pectinatus* group, *Pediococcus* group). For instance, the *Lactobacillus* group contains *L. perolens* and *L. harbinensis* since their DNA produces the same melting curve resulting in their combined detection.

### Statistical evaluation

The chi-squared test can be used to determine whether measurements are normally distributed or differ significantly. The formula for the chi-squared test is described as follows:

$$\chi^2 = \Sigma \frac{(e-0)^2}{e}$$

where $e$ is the expected value and $o$ is the observed value, whereby $e$ is the product of the assignable total number of positive samples for a species and the percentage class-specific total distribution (species-independent). For example, the assignable number of positive samples for *L. brevis* is 365, the percentage class-specific distribution for 'sample type I' is 35.2% (=0.352) and the resulting expected value $e_I$ = 128.5 (with I for sample type I). Accordingly, $e_{II}$ (with II for sample type II) is 365 × 0.648 (64.8% = percentage total distribution of sample type II) = 236.5.

**Table 1.** List of bacterial species recorded using the foodproof® beer screening kit

| Species/group | Described by |
|---|---|
| *L. brevis* | *(22)* |
| *L. lindneri* | *(23,24)* |
| *L. backii* | *(25)* |
| *L. (para-)casei* | *(26,27)* |
| *L.* group[a] | *(22,28,29)* |
| *L. collinoides* | *(30,31)* |
| *L. (para-)buchneri* | *(32,33)* |
| *L. rossiae* | *(34)* |
| *L. perolens/harbinensis* | *(35,36)* |
| *M. cerevisiae* | *(37)* |
| *P.* group[b] | *(38–40)* |
| *Pd. damnosus* | *(41)* |
| *Pd. inopinatus* | *(42–44)* |
| *Pd. claussenii* | *(45)* |
| *Pd.* group [c] | *(46–49)* |

[a] *L. (para-)plantarum, L. coryniformis;*
[b] *P. cerevisiiphilus, P. frisingensis, P. haikarae;*
[c] *Pd. parvulus, Pd. pentosaceus, Pd. acidilactici.*

Using relevant chi-squared tables, the calculated test value $\chi^2$ is compared with the defined significance thresholds for each species or group, together with the selected probability $P$ = 95% (90%) and the number of degrees of freedom ($n = k - 1$, where $k$ is the number of possible classes; here $k$ = 2 for sample type I/II or bottom-/top-fermented). If the calculated test value $\chi^2$ is greater than the significance threshold defined for the respective significance level (0.05 or 0.1), then the hypothesis that the tested parameter is normally distributed can be rejected *(50,51)*.

In this study, the samples were classified into two categories according to their chronological process step. Type I samples were taken during the brewing process pre-packaging but including flash pasteurization (early stages of the brewing process). Type II samples comprise swab samples from the filling area and

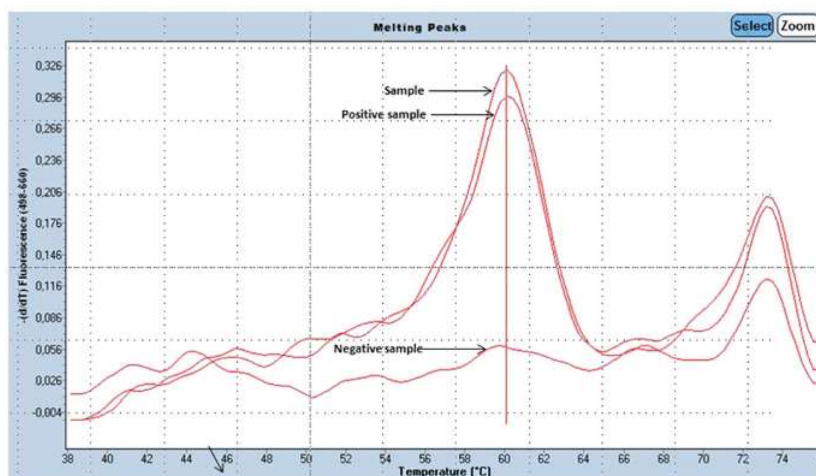



**Figure 2.** Example of a real-time PCR hybridization probe melting curve analysis. Figure is reproduced in colour in online version. [Colour figure can be viewed at wileyonlinelibrary.com]


Copyright © 2018 The Institute of Brewing & Distilling

packaged product samples (later stages of the brewing process). Two further categories were classified from the original sample as bottom or top fermented samples.

## Results and discussion

Of the 13,802 analysed samples, 11.8% (=1456) from 128 breweries in 22 European and non-European countries tested positive for beer spoilage bacteria. Screening for beer spoilage bacteria was positive in 550 cases, and were identified using the melting curve in 906 cases. As several detectable species could be found in some samples, the number of positive identified bacteria in this period reached a total of 1054. The distribution of the 13,802 individual analyses over the observed period can be seen in Fig. 3. Over the years, a rise was recorded in the number of samples investigated using molecular biology methods.

In addition to the percentage distribution of the individual findings to the analysed species and groups, more detailed insights can be obtained on the presence of certain species considering that multiple samples are often analysed within a short time frame per contamination. For example, a brewery had various intermediate product samples analysed within one month, which resulted in numerous PCR analyses with the same findings. Many individual findings are created this way even though they relate to the same case of contamination. In this study, a so-called 'incident' of this kind is defined if one species occurs in one brewery within a period of 6 months, independently of the sample types. There were 465 incidents in the assessed period, their distribution across 2010–2016 is presented in Table 2.

At >40% of all positively identified bacteria, *L. brevis* occurred most frequently, but only 34.5% of all contamination cases were

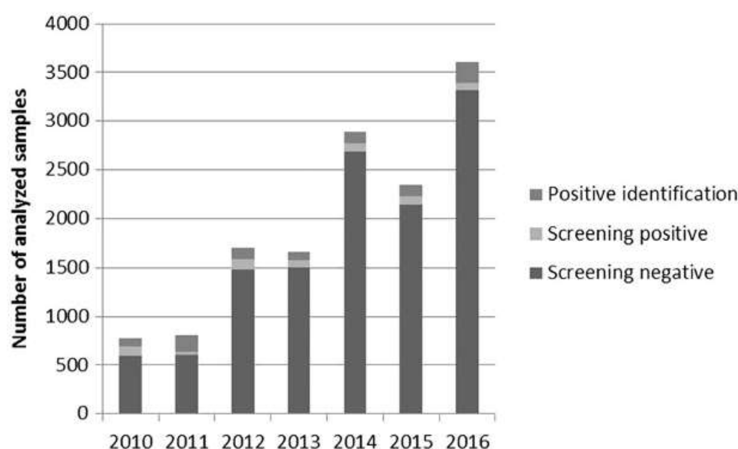



**Figure 3.** Distribution of the number of samples for real-time PCR detection of beer spoilage bacteria from 2010 to 2016.

**Table 2.** Number of findings and incidents (in brackets) per species or bacterial group

| | 2010 | 2011 | 2012 | 2013 | 2014 | 2015 | 2016 | Total |
|---|---|---|---|---|---|---|---|---|
| *L. brevis* | 43 (29) | 105 (23) | 58 (28) | 44 (21) | 57 (21) | 36 (19) | 88 (30) | 431 (161) |
| *L. lindneri* | 9 (6) | 12 (7) | 4 (3) | 0 (0) | 3 (2) | 21 (3) | 19 (8) | 68 (29) |
| *L. backii* | 4 (3) | 21 (9) | 15 (6) | 9 (5) | 16 (5) | 14 (7) | 23 (11) | 102 (46) |
| *L. (para-)casei* | 8 (5) | 20 (7) | 17 (10) | 5 (4) | 10 (4) | 25 (8) | 26 (10) | 111 (48) |
| *L.* group[a] | 4 (4) | 6 (3) | 4 (1) | 12 (4) | 8 (5) | 8 (5) | 22 (10) | 64 (32) |
| *L. collinoides* | 0 (0) | 2 (1) | 5 (5) | 1 (1) | 5 (3) | 2 (1) | 8 (7) | 23 (18) |
| *L. (para-)buchneri* | 3 (2) | 9 (5) | 1 (1) | 15 (5) | 13 (3) | 1 (1) | 10 (8) | 52 (25) |
| *L. rossiae* | 1 (1) | 0 (0) | 0 (0) | 1 (1) | 2 (1) | 2 (2) | 10 (8) | 16 (13) |
| *L. perolens/harbinensis* | 2 (2) | 8 (2) | 2 (1) | 4 (2) | 9 (4) | 11 (4) | 25 (11) | 61 (25) |
| *M. cerevisiae* | 5 (4) | 2 (1) | 2 (1) | 0 (0) | 3 (1) | 0 (0) | 16 (3) | 28 (10) |
| *P.* group[b] | 3 (2) | 7 (3) | 11 (3) | 0 (0) | 5 (3) | 7 (5) | 3 (2) | 36 (18) |
| *Pd. damnosus* | 1 (1) | 6 (3) | 18 (10) | 6 (3) | 5 (5) | 9 (5) | 10 (8) | 55 (35) |
| *Pd. inopinatus* | 0 (0) | 0 (0) | 1 (1) | 1 (1) | 0 (0) | 0 (0) | 0 (0) | 2 (2) |
| *Pd. claussenii* | 0 (0) | 2 (1) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 2 (1) |
| *Pd.* group[c] | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (1) | 2 (1) | 3 (2) |
| Total | 83 (49) | 200 (65) | 138 (70) | 98 (47) | 136 (57) | 137 (61) | 262 (116) | 1054 (465) |

[a]*L. (para-)plantarum, L. coryniformis;*
[b]*P. cerevisiiphilus, P. frisingensis, P. haikarae;*
[c]*Pd. parvulus, Pd. pentosaceus, Pd. acidilactici.*


Copyright © 2018 The Institute of Brewing & Distilling

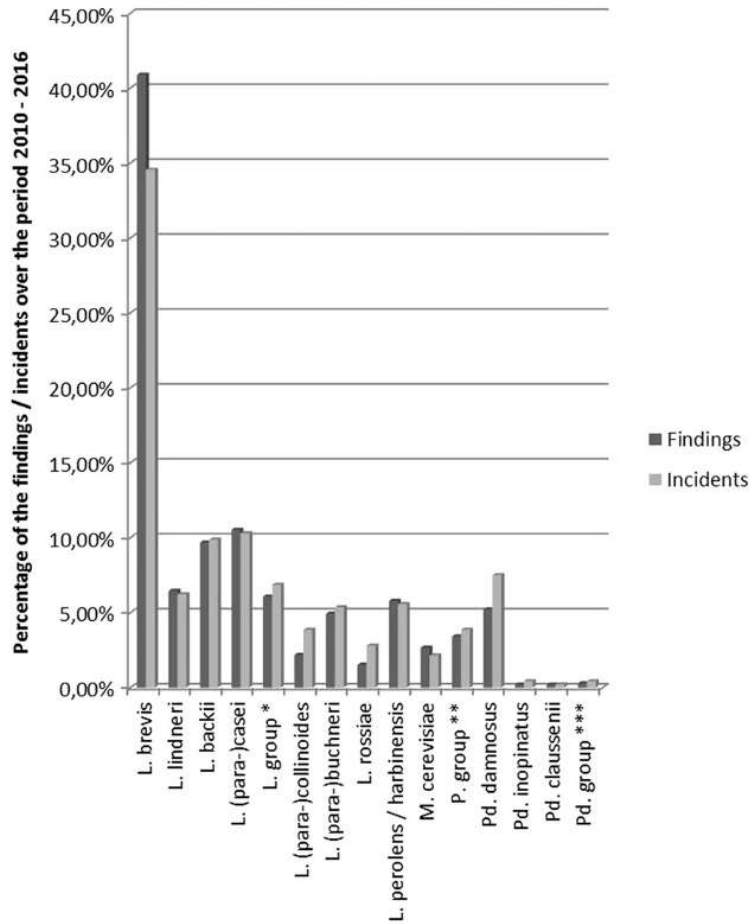



**Figure 4**. Mean values of the percentage distribution of findings or the incidents divided according to species or group between 2010 and 2016; * *L. (para-)plantarum, L. coryniformis*; ** *P. cerevisiiphilus, P. frisingensis, P. haikarae*; *** *Pd. parvulus, Pd. pentosaceus, Pd. acidilactici.*

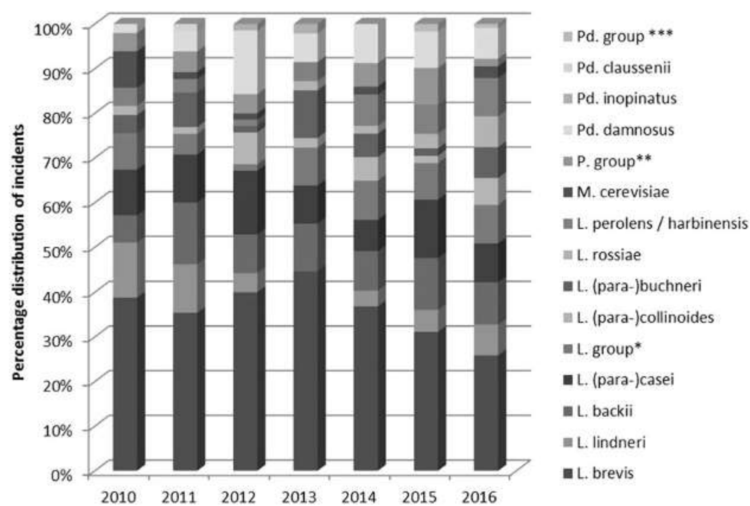



**Figure 5**. Percentage distribution of the species incidents in individual years; * *L. (para-)plantarum, L. coryniformis*; ** *P. cerevisiiphilus, P. frisingensis, P. haikarae*; *** *Pd. parvulus, Pd. pentosaceus, Pd. acidilactici.*



Copyright © 2018 The Institute of Brewing & Distilling

**Table 3.** Individual values of the positive assignable samples for the individual species and the calculated test values $\chi^2$ based on these for the test criteria sample type I/II

| | $o_I$ | $o_{II}$ | $\Sigma(o_I + o_{II})$ | $e_I$ | $e_{II}$ | $\chi^2$ |
|---|---|---|---|---|---|---|
| *L. brevis* | 136.00 | 229.00 | 365.00 | 128.53 | 236.47 | 0.67 |
| *L. lindneri* | 12.00 | 43.00 | 55.00 | 19.37 | 35.63 | 4.33 |
| *L. backii* | 40.00 | 49.00 | 89.00 | 31.34 | 57.66 | 3.69 |
| *L. (para-) casei* | 42.00 | 48.00 | 90.00 | 31.69 | 58.31 | 5.18 |
| *L.* group[a] | 11.00 | 40.00 | 51.00 | 17.96 | 33.04 | 4.16 |
| *L. collinoides* | 9.00 | 7.00 | 16.00 | 5.63 | 10.37 | 3.10 |
| *L. (para-) buchneri* | 8.00 | 26.00 | 34.00 | 11.97 | 22.03 | 2.03 |
| *L. rossiae* | 6.00 | 11.00 | 17.00 | 5.99 | 11.01 | 0.00 |
| *L. perolens/ harbinensis* | 15.00 | 35.00 | 50.00 | 17.61 | 32.39 | 0.60 |
| *M. cerevisiae* | 2.00 | 23.00 | 25.00 | 8.80 | 16.20 | 8.12 |
| *P.* group[b] | 2.00 | 24.00 | 26.00 | 9.16 | 16.84 | 8.63 |
| *Pd. damnosus* | 19.00 | 25.00 | 44.00 | 15.49 | 28.51 | 1.22 |
| *Pd.* group[c] | 4.00 | 3.00 | 7.00 | 2.46 | 4.54 | 1.48 |

[a] *L. (para-)plantarum, L. coryniformis;*
[b] *P. cerevisiiphilus, P. frisingensis, P. haikarae;*
[c] *Pd. parvulus, Pd. pentosaceus, Pd. acidilactici.*
*o*, Observed value; *e*, expected value; I, sample type I; II, sample type II.

attributed to this bacterium (see Fig. 4). The second and third most frequent causes of contamination are the species *L. (para-)casei* (10.3% of incidents) and *L. backii* (9.9% of incidents). The individual percentage frequency of all observed species and groups broken down over the years 2010–2016 is reported in the Supporting Information.

The distribution of the individual species incidents over the different years can be seen in Fig. 5. Within the samples whose description enabled definite assignment to one of the two categories, 35.2% of samples were analysed in the observed time frame could be classified as type I samples and 64.8% as type II samples. The trend towards more type II samples being analysed because information here about the presence or absence of beer-spoilage bacteria is critical to the further development of the beers, and the additional analysis help to pinpoint problems arising in package.

Table 3 gives the calculated values for the test value $\chi^2$ with reference to the distribution among type I and type II samples. It can be seen from the table that the significance threshold for a probability of 95% (90%) and a degree of freedom of $n = 1$ is $\chi^2 = 3.841$ (2.706) *(23)*. For species whose $\chi^2$ values are above the respective significance threshold, the hypothesis of normal distribution can be rejected.

Figure 6 shows how the percentage distribution among sample type I and II is broken down to the individual species

Species that deviate from the expected normal distribution (significance level 0.05) are *L. lindneri*, *L. (para-)casei*, the *Lactobacillus* group [= *L. (para-)plantarum* and *L. coryniformis*], *Megasphaera cerevisiae* and the *Pectinatus* group, where *L. (para-)casei* was significantly more frequent in type I samples. In contrast, the other species and groups were found predominantly in type II samples. At a significance level of 0.10, a further two species deviated from the normal distribution: *L. backii* and *L. collinoides* (significantly more frequent in sample type I). *M. cerevisiae* and the *Pectinatus* group deviated even on a significance level of 0.01 from the normal distribution ($\chi^2 \geq 6.635$) which reflects *Pectinatus* and *Megasphaera* being classical secondary contaminants in the brewing environment *(3,6)*. Also, the findings of *L. lindneri* predominately in type II samples are in agreement with reports of the difficulty to detect *L. lindneri* before it causes spoilage of finished beer *(3,52–54)*.

Table 4 reports the calculated values for the test value $\chi^2$ with reference to the distribution bottom- and top-fermented samples. The values $\geq 3.841$ and $\geq 2.706$, respectively apply again as significance thresholds ($P = 95\%$ and $P = 90\%$; $n = 1$). Among the samples, similar proportions were bottom fermented (52.4%) and top fermented (47.6%). Top-fermented beers (excluding strong hopped craft beers like Indian Pale Ale) are generally more susceptible to beer-spoilage bacteria as they tend to contain more

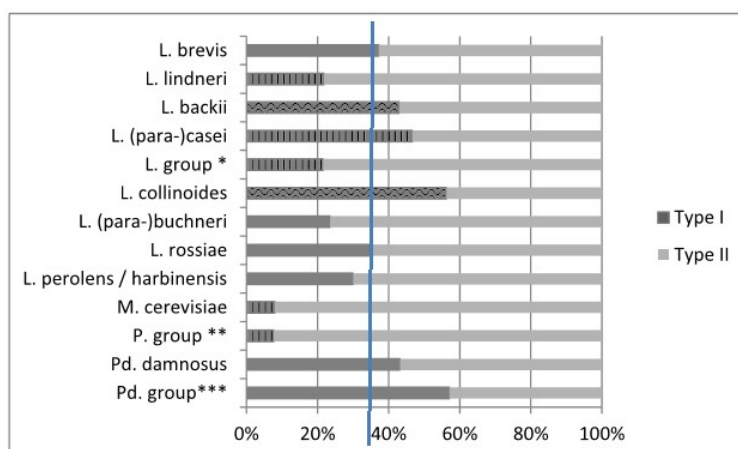



**Figure 6.** Percentage distribution of the individual species among type I and type II samples. Hatched bars, species that deviate from the normal distribution of samples at a significance level of 0.05; bars with jagged lines, species that deviate from the normal sample distribution at a significance level of 0.10; blue bar, overall percentage of 35.21% type I samples and 64.79% type II samples. * *L. (para-)plantarum, L. coryniformis*; ** *P. cerevisiiphilus, P. frisingensis, P. haikarae*; *** *Pd. parvulus, Pd. pentosaceus, Pd. acidilactici.* Figure is reproduced in colour in online version. [Colour figure can be viewed at wileyonlinelibrary.com]


Copyright © 2018 The Institute of Brewing & Distilling

**Table 4.** Individual values of the positive assignable samples for the individual species and the calculated test values $\chi^2$ based on these for the test criteria bottom/top-fermented sample

| | $o_b$ | $o_t$ | $\Sigma(o_b + o_t)$ | $e_b$ | $e_t$ | $\chi^2$ |
|---|---|---|---|---|---|---|
| *L. brevis* | 71.00 | 134.00 | 205.00 | 107.47 | 97.53 | 26.02 |
| *L. lindneri* | 45.00 | 2.00 | 47.00 | 24.64 | 22.36 | 35.36 |
| *L. backii* | 25.00 | 31.00 | 56.00 | 29.36 | 26.64 | 1.36 |
| *L. (para-)casei* | 20.00 | 9.00 | 29.00 | 15.20 | 13.80 | 3.18 |
| *L.* group[a] | 0.00 | 13.00 | 13.00 | 6.82 | 6.18 | 14.33 |
| *L. collinoides* | 3.00 | 1.00 | 4.00 | 2.10 | 1.90 | 0.82 |
| *L. (para-) buchneri* | 10.00 | 3.00 | 13.00 | 6.82 | 6.18 | 3.13 |
| *L. rossiae* | 1.00 | 1.00 | 2.00 | 1.05 | 0.95 | 0.00 |
| *L. perolens/ harbinensis* | 4.00 | 5.00 | 9.00 | 4.72 | 4.28 | 0.23 |
| *M. cerevisiae* | 9.00 | 1.00 | 10.00 | 5.24 | 4.76 | 5.66 |
| *P.* group[b] | 16.00 | 2.00 | 18.00 | 9.44 | 8.56 | 9.60 |
| *Pd. damnosus* | 23.00 | 1.00 | 24.00 | 12.58 | 11.42 | 18.13 |
| *Pd.* group[c] | 0.00 | 3.00 | 3.00 | 1.57 | 1.43 | 3.31 |

[a]*L. (para-)plantarum, L. coryniformis;*
[b]*P. cerevisiiphilus, P. frisingensis, P. haikarae;*
[c]*Pd. parvulus, Pd. pentosaceus, Pd. acidilactici.*
*o*, Observed value; *e*, expected value; b, bottom-fermented; t, top-fermented.

utilizable carbohydrates and are less well hopped *(1,3,55)*. When a bacterium is predominantly found in the top fermented products, it can be assumed that its beer spoilage potential is lower than that of a bacterium found in the bottom fermented samples. Those mainly found in the bottom fermented samples are the *Pediococcus* group, *L. perolens/harbinensis*, *L. rossiae*, *L. backii* and *L. brevis* (see Fig. 7). All other species and groups occur more in the top fermented samples. At a probability of 95%, *Pd. damnosus*, the *Pectinatus* group, *M. cerevisiae*, the *Lactobacillus* group and *L. lindneri* favour bottom fermented beers. In contrast, intermediate products and *L. brevis* in contrast favour the top fermented samples. At a significance level of 0.10, three further trends can be established: the *Pediococcus* group occurs more frequently in top fermented samples and *L. buchneri* together with *L. (para-)casei* occur more frequently in bottom-fermented samples.

## Conclusion

PCR technology has revolutionized routine analysis in brewery laboratories. It is possible within a short period of time to obtain information on the quantity of DNA and the identity of a contaminating bacterium. This provides breweries with key information on the level of hygiene in their brewery and the condition of the samples. Regular sampling in accordance with a structured plan may help quality assurance identify the source of contamination, whether it can be removed by targeted cleaning or whether there may be different contamination sources in spoilage cases. The increase in samples being sent to our laboratory suggests that breweries are increasingly relying on this tool and species identification is becoming particularly important.

The overview of the period 2010–2016 shows *L. brevis* to be the most frequently occurring beer spoiler, followed by *L. (para-)casei* and *L. backii*. However, there are fluctuations between the individual years. For example, *L. lindneri*, an obligate beer spoiler, was not identified in 2013 whereas in 2010 it was found in >10% of contaminations.

The definite assignment of contamination to different stages of the brewing process is not always possible. Nevertheless, several statistical insights can be gained that relate to the increased incidence of a beer-spoilage species or a group in a specific sector. *L. lindneri*, the *Lactobacillus* group (*L. plantarum* and *L. coryniformis*), *M. cerevisiae* and the *Pectinatus* group (*P. cerevisiiphilus*, *P. frisingensis*, *P. haikarae*) are more frequently found in later production steps (type II samples) and *L. (para-)casei* in early steps (significance level 0.05).

In the bottom fermented samples, there is an increase in the frequency of *L. lindneri*, the *Lactobacillus* group (*L. plantarum* and *L. coryniformis*), *M. cerevisiae*, the *Pectinatus* group (*P. cerevisiiphilus*, *P. frisingensis*, *P. haikarae*) and *Pd. damnosus*. Conversely, *L. brevis* favours the top fermented sector.

Building on the work reported here, it will be useful to evaluate the PCR results over the next few years with the same PCR methods under the same culture and PCR conditions.

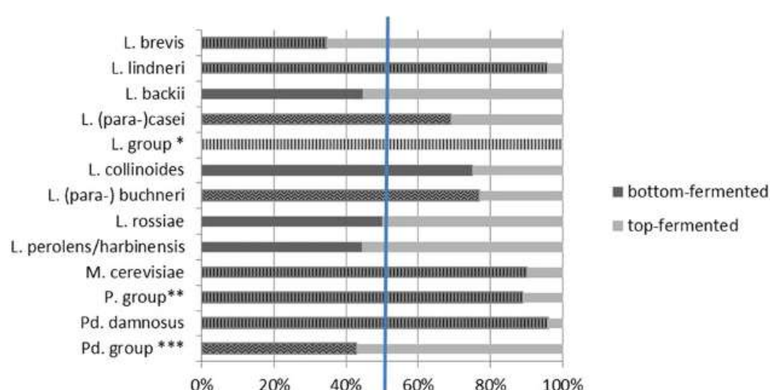



**Figure 7.** Percentage distribution of the individual species for bottom fermented and top fermented samples. Hatched bars, species that deviate from the normal distribution of samples at a significance level of 0.05; bars with jagged lines, species that deviate from the normal distribution of samples at a significance level of 0.10; blue bar, overall percentage of 52.4% bottom fermented and 47.6% top fermented samples. * *L. (para-)plantarum, L. coryniformis;* ** *P. cerevisiiphilus, P. frisingensis, P. haikarae;* *** *Pd. parvulus, Pd. pentosaceus, Pd. acidilactici.* Figure is reproduced in colour in online version. [Colour figure can be viewed at wileyonlinelibrary.com]


Copyright © 2018 The Institute of Brewing & Distilling

Consequently, key findings can be obtained over time about the bacteria capable of spoiling beer, which could become important in their control and characterization. Furthermore, novel beer-spoilers like *L. cerevisiae (56)*, *L. acetotolerans (57)* and *L. curtus (58)* as well as known beer spoilers with less distinct beer spoilage potential (like acetic acid bacteria, wort bacteria and enterobacteria) have to be taken into account to complete the overview.

## References


1. Back, W., and Bohak, I. (2005) *Ausgewählte Kapitel der Brauereitechnologie*, Hans Carl Verlag, Nürnberg.
2. Back, W. (1980) Bierschädliche Bakterien, *Brauwelt 43*, 1562–1569.
3. Back, W. (1994) *Farbatlas und Handbuch der Getränkebiologie–Part 1*, 1st ed., Hans Carl Verlag, Nürnberg.
4. Back, W. (2000) *Farbatlas und Handbuch der Getränkebiologie–Part 2*, 1st ed., Hans Carl Verlag, Nürnberg.
5. Back, W., Breu, S., and Weigand, C. (1988) Infektionsursachen im Jahre 1987, *Brauwelt 31*, 1358–1362.
6. Back, W. (1994) Sekundärkontaminationen im Abfüllbereich, *Brauwelt 16*, 686–695.
7. Ault, R. G. (1965) Spoilage bacteria in brewing – A review, *J. Inst. Brew. 71*, 376–391.
8. Back, W. (1981) Bacteria noxious for the beer, *Monatsschr. Brauwiss. 34*, 267–276.
9. Ingledew, W. M. (1979) Effect of bacterial contaminants on beer. A review, *J. Am. Soc. Brew. Chem. 37*, 145–150.
10. Dolezil, L., and Kirsop, B. H. (1980) Variations amongst beers and lactic-acid bacteria relating to beer spoilage, *J. Inst. Brew. 86*, 122–124.
11. Rainbow, C. (1973) Beer spoilage lactic acid bacteria, in *Lactic Acid Bacteria in Beverages and Food*, (Carr, J. G., and Cutting, C. V., and Whiting, G. C. Eds.), Academic Press, New York.
12. Sakamoto, K., and Konings, W. N. (2003) Beer spoilage bacteria and hop resistance, *Int. J. Food Microbiol. 89*, 105–124.
13. Suzuki, K., Asano, S., Iijima, K., and Kitamoto, K. (2008) Sake and beer spoilage lactic acid bacteria – A review, *J. Inst. Brew. 114*, 209–223.
14. Campbell, I. (2003) Microbiological aspects of brewing, in *Brewing Microbiology*, (Priest, F. G., and Campbell, I. Eds.), Kluwer Academic/Plenum, New York.
15. Hutzler, M., Koob, J., Grammer, M., Riedl, R., and Jacob, F. (2012) Statistische Auswertung der PCR-Analyse bierschädlicher Bakterien in den Jahren 2010 und 2011, *Brauwelt 152*, 546–547.
16. Koob, J., Jacob, F., Grammer, M., Riedl, R., and Hutzler, M. (2014) PCR-Analysen bierschädlicher Bakterien 2012 und 2013, *Brauwelt 10*, 288–290.
17. Malorny, B., Tassios, P. T., Radstrom, P., Cook, N., Wagner, M., and Hoorfar, J. (2003) Standardization of diagnostic PCR for the detection of foodborne pathogens, *Int. J. Food Microbiol. 83*, 39–48.
18. Brandl, A. (2006) *Entwicklung und Optimierung von PCR-Methoden zur Detektion und Identifizierung von brauereirelevanten Mikroorganismen zur Routine-Anwendung in Brauereien*, thesis, TU München, Freising.
19. Rupens, N. P., and Herman, L. M. F. (2002) Molecular methods for identification and detection of bacterial food pathogens, *J. AOAC Int. 85*, 984–995.
20. Haakensen, M. (2009) *Genetic markers for beer-spoilage by lactobacilli and pediococci*, thesis, University of Saskatchewan, Saskatoon.
21. Methner, F. J., Schuster, E., and Schackmann, A. (2004) Screening of beer-spoilage bacteria using the LightCycler PCR workflow system, *Biochemica 1*, 9–11.
22. Orla-Jensen, S. (1919) *The Lactic Acid Bacteria*, Host, Copenhagen.
23. Henneberg, W. (1901) Zur Kenntnis der Milchsäurebakterien der Brennereimaische, der Milch, und des Bieres, *Wochenschr. Brauwiss. 18*, 381–384.
24. Back, W., Bohak, I., Ehrmann, M., Ludwig, W., and Schleifer, K. H. (1996) Revival of the species *Lactobacillus lindneri* and the design of a species specific oligonucleotide probe, *Syst. Appl. Microbiol. 19*, 322–325.
25. Tohno, M., Kitahara, M., Irisawa, T., Masuda, T., Uegaki, R., Ohkuma, M., and Tajima, K. (2013) Description of *Lactobacillus iwatensis* sp nov., isolated from orchardgrass (*Dactylis glomerata* L) silage, and *Lactobacillus backii* sp nov, *Int. J. Syst. Evol. Microbiol. 63*, 3854–3860.
26. Orla-Jensen, S. (1916) *Milchwirtschaftliches Zentralblatt 9*, 129.
27. Collins, M. D., Phillips, B. A., and Zanoni, P. (1989) Deoxyribonucleic acid homology studies of *Lactobacillus casei*, *Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus* sp. nov., comb. nov., *Int. J. Syst. Bacteriol. 39*, 105–108.
28. Curk, M. C., Hubert, J. C., and Bringel, F. (1996) *Lactobacillus paraplantarum* sp. nov., a new species related to *Lactobacillus plantarum*, *Int. J. Syst. Bacteriol. 46*, 595–598.
29. Aboelnaga, I. G., and Kandler, O. (1965) Zur Charakterisierung der in der Milch verbreiteten Staphylokokken, *Zentralblatt Fur Bakteriologie Parasitenkunde Infektionskranheiten Und Hygiene 196*, 438.
30. Carr, J. G., and Davies, P. A. (1972) Ecology and classification of strains of *Lactobacillus collinoides* nov. spec. – Bacterium commonly found in fermenting apple juice, *J. Appl. Bacteriol. 35*, 463.
31. Suzuki, K., Funahashi, W., Koyanagi, M., and Yamashita, H. (2004) *Lactobacillus paracollinoides* sp nov., isolated from brewery environments, *Int. J. Syst. Evol. Microbiol. 54*, 115–117.
32. Bergey, D. H., Harrison, D. C., Breed, R. S., Hammer, B. W., and Huntoon, F. M. (1923) *Bergey's Manual of Determinative Bacteriology*, Williams & Wilkins, Baltimore, MD.
33. Farrow, J. A. E., Phillips, B. A., and Collins, M. D. (1988) Nucleic acid studies on some heterofermentative lactobacilli – Description of *Lactobacillus malefermentans* sp. nov. and *Lactobacillus parabuchneri* sp. nov., *FEMS Microbiol. Lett. 55*, 163–167.
34. Di Cagno, R., De Angelis, M., Gallo, G., Settanni, L., Berloco, M. G., Siragusa, S., Parente, E., Corsetti, A., and Gobbetti, M. (2007) Genotypic and phenotypic diversity of *Lactobacillus rossiae* strains isolated from sourdough, *J. Appl. Microbiol. 103*, 821–835.
35. Back, W., Bohak, I., Ehrmann, M., Ludwig, T., Pot, B., and Schleifer, K. H. (1999) Lactobacillus perolens sp nov., a soft drink spoilage bacterium, *Syst. Appl. Microbiol. 22*, 354–359.
36. Miyamoto, M., Seto, Y., Hao, D. H., Teshima, T., Sun, Y. B., Kabuki, T., Yao, L. B., and Najajima, H. (2005) *Lactobacillus harbinensis* sp nov., consisted of strains isolated from traditional fermented vegetables 'Suan cai' in Harbin, Northeastern China and *Lactobacillus perolens* DSM 12745, *Syst. Appl. Microbiol. 28*, 688–694.
37. Engelmann, U., and Weiss, N. (1985) *Megasphaera cerevisiae* sp. nov.: A new Gram-negative obligately anaerobic coccus isolated from spoiled beer, *Syst. Appl. Microbiol. 6*, 287–290.
38. Lee, S. Y., Mabee, M. S., and Jangaard, N. O. (1978) Pectinatus, a new genus of family Bacteroidaceae, *Int. J. Syst. Bacteriol. 28*, 582–594.
39. Schleifer, K. H., Leuteritz, M., Weiss, N., Ludwig, W., Kirchhof, G., and Seidelrufer, H. (1990) Taxonomic study of anaerobic, Gram-negative, rod-shaped bacteria from breweries – Emended description of *Pectinatus cerevisiiphilus* and description of *Pectinatus frisingensis* sp. nov., *Selenomonas lacticifex* sp. nov., *Zymophilus raffinosivorans* gen. nov., and *Zymophilus paucivorans* sp. nov., *Int. J. Syst. Bacteriol. 40*, 19–27.
40. Juvonen, R., and Suihko, M. L. (2006) *Megasphaera paucivorans* sp. nov., *Megasphaera sueciensis* sp. nov. and *Pectinatus haikarae* sp. nov., isolated from brewery samples, and emended description of the genus *Pectinatus*, *Int. J. Syst. Evol. Microbiol. 56*, 695–702.
41. Claussen, N. H. (1903) Études sur les bactéries dites sarcines et sur les maladies quelles provoquent dans la bière, *CR Trav Lab Carlsberg 6*, 64–83.
42. Back, W. (1978) Zur Taxonomie der Gattung Pediococcus. Phänotypische und genotypische Abgrenzung der bisher bekannten Arten sowie Beschreibung einer neuen bierschädlichen Art: Pediococcus inopinatus. Teil 1, *Monatsschr. Brauwiss. 31*(9), 237–250.
43. Back, W. (1978) Zur Taxonomie der Gattung Pediococcus. Phänotypische und genotypische Abgrenzung der bisher bekannten Arten sowie Beschreibung einer neuen bierschädlichen Art: Pediococcus inopinatus. Teil 2, *Monatsschr. Brauwiss. 31*(11), 312–320.
44. Back, W. (1978) Zur Taxonomie der Gattung Pediococcus. Phänotypische und genotypische Abgrenzung der bisher bekannten Arten sowie Beschreibung einer neuen bierschädlichen Art: Pediococcus inopinatus. Teil 3, *Monatsschr. Brauwiss. 31*(12), 336–343.
45. Dobson, C. M., Deneer, H., Lee, S., Hemmingsen, S., Glaze, S., and Ziola, B. (2002) Phylogenetic analysis of the genus *Pediococcus*, including *Pediococcus claussenii* sp. nov., a novel lactic acid bacterium isolated from beer, *Int. J. Syst. Evol. Microbiol. 52*, 2003–2010.
46. Gunther, H. L., Coster, E., and White, H. R. (1962) Designation of the type strain of *Pediococcus parvulus* Gunther and White, *Int. Bulletin Bacteriol. Nomenclature and Taxonomy 12*, 189–190.
47. Dobrogosz, W. J., and Stone, R. W. (1962) Oxidative metabolism in *Pediococcus pentosaceus* – 1. Role of oxygen and catalase, *J. Bacteriol. 84*, 716.



Copyright © 2018 The Institute of Brewing & Distilling

48. Dobrogosz, W. J., and Stone, R. W. (1962) Oxidative metabolism in *Pediococcus pentosaceus* – 2. Factors controlling formation of oxidative activities, *J. Bacteriol. 84*, 724.
49. Sakaguchi, K. (1960) Vitamin and amino acid requirements of *Pediococcus soyae* and *Pediococcus acidilactici* Kitahara strain – 9. Studies on the activities of bacteria in soy sauce brewing, *Bull. Agric. Chem. Soc. Japan 24*, 638–643.
50. Gruber, U., and Klein, W. (Eds) (2000) *Statistik für Anwender*, Wiley-VCH, Berlin.
51. Lancaster, H. O., and Seneta, E. (2005) *Chi-Square Distribution–Encyclopedia of Biostatistics*, John Wiley, Weinheim.
52. Suzuki, K., Asano, S., Iijima, K., Kuriyama, H., and Kitagawa, Y. (2008) Development of detection medium for hard-to-culture beer-spoilage lactic acid bacteria, *J. Appl. Microbiol. 104*, 1458–1470.
53. Suzuki, K., Iijima, K., Asano, S., Kuriyama, H., and Kitagawa, Y. (2006) Induction of viable but nonculturable state in beer spoilage lactic acid bacteria, *J. Inst. Brew. 112*, 295–301.
54. Back, W. (2005) *Colour Atlas and Handbook of Beverage Biology*, Hans Carl Verlag, Nürnberg.
55. Riedl, R., Goderbauer, P., Brandl, A., Jacob, F., and Hutzler, M. (2017) Bavarian wheat beer, an example of a special microbe habitat – Cultivation, detection, biofilm formation, characterization of selected lactic acid bacteria hygiene indicators and spoilers, *Brew. Sci. 70*, 39–50.
56. Koob, J., Jacob, F., Wenning, M., and Hutzler, M. (2017) *Lactobacillus cerevisiae* sp. nov., isolated from a spoiled brewery sample, *Int. J. Syst. Evol. Microbiol. 67*, 3452–3457.
57. Entani, E., Masai, H., and Suzuki, K. I. (1986) *Lactobacillus acetotolerans*, a new species from fermented vinegar broth, *Int. J. Syst. Bacteriol. 36*, 544–549.
58. Asakawa, Y., Takesue, N., Asano, S., Shimotsu, S., Iijima, K., Suzuki, K., Motoyama, Y., and Aizawa, M. (2017) *Lactobacillus curtus* sp. nov., isolated from beer in Finland, *Int. J. Syst. Evol. Microbiol. 67*, 3899–3906.


## Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

 Copyright © 2018 The Institute of Brewing & Distilling 

## CHAPTER A – Supplementary Material

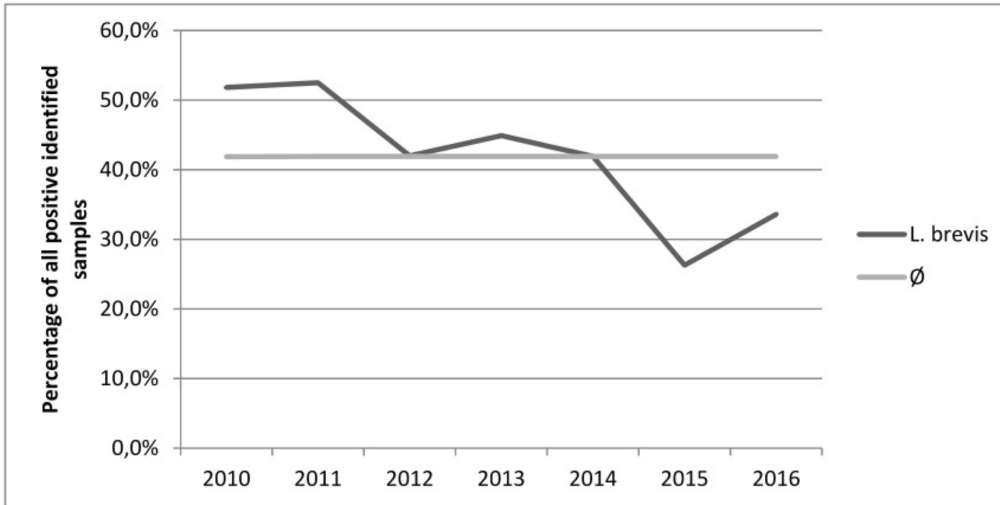



Figure S1: Percentage of samples positive for *L. brevis* from 2010 – 2016 including 7-year mean value

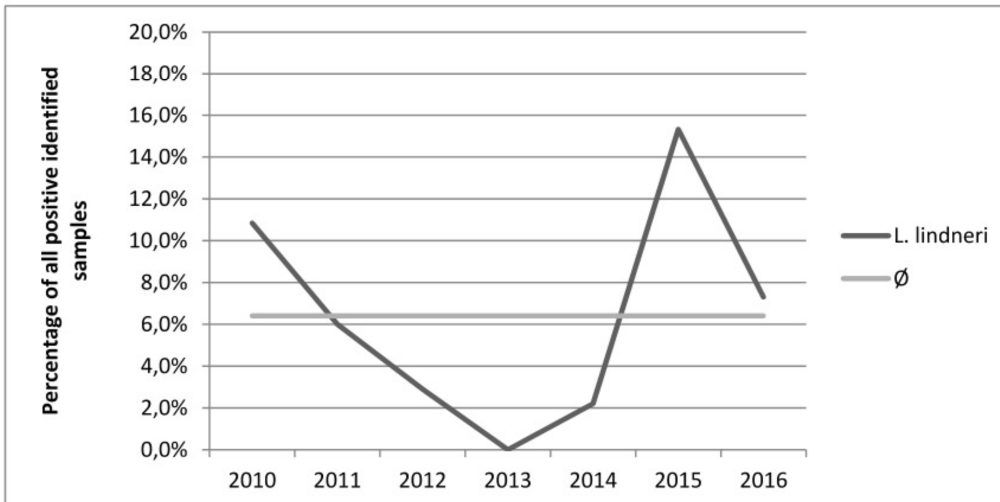



Figure S2: Percentage of samples positive for *L. lindneri* from 2010 – 2016 including 7-year mean value

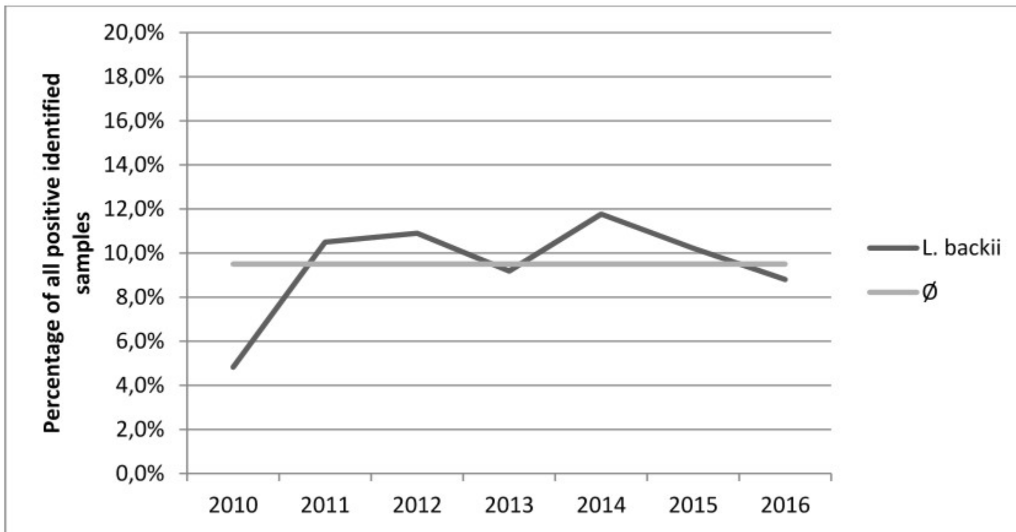



Figure S3: Percentage of samples positive for *L. backii* from 2010 – 2016 including 7-year mean value

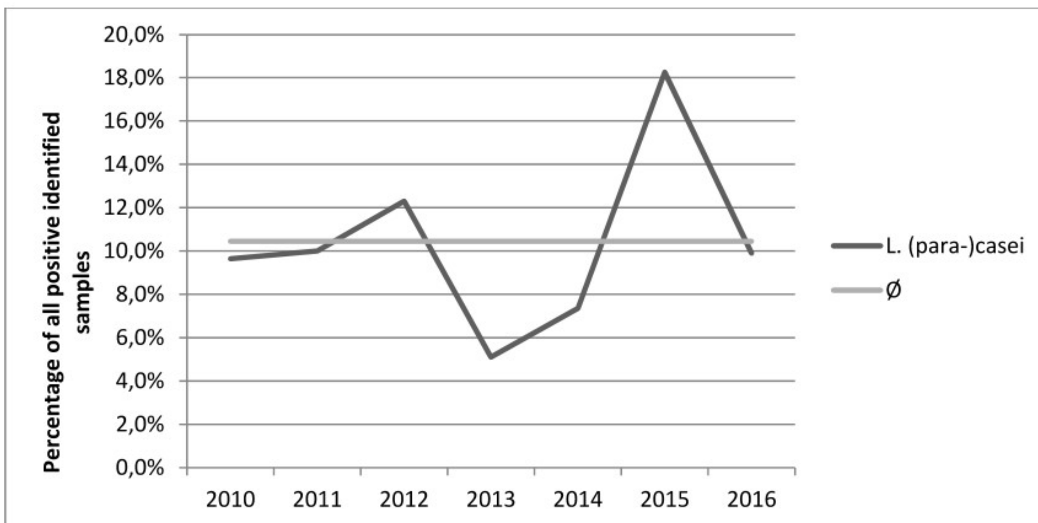



Figure S4: Percentage of samples positive for *L. (para-)casei* from 2010 – 2016 including 7-year mean value

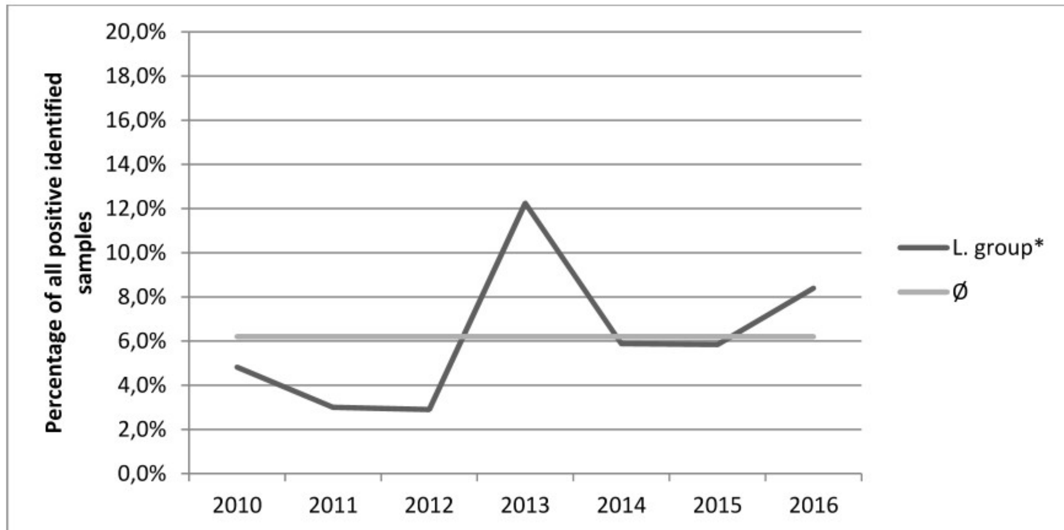



Figure S5: Percentage of samples positive for *L.* group (*= *L. plantarum* and *L. coryniformis*) from 2010 – 2016 including 7-year mean value

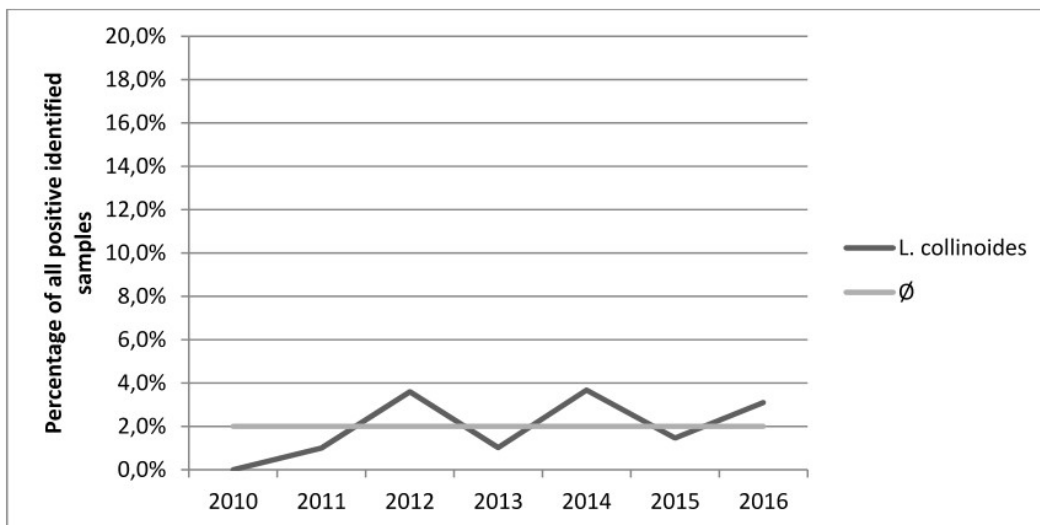



Figure S6: Percentage of samples positive for *L. collinoides* from 2010 – 2016 including 7-year mean value

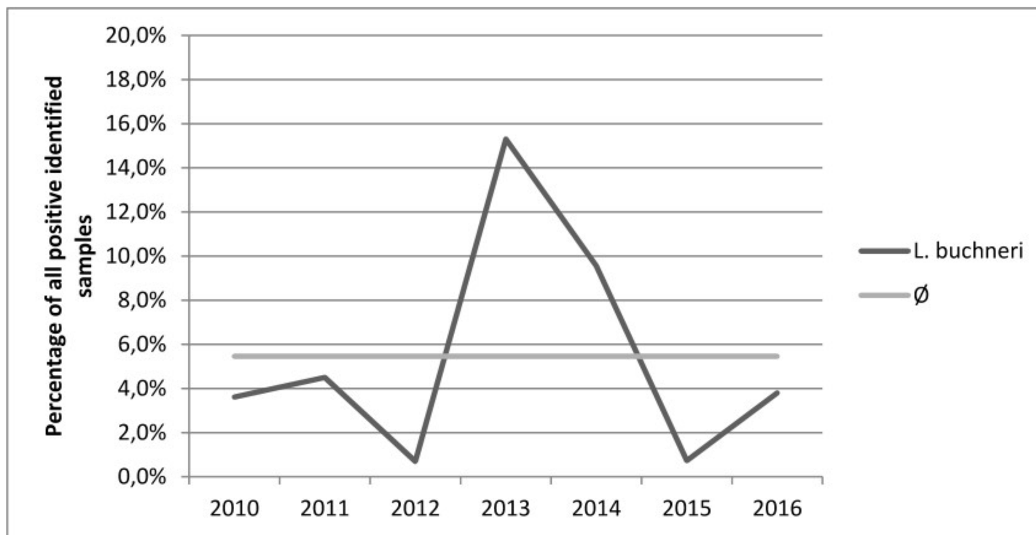



Figure S7: Percentage of samples positive for *L. buchneri* from 2010 – 2016 including 7-year mean value

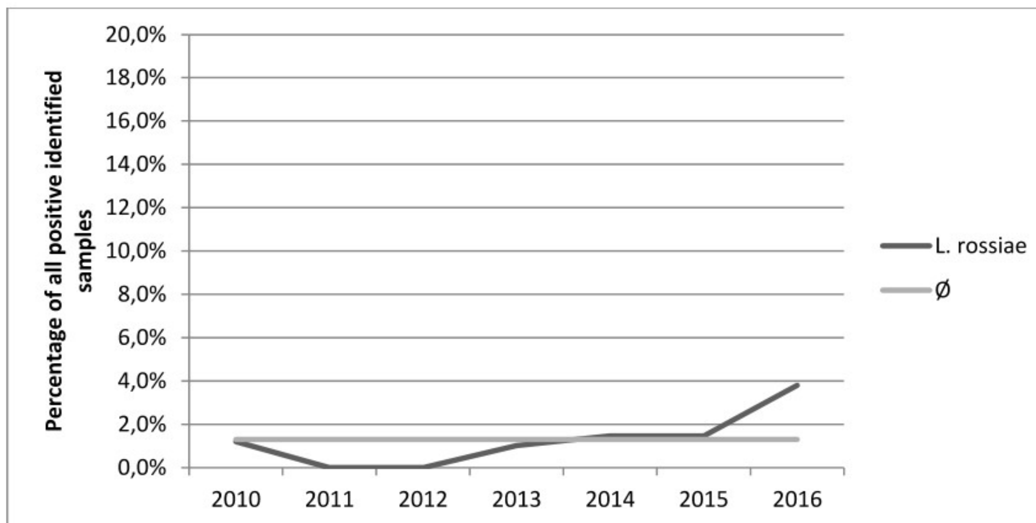



Figure S8: Percentage of samples positive for *L. rossiae* from 2010 – 2016 including 7-year mean value

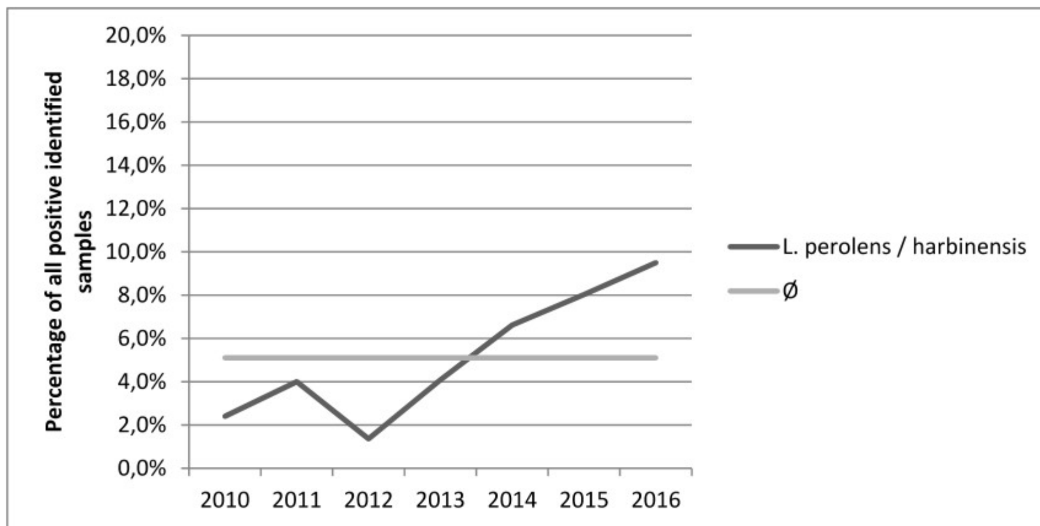



Figure S9: Percentage of samples positive for *L. perolens / harbinensis* from 2010 – 2016 including 7-year mean value

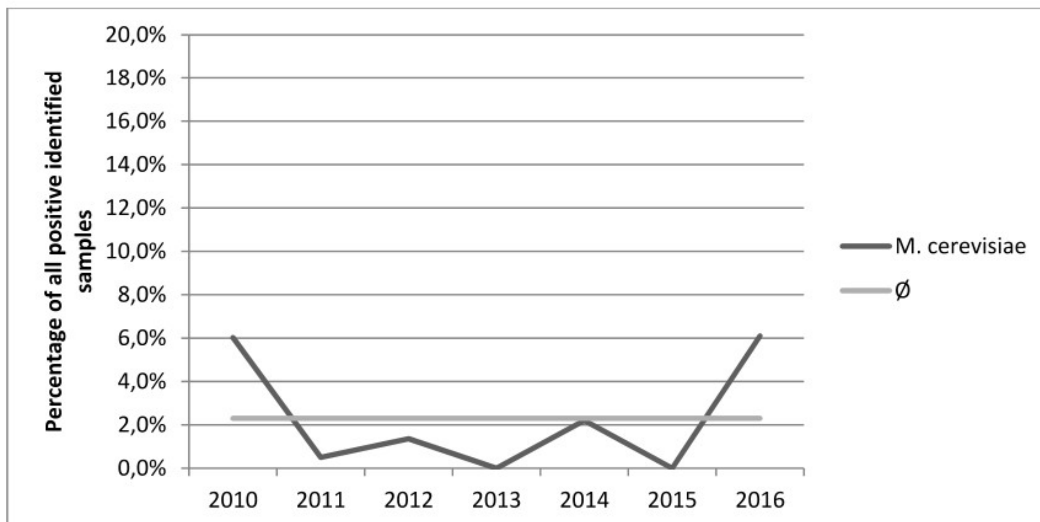



Figure S10: Percentage of samples positive for *M. cerevisiae* from 2010 – 2016 including 7-year mean value

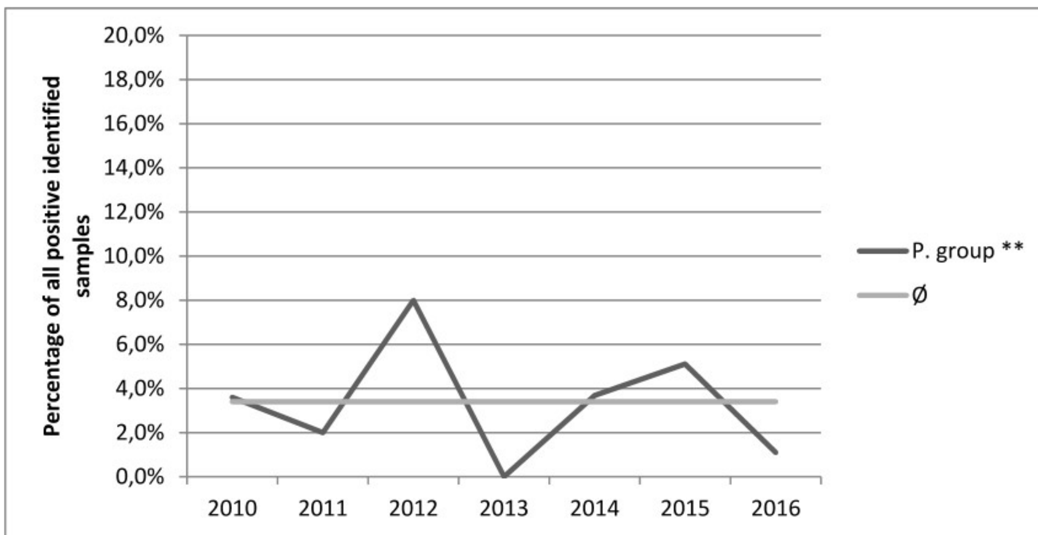



Figure S11: Percentage of samples positive for P. group (**= *P. frisingensis, P. cerevisiiphilus and P. haikarae*) from 2010 – 2016 including 7-year mean value

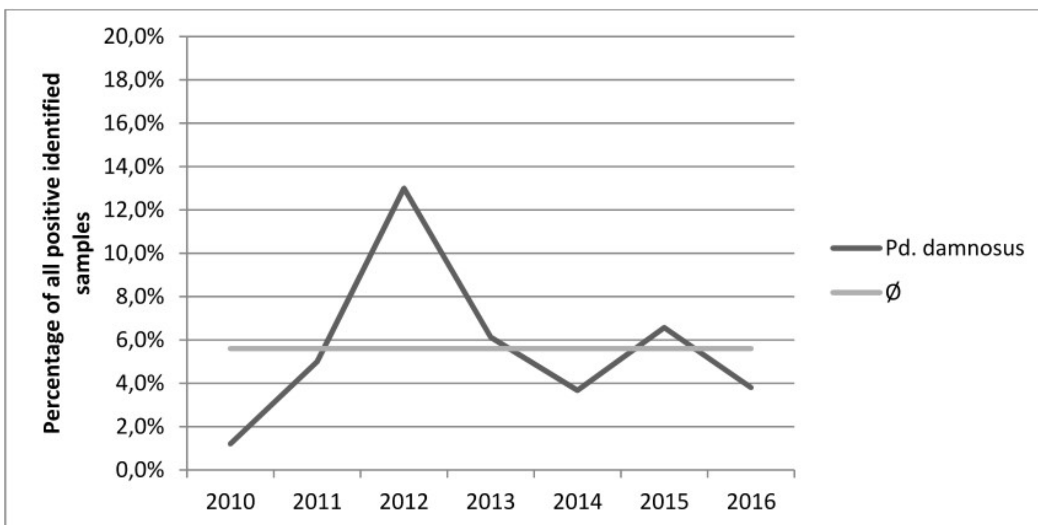



Figure S12: Percentage of samples positive for *Pd. damnosus* from 2010 – 2016 including 7-year mean value

## 3.3 CHAPTER B – Enhanced cultivation of beer spoilage bacteria by enforced yeast suppression

BrewingScience September / October 2017 (Vol. 70) 142

J. Schneiderbanger (former Koob), H. Schneiderbanger, F. Jacob and M. Hutzler

# Enhanced Cultivation of Beer Spoilage Bacteria in Propagation Yeast by Enforced Yeast Suppresion

**It is enormously important to be able to detect beer spoilage bacteria during the brewing process. These bacteria have the potential, for instance, to cause turbidity, acidity, and detrimental flavor changes in the product. It is extremely difficult to detect these beer spoilage organisms, in particular in the pure yeast culture or even in the yeast crop, as their growth is suppressed by the yeast. Furthermore, these bacteria are often only present as trace contaminants. In this study, a method was developed to more reliably and more quickly detect beer spoilage bacteria in pure yeast cultures than by previous methods. A natural antibiotic called Natamax® (Danisco, Niebull, Germany), which reliably kills the yeast cells but does not affect the bacteria, was added to the yeast samples. This made it possible to detect a significantly higher quantity of beer spoilage organisms than is possible without adding this antibiotic.**

Descriptors: beer spoilage bacteria, lactic acid bacteria, Saccharomyces cerevisiae, yeast, natamycin, Natamax®

## 1 Introduction

Quality assurance plays a decisive role in every brewery. Besides technical chemical parameters, the microbiological safety of the beer is critical. If the microbiological purity of the beer is not ensured, this can lead to negative aroma changes, haze, acidification, right through to consistency changes [2, 4, 10]. For this reason, breweries constantly endeavor to subject sensitive areas of production to regular microbiological testing by means of stage-by-stage controls. In the field of brewery microbiology, a distinction is made between bacteria that are optimally adapted to the beer environment and can multiple within it without adaptation (obligate beer spoilage bacteria), and bacteria that can proliferate only after a certain adaptation phase, or only in certain beer types with reduced selective properties (potential beer spoilage bacteria) [3].

Many breweries use the traditional method of applying the product samples, rinse water samples, or wipe samples to several different culture media and incubating these at optimum temperatures for bacteria (28 ± 1 °C) in order to concentrate any contaminating bacteria [11, 14]. Depending on the type of sample, this kind of testing can take between 7 and 21 days, sometimes even longer, until the number of contaminants reaches the relevant detection limit (microscope: $10^5$ cells/mL; real-time PCR (polymerase chain reaction): $10^2$–$10^3$ cells/mL) and a reliable result can be obtained.

**https://doi.org/10.23763/BrSc17-14Schneiderbanger**

**Authors**

Jennifer Schneiderbanger (former Koob), Hubertus Schneiderbanger, Fritz Jacob, Mathias Hutzler, Research Center Weihenstephan for Brewing and Food Quality, Technical University of Munich, Freising, Germany; corresponding author: hutzler@wzw.tum.de

These days larger breweries in particular employ PCR-based methods that provide a fast and reliable means of detecting selected harmful bacteria at low concentrations.

Regardless of the detection method used, the detection of beer spoilage microorganisms in culture yeast poses particular problems for breweries. In thick yeast slurry, e.g. in propagation yeast and especially in the yeast crop, beer spoilage bacteria can “hide” and be extremely difficult to detect amid the high number of yeast cells (e.g. $10^2$ cells/mL bacteria per $10^8$ cells/mL yeast). Vital yeast also suppresses the growth of bacteria, so the essentially small number of bacteria present in the sample will increase very little in the presence of the yeast cells, even under optimum conditions. This is why several growth media (for example NBB media (Doehler)) contain a yeast-repressing adding. However, the detection of beer spoiling bacteria remains difficult. Furthermore, another difficulty with yeast samples is that the contaminating bacteria cannot be mechanically concentrated, as is possible, for example, for bright beer tank samples using a membrane filter. Repeated incubation in specific culture media is therefore the only option available to conventional brewery microbiology. It is also not always possible to reliably detect beer spoilage bacteria using modern molecular biological methods as the necessary detection limits cannot be reached due to the suppressive effect of the brewing yeast.

The natural antibiotic Natamax® from Danisco is used to specifically kill yeast cells [7]. Natamax® contains natamycin, which is produced during fermentation by the bacterium *Streptomyces natalensis* and mixed with lactose. This agent is typically used in cheese manufacture or for other non-sterile foods where it provides a reliable means of killing undesirable yeasts and molds [1]. It is also used in other areas of the food industry such as in the production of fish and meat products. Natamycin inhibits endocytosis (the active uptake of membrane vesicles into the cell) in yeast cells by binding to ergosterol [9, 12, 17]. In contrast to

other fungicidal agents such as filipin and nystatin, natamycin does not increase the permeability of the cytoplasmic membrane [17, 20]. Natamycin prevents the growth of yeasts and molds and causes these cells to die, though not due to the leakage of vital cell components as a result of an increase in membrane permeability. The precise mode of action of so-called polyene antimycotics is not yet fully understood but has been extensively discussed [6, 8, 12, 13, 16-20]. The growth of lactic acid bacteria (LAB) is not affected by the addition of natamycin [15].

**Table 1: Yeast and bacterial strains used**

| Microorganism | Characterization | Culture collection number |
|---|---|---|
| *Lactobacillus backi* | Obligate beer-spoiling | TUM BP 120123021-2124 |
| *Lactobacillus brevis* | Obligate beer-spoiling | TUM BP 120711011-2578 |
| *Lactobacillus casei* | Potential beer-spoiling | TUM BP 120509129-2360 |
| *Lactobacillus harbinensis* | Potential beer-spoiling | TUM BP 120906016-2993 |
| *Lactobacillus lindneri* | Obligate beer-spoiling | TUM BP 120703011-2512 |
| *Pediococcus damnosus* | Obligate beer-spoiling | TUM BP 121012022-3041 |
| *Saccharomyces pastorianus* | Bottom-fermenting beer yeast | TUM 34/70 |

The aim of this study was to develop a method of reliably killing yeast cells in high concentrations without, however, adversely affecting the growth of beer spoilage bacteria to improve and speed up the their detection in yeast samples. Even if the subsequent enrichment medium contains a yeast-suppressing adding, the guarantee of 100 % dead yeast cells in a short time is advantageous for a secure identification. The objective was to ensure that the growth of the bacteria starts significantly earlier thanks to reduced competition from the yeast cells. Various test approaches comprising both culture and molecular biological methods were used to verify whether the detection of beer spoilage bacteria in yeast containing samples can be accelerated by adding Natamax®. Any time saving achieved would provide a practical benefit to the brewing microbiology lab and, ultimately, the brewery commissioning the testing who will receive the results more quickly and be able to react accordingly.

## 2 Material and Methods

### 2.1 Yeast and bacterial strains

The most frequently used bottom-fermented yeast *Saccharomyces* (S.) *pastorianus* TUM 34/70 and six different beer spoilage bacteria (4 obligate beer spoiling; 2 potentially beer spoiling) of the genera *Lactobacillus* (L.) and *Pediococcus* (P.) were used (Tab. 1). The yeast was obtained from the Yeast Center at the Research Center Weihenstephan for Brewing and Food Quality, where they are routinely checked for the presence of microorganisms using molecular biological methods. All bacterial strains used were isolated from beer samples, separated, identified using molecular biological methods, and preserved in a strain collection at – 80 °C in a cryobank.

After cultivating the yeast and bacterial strains in suitable culture media at 28 °C, isolation streaks of all the cultures were prepared and 10 individual colonies from each were combined to produce corresponding initial suspensions. These were tested for contamination using real-time PCR (see 2.4).

### 2.2 Cell count determination

The cell count determination was performed in triplicate and using a Thoma counting chamber (Bast, 1999). For each test, sterile wort was used to adjust the yeast cell count to $10^8$ cells/mL, which is comparable to the cell count in a sample of thick yeast slurry. After counting, the bacterial suspensions were adjusted to the desired cell count ($10^1$, $10^2$, $10^3$, etc. cells/mL) using a 2 % saline solution.

### 2.3 Culture methods

Culture methods in brewery microbiology employ a variety of enrichment media that are tailored to the growth requirements of the type of microorganism being cultivated. Bottom- and top-fermenting brewing yeast is particularly well adapted to the wort medium, which is why liquid cultures are grown in sterile wort. To prepare the agar plates, 2 % agar-agar, and tetracycline to kill off undesired bacteria, were added to wort (wort agar).

For the beer spoilage bacteria, a detection medium was used that fulfils the most important nutrient and growth requirements of this small group of bacteria that is well-adapted to the beer environment (NBB®, detection medium for beer spoilage bacteria, Döhler). NBB® broth was used for liquid enrichment, and NBB® agar for quantitative analyses. The growth of the yeast cells was investigated in an aerobic atmosphere, and the growth of the bacteria in an anaerobic atmosphere, in each case at 28 °C.

### 2.4 Molecular biological methods

Extraction was performed using the InstaGene™ Matrix (Bio-Rad) as per the manufacturer's instructions for gram-positive microorganisms. The sample volumes were 1 mL for liquid samples, and 1 colony for agar plate samples. Identification and quantification of the bacteria was performed using the LightCycler® 480 II (Roche) and foodproof® beer screening kits (Biotecon Diagnostics). The beer spoilage bacteria were identified on the basis of a melting curve analysis. The quantity of DNA present in the sample was determined by means of the so-called cycle threshold (Ct) value, which identifies the point at which exponential growth of the PCR products begins. The lower this Ct value, the more DNA was present initially.

### 2.5 Experimental

*Determination of the exposure time*

In a triplicate series of tests, 50 ml of yeast suspension ($10^8$ cells/mL) was added to an Erlenmeyer flask and mixed with *L. brevis* ($10^2$, $10^3$ and $10^4$ cells/mL) and 5 g/L Natamax®. A parallel sample without Natamax® was prepared. The samples were stored in an aerobic atmosphere at 20 °C.

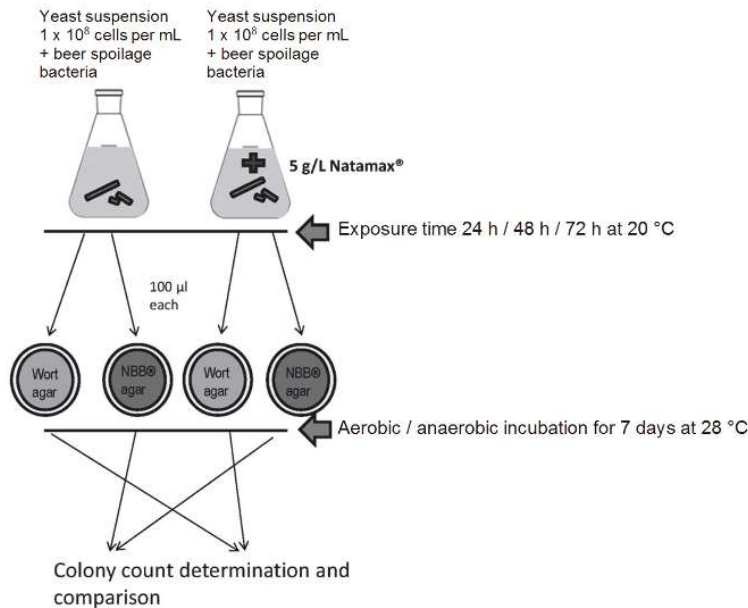



**Fig. 1** **Schematic drawing of cultural approach**

After 24 h, 48 h and 72 h, 100 µl of each test solution was smeared onto wort agar and NBB® agar using a spatula. The wort agar plates and NBB® agar plates were evaluated after 7 days of aerobic or anaerobic incubation at 28 °C. At the same time, the required exposure time for Natamax® to reliably kill the $10^8$ cells/mL of yeast cells without reducing the bacterial cell count was also compared.

*Culture method*

Six different strains of beer spoilage bacteria were inoculated in yeast suspensions with cell counts of $10^1$ and $10^3$ cells/mL in each case with and without 5 g/L antimycotic. An exposure time of 48 h was selected. 100 µL of each suspension was then smeared onto wort agar to determine the yeast concentration, and 100 µL of each suspension as well as 2 dilutions (1:10 with sterile water) onto NBB® agar to determine the bacterial concentration. The agar plates were then incubated aerobically (for wort agar) and anaerobically (for NBB® agar) for 7 days at 28 °C. If the addition of the antimycotic suppresses the growth of the yeast cells and promotes the growth of the inoculated bacteria, the bacterial cell count in the suspension with added Natamax® ought be higher than in the comparison sample, and the yeast cell count lower (Fig. 1).

*PCR method*

As the PCR method is often used nowadays in brewery microbiology to test for the absence of bacteria in product, a further real-time PCR-based test was performed in this study. Instead of smearing the bacteria/ yeast suspension onto culture mediums after the exposure time, 1 mL of each sample underwent molecular biological analysis. Comparing the Ct values provides information on the growth of the used bacteria.

## 3 Results and Discussion

*Effect of exposure time*

Following incubation on wort agar, all samples to which no Natamax® had been added were overgrown with yeast, thus making it impossible to determine specific cell counts. As a result of adding 5 g/L of the antibiotic and after an exposure time of just 24 h, no yeast colonies were identifiable after aerobic incubation on wort agar.

To detect the beer spoilage bacteria, 100 µl was taken from the same samples after 24 h, 48 h and 72 h, smeared onto NBB® agar, and incubated under anaerobic conditions at 28 °C (Fig. 2).

As the exposure time to the antimycotic increases, so too does the number of colonies on the NBB® agar. An increase in colonies depending on the initial bacterial count used was also observed. The low recovery rate (e.g. initial bacterial count: $10^4$ bacteria/mL; bacterial count after 48 h: 150 colonies / 100 µL = 1.5 x $10^3$ cells/mL) is explained by the exposure of the bacterial cells to stress, triggered by the yeast-containing medium, and by the fact that one colony on agar does

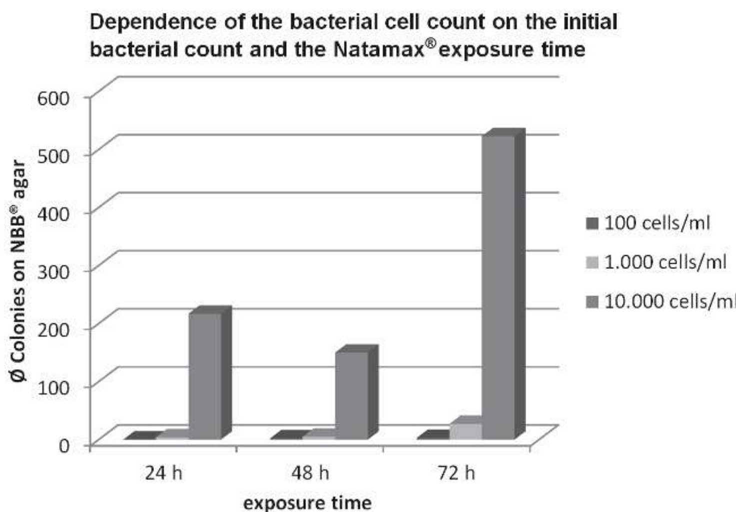



**Fig. 2** **Change in cell count of L. brevis depending on the initial bacterial count and exposure time to Natamax®**

not necessarily correspond to one cell, but usually several cells in a conglomerate.

While the initial bacterial count of $10^4$ cells/mL gave the best results, this scenario is not representative, however, of the problem to be solved in practice. The addition of antibiotic is intended, in particular, to provide a faster and more reliable method of detecting trace contaminants, so bacterial cell counts of $10^1$ and $10^3$ cells/mL were selected in subsequent tests.

*Test approach using cultures*

With an initial cell count of $10^3$ cells/mL, no significant increase in cell count as a result of adding the antimycotic could be determined for the *L. brevis* bacterium as the agar plates were completely overgrown in both preparations. *L. casei*, on the other hand, could no longer be cultivated in either test preparation (with or without Natamax®) after a 48 hour exposure time. For the four other species, a significant increase in the bacterial counts could be achieved by adding Natamax® (Fig. 3).

When using only 10 cells/mL, *L. brevis* again grew the fastest and overgrew the corresponding agar plates in both the preparations with and without the addition of Natamax®. *L. casei* and *P. damnosus* could not be cultivated under the selected conditions. The final bacterial count of *L. backi* was not significantly different for the preparations with or without antibiotic. A significant increase in cell count was observed for the *L. harbinensis* and *L. lindneri* bacteria (Fig. 4).

*Test approach using real-time PCR*

Having demonstrated using the culture detection method that a higher bacterial count could be detected by adding Natamax®, the next step was to perform verification using PCR. Because conventional PCR systems require a minimum bacterial count of 100–1000 bacteria for reliable detection, and since it was necessary to maintain the same basic cultivation conditions as in the preceding tests, an initial bacterial count of 100 cells per milliliter was used. The Ct values obtained are shown in tables 2 and 3 (see page 146), where a Ct value of 40 represents the maximum possible Ct value for the PCR system used. A Ct value of 40 means that the quantity of DNA present in the sample is insufficient to be detected.

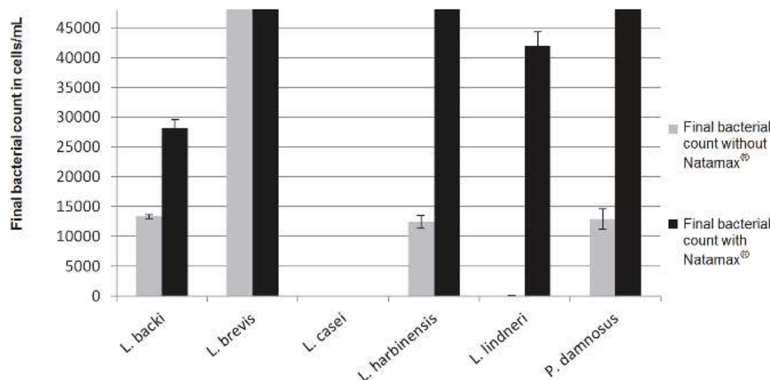



**Fig. 3** **Final bacterial count in the samples with and without the addition of Natamax® for an initial bacterial concentration of 103 cells/mL**

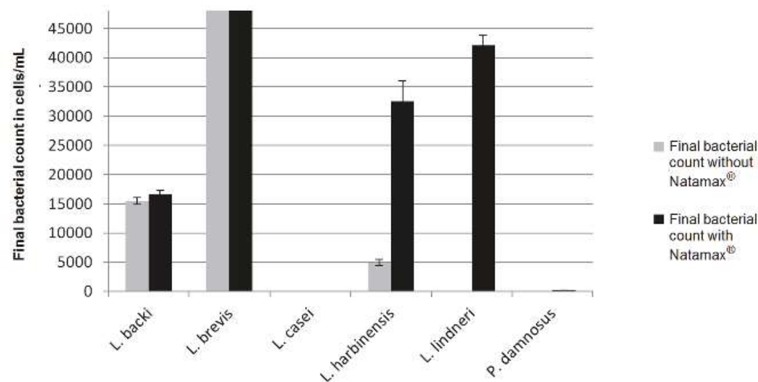



**Fig. 4** **Final bacterial count in the samples with and without the addition of Natamax® for an initial bacterial concentration of 101 cells/mL**

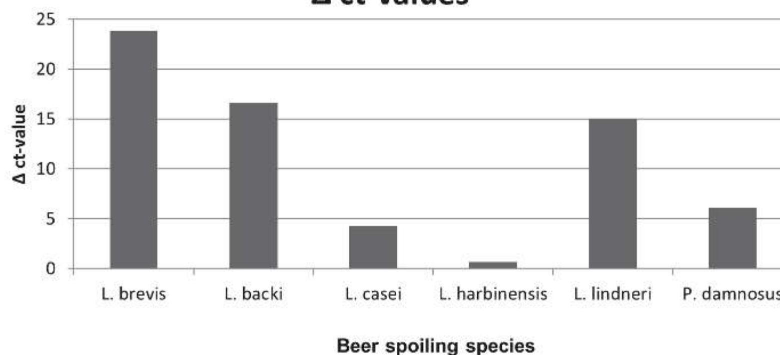



**Fig. 5** **Delta Ct values (= Ct value without Natamax® – Ct value with Natamax®)**

**Table 2** Ct values for the series of tests without the addition of Natamax®

| Bacterial strain | Original cell amount [cells/mL] | Ct value 1 without Natamax® | Ct value 2 without Natamax® | Ct value 3 without Natamax® | Average Ct value | Standard deviation | Confidence interval |
|---|---|---|---|---|---|---|---|
| *L. brevis* | 100 | 40.0 | 40.0 | 40.0 | 40.0 | 0.0 | 0.0 |
| *L. backi* | 100 | 33.2 | 31.2 | 40.0 | 34.8 | 3.8 | 4.3 |
| *L. casei* | 100 | 40.0 | 40.0 | 40.0 | 40.0 | 0.0 | 0.0 |
| *P. damnosus* | 100 | 40.0 | 40.0 | 31.1 | 37.0 | 4.2 | 4.8 |
| *L. harbinensis* | 100 | 32.7 | 31.8 | 32.3 | 32.3 | 0.4 | 0.5 |
| *L. lindneri* | 100 | 40.0 | 23.5 | 31.5 | 31.7 | 6.7 | 7.6 |

**Table 3** Ct values for the series of tests with the addition of Natamax®

| Bacterial strain | Original cell amount [cells/mL] | Ct value 1 with Natamax® | Ct value 2 with Natamax® | Ct value 3 with Natamax® | Average Ct value | Standard deviation | Confidence interval |
|---|---|---|---|---|---|---|---|
| L. brevis | 100 | 15.5 | 16.5 | 16.6 | 16.2 | 0.5 | 0.6 |
| L. backi | 100 | 17.7 | 18.5 | 18.1 | 18.1 | 0.3 | 0.4 |
| L. casei | 100 | 40.0 | 34.0 | 33.2 | 35.7 | 3.0 | 3.4 |
| P. damnosus | 100 | 29.2 | 31.8 | 31.9 | 31.0 | 1.3 | 1.4 |
| L. harbinensis | 100 | 32.3 | 30.4 | 32.2 | 31.6 | 0.9 | 1.0 |
| L. lindneri | 100 | 16.8 | 16.7 | 16.5 | 16.7 | 0.1 | 0.2 |

As can be seen from tables 2 and 3 and figure 5, the results from the tests using culture enrichment (Fig. 3 and 4) could be verified. The DNA of all beer spoilage organisms tested could be detected in higher concentrations by adding Natamax®. All Ct values were lower than for the test samples with no antibiotic added to them. While the Ct values for the *L. casei*, *L. harbinensis* and *P. damnosus* samples were quite high, they were still within the detectable range. This was not possible without adding Natamax®.

## 4 Conclusion

As a result of using the natamycin-containing antibiotic, the cultured yeast was able to be reliably killed at a concentration of $10^8$ cells/mL after 48 h. The addition of Natamax® proofed to be a practical tool for a secured and quicker identification of beer-spoiling bacteria in yeast samples. Especially the addition before the cultivation in a growth medium can speed up the identification of bacteria since 100 % dead yeast cells can be guaranteed. The growth of the most relevant beer spoilage organisms was not affected by the addition of Natamax® (Fig. 3). A contact time of 24 h with Natamax® led to a significantly higher number of detectable beer spoilage bacteria on agar plates. These results could be confirmed in PCR tests. Why some *Lactobacillus* strains were not detected in significantly higher number under the natamycin influence should be investigated in further studies. Also, a broader range of beer spoiling species and strains could be evaluated. However, adding 5 g/L Natamax® to conventional yeast samples enables trace contaminants to be detected more quickly and reliably. This does not impose any significant additional effort in everyday brewery laboratory practice, but leads to an optimized quality assurance process.

## 5 Notes

The authors declare no competing financial interest.

## 6 Literature


1. Aparicio, J.F.; Colina, A.J.; Ceballos, E. and Martin, J.F.: The biosynthetic gene cluster for the 26-membered ring polyene macrolide pimaricin – A New polyketide synthase organization encoded by two subclusters separated by functionalization genes, Journal of Biological Chemistry, **274** (1999), pp. 10133-10139.
2. Back, W.: Farbatlas und Handbuch der Getränkebiologie, Fachverlag Hans Carl, Nürnberg, Germany, 1994.
3. Back, W.: Ausgewählte Kapitel der Brauereitechnologie, Band 1, Fachverlag Hans Carl, Nürnberg, Germany, 2005.
4. Bast, E.: Mikrobiologische Methoden, Spektrum Akademischer Verlag, Berlin, Germany, 1999.
5. Blackburn, C.D.W.: Food spoilage microorganisms, Woodhead Publishing Ltd. Cambridge, England, 2006.
6. Bolard, J.: How do the polyene macrolide antibiotics affect the cellular membrane-properties, Biochimica Et Biophysica Acta, **864** (1986), pp. 257-304.
7. Danisco Deutschland GmbH: Natamax® product description, 2010.
8. Hamilton-Miller, J.M.T.: Chemistry and biology of polyene macrolide antibiotics, Bacteriological Reviews, **37** (1973), pp. 166-196.
9. Heese-Peck, A.; Pichler, H.; Zanolari, B.; Watanabe, R.; Daum, G. and Riezman, H.: Multiple functions of sterols in yeast endocytosis, Molecular Biology of the Cell, **13** (2002), pp. 2664-2680.
10. Hill, A.E.: Brewing Microbiology – Managing Microbes, ensuring quality and valorising waste, Woodhead Publishing Ltd. Cambridge, England, 2015.

147 September / October 2017 (Vol. 70) BrewingScience

11. Methner, F.J.: Microbreweries – from project planning to quality assurance, author's edition of MEBAK, Freising, Germany, 2017.
12. Munn, A.L.; Heese-Peck, A.; Stevenson, B.J.; Pichler, H. and Riezman, H.: Specific sterols required for the internalization step of endocytosis in yeast, Molecular Biology of the Cell, **10** (1999), pp. 3943-3957.
13. Operakova, M. and Tanner, W.: Membrane Transport Inhibition as Mode of Action of Polyene Antimycotics: Recent Data Supported by Old Ones, Food Technology and Biotechnology, **52** (2014), pp. 8-12.
14. Riedl, R.; Goderbauer, P.; Brandl, A.; Jacob, F. and Hutzler, M.: Bavarian wheat beer, an example of a special microbe habitat – Cultivation, detection, biofilm formation, characterization of selected lactic acid bacteria hygiene indicators and spoilers, BrewingScience, **70** (2017), no. 1/2, pp. 39-50.
15. Romero-Gil, V.; Garcia-Garcia, P.; Garrido-Fernandez, A. and Arroyo-Lopez, F.N.: Susceptibility and resistance of lactic acid bacteria and yeasts against preservatives with potential application in table olives, Food Microbiology, **54** (2016), pp. 79-86.
16. Streekstra, H.; Verkennis, A.E.E.; Jacobs, R.; Dekker, A.; Stark, J. and Dijksterhuis, J.: Fungal strains and the development of tolerance against natamycin, International Journal of Food Microbiology, **238** (2016), pp. 15-22.
17. Van Leeuwen, M.R.; Golovina, E.A. and Dijksterhuis, J.: The polyene antimycotics nystatin and filipin disrupt the plasma membrane, whereas natamycin inhibits endocytosis in germinating conidia of Penicillium discolor, Journal of Applied Microbiology, **106** (2009), pp. 1908-1918.
18. Van Leeuwen, M.R.; Smant, W.; De Boer, W. and Diksterhuis, J.: Filipin is a reliable in situ marker of ergosterol in the plasma membrane of germinating conidia (spores) of Penicillium discolor and stains intensively at the site of germ tube formation, Journal of Microbiological Methods, **74** (2008), pp. 64-73.
19. Van Leeuwen, M.R.; Van Doorn, T.M.; Golovina, E.A.; Stark, J. and Dijksterhuis, J.: Water- and Air-Distributed Conidia Differ in Sterol Content and Cytoplasmic Microviscosity, Applied and Environmental Microbiology, **76** (2010), pp. 366-369.
20. Welscher, Y.M.T.; Ten Napel, H.H.; Balague, M.M.; Souza, C.M.; Riezman, H.; De Kruijff, B. and Breukinko, E.: Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane, Journal of Biological Chemistry, **283** (2008), pp. 6393-6401.

*Received 6 September 2017, accepted 18 October 2017*

## 3.4 CHAPTER C – Detection of a new bacterial species with beer-spoilage potential

### Section 1 *Lactobacillus* sp. brewery isolate: A new threat to the brewing industry?

BrewingScience July / August 2016 (Vol. 69) 42

J. Koob, F. Jacob, F.-J. Methner and M. Hutzler

# *Lactobacillus* sp. brewery isolate: A new threat to the brewing industry?

**Only a restricted group of bacterial species is known to be capable to spoil beer. To maintain a good microbiological quality control it is important to know which microbes are hazardous for the brewing industry.**
**Three *Lactobacillus (L.)* isolates that could not be identified by a commercial realtime PCR system for the detection and identification of beer-spoilage bacteria (Foodproof® Beer Screening Kit, Biotecon Diagnostics, Germany) were obtained from brewery samples. A multivariate study was conducted on the basis of phenotypic, genotypic and beer-related characteristics. The tests were carried out with regard to the two most probable species, *L. brevis* and *L. parabrevis*.**
**The comparison of the 16S rRNA gene and the *pheS* housekeeping gene sequences revealed that the three isolates belong to one species or one operational taxonomic unit (OTU). Furthermore, the unknown brewery isolate could be differentiated from the two most probable species by gene sequence comparisons and DNA-DNA hybridizations. The evaluation of physiological characteristics of the unknown brewery isolate did not demarcate it clearly. The Lactobacillus isolate contained two hop resistance genes and was able to grow in four different beer types resulting in significant compound concentration changes.**
**It was determined that the unknown bacterium did not belong to the species *L. brevis* or *L. parabrevis.* In parallel, a species description is submitted to the International Journal of Systematic and Evolutionary Microbiology. Therein, further discrimination of the isolated bacterium from genetically related species (*L. yonginensis, L. koreensis, L. hammesii)* with no relation to the brewing sector is shown.**

Descriptors: lactic acid bacteria, beer spoilage, brewery isolate, *Lactobacillus brevis*, *Lactobacillus parabrevis*, multivariate study

## 1 Introduction

Beer is known to be microbiologically stable. Only a few bacteria and yeasts are able to tolerate the hop and alcohol content, the anaerobic atmosphere and the altered nutrient composition compared to wort. The largest group of beer spoilage microorganisms belongs to the genus *Lactobacillus (L.)* with the most frequent spoilage species being *L. brevis.* In some years, *L. brevis* causes more than 40 % of all bacterial spoilage incidents in breweries [5, 3, 4, 26, 32].

Typical consequences of microorganism growth in beer are sediment formation, increased turbidity and acidity, off-flavors and, in the worst case, slime formation. Brewing microbiologists are still trying to develop new strategies to detect contaminations faster and to identify the spoiling bacterium down to strain level, if necessary. The characteristics a bacterium requires to be able to spoil beer are not yet fully understood. Every now and then new species show up in the brewing environment, which, after adapting to the adverse properties of beer, enlarge the group of beer-spoilage organisms. Recently included species in this restricted collection of bacteria are *L. backii, L. rossii, L. paucivorans* and *L. acetotolerans* [8, 11, 16, 17, 36, 56].

In 2013 and 2014, three bacterial isolates were obtained from turbid beer samples of a German brewery that could not be identified by the commercially available realtime PCR foodproof® beer screening kit of Biotecon Diagnostics (Potsdam, Germany). Subsequent 16S rRNA gene sequencing and BLAST (Basic Local Alignment Tool) analysis revealed the species with the highest values of sequence homology to be *L. koreensis* (99.45 %), *L. yonginensis* (98.82 %), *L. parabrevis* (98.71 %), *L. brevis* (98.69 %) and *L. hammesii* (98.24 %).

Except for *L. brevis*, none of these species was known to have beer-spoilage potential. Due to the fact that *L. parabrevis* was originally classified as *L. brevis* and the latter is the most dangerous and most common beer-spoilage bacterium [42, 57], it was assumed that the unknown Lactobacillus isolates would belong either to the species *L. brevis* or to *L. parabrevis.* Additional reasons for the inclusion of *L. parabrevis* in further analyses were that the type strain derived from wheat and its malt is a raw material used for wheat beer production in German breweries. Furthermore, the *L. parabrevis* type strain was isolated in Europe. In 2011, a contamination incident with *L. parabrevis* was reported to the Research Center Weihenstephan

**Authors**

Jennifer Koob[a], Prof. Dr. Fritz Jacob[a], Prof. Dr.-Ing. Frank-Jürgen Methner[b], Dr. Mathias Hutzler[a];
[a]Technische Universität München, Research Center Weihenstephan for Brewing and Food Quality, Freising, Germany;
[b]Technische Universität Berlin, Institute of Food Technology and Food Chemistry, Department Brewing Science, Berlin, Germany;
corresponding author: hutzler@wzw.tum.de

for Brewing and Food Quality, TU München, which was identified by another brewing microbiology laboratory. Unfortunately, the isolated beer-spoilage strain was not stored and the identification could not be reconfirmed by the Research Center Weihenstephan. In summary, these facts contributed to the decision that *L. brevis* and *L. parabrevis* were further analyzed for their beer-spoilage ability. To verify the brewery isolates' identity and to demarcate it from two different strains of *L. brevis* as well as the *L. parabrevis* type strain, physiological and molecular biological methods were executed in a multivariate study. By beer incubation test it was determined in which beer type the newly obtained brewery isolate was able to grow using a standard method of the Research Center. This beer incubation test was performed under standardized conditions minimizing the inoculation time when oxygen and carbon-dioxide concentrations of the bottles are manipulated and minimizing the oxygen uptake by manual stimulation to foam over. This method is state of the art to test if a product is susceptible for a certain microbe that is inoculated in a defined cell concentration. In case of growth, the impact of the bacterial metabolism on the respective beer type was evaluated by physico-chemical analyses. Additionally, the presence of genes was checked that were discussed to be related to hop resistance.

## 2 Material and methods

### 2.1 Strains

The three *Lactobacillus* brewery isolates were obtained from a lager tank sample, a beer sample just before filling and a bright beer tank sample of the same brewery at three different points in time within a period of eight months. Since the isolates showed 100 % similarity in 16S rRNA gene as well as in pheS (phenylalanyl-tRNA synthase alpha subunit) housekeeping gene sequence (Fig. 1 and 2), all further analyses were performed using only one of the isolates (L. sp. 2301; Table 1) which derived from the bright beer tank sample.

The *L. parabrevis* and *L. brevis* type strains as well as one highly beer-spoiling *L. brevis* brewery isolate were included in the analyses.

**Table 1 List of species used in this study**

| Designation | Reference number | Characteristics |
|---|---|---|
| *L.* sp. 1872 | TUM BP 130919043-2789[a] | Brewery isolate, lager tank sample |
| *L.* sp. 1921 | TUM BP 131010000-2400[a] | Brewery isolate, sample before filling |
| *L.* sp. 2301 | TUM BP 140423000-2250[a] | Brewery isolate, bright beer tank sample |
| *L. parabrevis* | LMG 11984[T b] | Type strain |
| *L. brevis* | DSM 20054[T c] | Type strain |
| *L. brevis* 31 | TUM BP 111115005-2022[a] | Brewery isolate, finished Pilsener beer |

[a] = Culture collection of the Research Center Weihenstephan, Freising, Germany; [b] = purchased from the Belgian Coordinated Collections of Microorganisms (BCCM™), Ghent, Belgium; [c] = purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany

### 2.2 Microorganism cultivation and DNA isolation

The brewery isolates and the strains of the culture collections were fractionated streaked on MRS agar and incubated anaerobically [13]. Ten colonies of each strain were picked and pooled to form the initial cultures. The pooled pure cultures were re-suspended in MRS broth and stored in glycerol at –80°C in a cryobank. Active strains were cultivated in MRS broth and weekly transferred into fresh medium. Strain identity was monthly checked using a $GTG_5$ PCR capillary electrophoresis typing with subsequent Bionumerics fingerprint analysis. The strain patterns remained stable over the period of the study (data not shown).

Bacterial DNA was extracted using the InstaGene™ matrix according to the manufacturer's instructions for bacteria (Bio-rad Laboratories, Munich, Germany). The DNA content was determined by spectrometric analysis using the NanoDrop ND 1000 (Thermo Scientific Fisher, Wilmington, USA) and adjusted with Ampuwa® (Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany) to the initial concentration of 100 ng/µL.

### 2.3 Molecular biological methods

The complete 16S rRNA gene sequence was determined using the primer pairs 27f / 1492r and 933f / 1541r (Table 2). The PCR procedure consisted of an initial denaturation step at 95 °C for 5 min 35 cycles of 95 °C/25 sec, 55 °C/40 sec, 72 °C/2 min and one final extension step at 72 °C for 5 min. The PCR reaction mixtures (50 µL in total) contained 25 µL 2-fold RedTaq mastermix containing

**Table 2 Primers used in this study**

| Primer | Sequence (5' → 3') | Source |
|---|---|---|
| 27f | AGA GTT TGA TCM TGG CTC AG | [44] |
| 933f | GCA CAA GCG GTG GAG CAT GTG G | [31] |
| 1492r | TAC GGY TAC CTT GTT ACG ACT T | [35] |
| 1541r | AAG GAG GTG ATC CAG CCG CA | [33] |
| *pheS*-forward | CAS GAT ACS TTC TAC ATY AC | [15] |
| *pheS*-reverse | ACC ATA CCR GCA CCY ACT TC | [15] |
| *rpoA*-21-F | ATG ATY GAR TTT GAA AAC C | [34] |
| *rpoA*-23-R | ACH GTR TTR ATD CCD GCR CG | [34] |
| *horA*-F | GGT CAA GGA ACT GTT GGC CA | This study |
| *horA*-R | TAA GAC CAA TGC GCC AAC CA | This study |
| *horA*-P[a] | TTC GGT TCC CAA AAC CGC AAC TTC G | This study |
| *horC*-F | TGA ATG CTC AAA TAT CGC AAT TG | This study |
| *horC*-R | CAC TTT GTT GCT GTG CGC TAA | This study |
| *horC*-P[a] | TAT CCC AAG CAC TTC CTA AGA TTG CAA ATG C | This study |
| 28F (*hitA*) | AGC GTA GCA GAA GAA CCT AAG | [20] |
| 207R (*hitA*) | CAA TTA CCA GGA TCC ATG TAC C | [20] |

[a] modified for realtime PCR application: 5' end reporter: 6-FAM, 3' end quencher: BHQ-1; P = probe

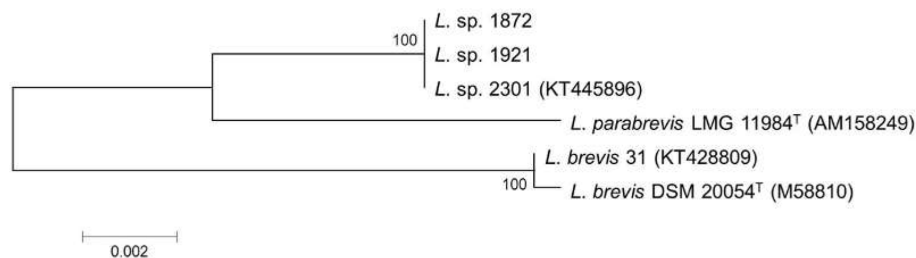



**Fig. 1 Neighbor-joining tree based on the 16S rRNA gene sequences of the three *Lactobacillus* sp. brewery isolates 1872, 1921 and 2301 (*L.* sp. 2301 GenBank accession no. KT445896), of the *L. parabrevis* type strain (AM158249), the *L. brevis* type strain (M58810) and the *L. brevis* brewery isolate 31 (KT428809). Bootstrap percentages > 50 % after 1000 simulations are shown [18]. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were executed using MEGA6 [53, 54]**

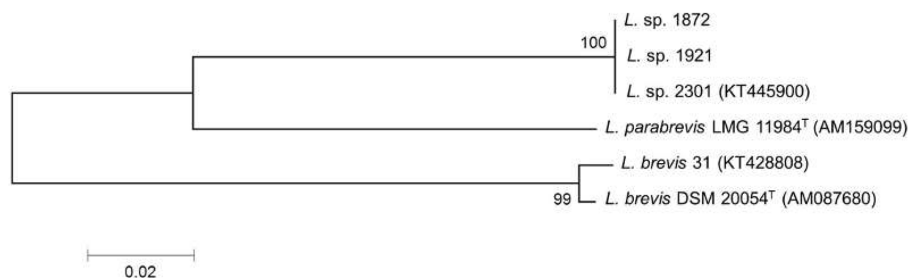



**Fig. 2 Neighbor-joining tree reconstructed from a comparative analysis of pheS gene sequences including the three *Lactobacillus* sp. brewery isolates 1872, 1921 and 2301 (L. sp. 2301 GenBank accession no. KT445900), *L. parabrevis* LMG 11984$^T$ (AM159099), *L. brevis* DSM 20054 (AM087680) and the *L. brevis* beer-spoilage isolate 31. Bootstrap values (expressed as percentages of 1000 replicates) > 50 % are shown [18]. Distances were computed using the Maximum Composite Likelihood method [53]. Bar, 0.02 substitutions per nucleotide position**

*Taq* polymerase, $MgCl_2$ and dNTP's (Genaxxon Bioscience GmbH, Ulm, Germany), 20 pmol of each primer, 5 µL DNA extract (100 ng/µL) and water (Ampuwa®, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany).

The sequence of the housekeeping gene *pheS* (product: phenylalanyl-tRNA synthase alpha subunit) was obtained by PCR using the primer pair *pheS*-forward / *pheS*-reverse (Table 2) as postulated by *Ehrmann* et al. [16]. The PCR mix for *pheS* gene amplification was composed of 25 µL 2-fold RedTaq mastermix, 25 pmol of each primer, 2.5 µL DNA extract and PCR-clean water adding up to a total volume of 50 µL. The temperature protocol for *pheS* gene PCR adopted from *Naser* et al. [34] was modified by changing the annealing temperature to 54 °C [16].

The PCR mix for the amplification of the *rpoA* housekeeping gene (product: RNA polymerase alpha subunit) contained in a total volume of 100 µL per reaction: 50 µL 2-fold RedTaq mastermix, 25 pmol of each primer (Tab. 2), 5 µL DNA template and sterile water. The PCR protocol was adopted from Naser et al. [34].

The size of the amplified fragments was checked by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). If electrophoresis displayed a single band of the expected size (for *pheS*: 300–350 bp; for *rpoA*: 700–800 bp), the PCR product was purified using the QIAquick® PCR purification kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer's instructions. The sequencing was performed by GATC Biotech AG (Constance, Germany). The obtained 16S rRNA sequences were trimmed and combined using the EditSeq software (DNASTAR, Madison, USA) followed by the comparison with stored type strain sequences using the neighbor-joining method of the MEGA6 (Molecular Evolutionary Genetics Analysis) software [18, 53, 54].

DNA-DNA hybridization is a method to differentiate two microbial strains sharing more than 97 % 16S rRNA gene homology [43, 55]. According to the recommendation of *Wayne* et al., 70 % DNA-DNA homology represent the threshold value for the definition of a new species [59]. DNA-DNA hybridizations were carried out at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) considering the pairings: *L.* sp. 2301 x *L. parabrevis* LMG 11984$^T$ and *L.* sp. 2301 x *L. brevis* DSM 20054$^T$ [10, 12, 25].

## 2.4 Physiological characterization

Sugar fermentation patterns were analyzed using the API CHL 50 system with *Lactobacillus paracasei* (BAA-52TM*) as the quality control organism (Biomerieux, Nürtingen, Germany).

Additional physiological characteristics were analyzed in MRS broth inoculated with 100 µL of overnight bacterial suspension in triplicates [13]. Alcohol tolerance (2.5–8.0 % (v/v) undenatured ethanol), salt tolerance (4.0, 6.5 and 8.0 % (w/w) NaCl), acid tolerance (pH values 4.0, 5.0, 7.2 and 7.8, adjusted with lactic acid or sodium hydroxide, respectively) and temperature tolerance (10, 15, 40 and 45 °C) were evaluated by visual control of turbidity and sediment formation in the test tubes over a period of four weeks. Incubation was carried out anaerobically using the Anaerocult®

**Table 3 Beer types and their properties for the beer incubation test**

| Beer type | Alcohol content [% (v/v)] | Bitter units [EBC units] | pH value | $CO_2$ content [% (v/v)] | Fermentable sugars[a] [g/100 mL] |
|---|---|---|---|---|---|
| Lager beer | 5.10 | 19.9 | 4.46 | 0.53 | 0.28 |
| Alcohol-free lager beer | 0.38 | 19.2 | 4.41 | 0.57 | 0.27 |
| Wheat beer | 5.64 | 13.0 | 4.61 | 0.68 | 0.17 |
| Alcohol-free wheat beer | 0.47 | 12.6 | 4.51 | 0.63 | 0.16 |
| Filtered wheat beer „Kristallweizen“ | 5.53 | 13.1 | 4.59 | 0.64 | 0.21 |

[a] sum of glucose, fructose, sucrose, maltose and maltotriose

system (Merck KGaA, Darmstadt, Germany) at 28 ± 1°C, if not indicated otherwise (i.e. temperature tolerance).

### 2.5 Genes associated with hop tolerance

The ability to tolerate the antibacterial properties of hop compounds is a main criterion for bacteria to grow in and spoil beer. Many studies have focused on the identification and application of different genes associated with hop resistance [6, 7, 19–24, 27–29, 38–41, 45–52, 58]. The plasmid-localized genes *horA* and *horC* that encode for multidrug transporters inserted into the cytoplasma membrane of gram-positive bacteria proved to be particularly good indicators for the ability to tolerate hop acids. The presence of *horA* and *horC* was analyzed by realtime PCR at the Research Center Weihenstephan for Brewing and Food Quality. The method was created to be compatible with the bacteria and yeast identification systems developed by *Brandl* and was modified using the *horA*- and *horC*-specific primers and probes listed in table 2 [9].

It was also proposed that the gene *hitA* confers hop tolerance to bacteria [24, 46]. Its product is homologous to divalent-cation transporters that can be found in many organisms. The endpoint PCR method for the detection of *hitA* was adopted from Haakensen and modified [20]. PCR mixes consisted of 12.5 µL 2-fold RedTaq mastermix, 5 pmol of the primers 28F and 207R (Table 2), 1 µL bacterial DNA and sterile water up to a final volume of 25 µL per reaction. The PCR procedure was composed of one cycle at 95 °C for 5 min, 35 cycles of 94 °C/45 sec, 52 °C/45 sec, 72 °C/50 sec and one final extension step at 72 °C for 5 min. Amplicons of the expected size (approx. 179 bp) were detected by capillary gel electrophoresis as described above.

### 2.6 Beer incubation test

To investigate the extent of the strain *L.* sp. 2301's beer-spoilage potential, five different beer types were inoculated with microorganisms, incubated at 28 ± 1 °C and visually evaluated for a six-week period with regard to turbidity or sediment formation. All strains were adapted to the different beers in flasks containing 75 % target beer and 25 % 2-fold MRS broth [13]. After determining the cell count using a Thoma counting chamber, each microorganism suspension was inoculated in three beer bottles resulting in a final concentration of $1x10^5$ cells per bottle (or 200 cells per mL). Table 3 shows the beer types with their physico-chemical properties that were chosen for the incubation test. Chemical analyses were carried out according to MEBAK (Central European Brewing Committee for Analysis) instructions [1, 14, 30].

The inoculated bacteria were checked for strain identity before and after incubation by 16S rRNA gene sequencing. After incubation, all bottles showing haze, turbidity or sediments were checked for the present bacterial cell count using the Thoma counting chamber and compared with the originally inoculated cell concentration. From all samples showing no signs of microorganism growth, 1 mL beer was poured into a petri dish, mixed with MRS agar and incubated anaerobically for 7 days at 28 ± 1°C.

### 2.7 Physico-chemical analyses

Physico-chemical analyses were executed at the Research Center Weihenstephan for Brewing and Food Quality in advance and after the six-week storage period at 28 ± 1°C with all beer samples of the incubation test that were positive for microorganism growth. In parallel, un-inoculated samples that were also opened, stimulated to foam over and exposed to the same storage conditions were additionally analyzed providing reference values with regard to amino acid, fermentation by-product, organic acid, vicinal diketone, fatty acid, and fermentable sugar content [1, 14, 30].

## 3 Results

### 3.1 Phylogeny

*16S rRNA gene sequence comparison*

On the basis of a multiple alignment similarity matrix, a phylogenetic tree (Fig. 1) was constructed using the neighbor-joining method in the MEGA6 software [37]. The statistical reliability of the tree was tested using 1000 bootstrap replicates [18]. NCBI (National Center for Biotechnology Information) accession numbers are shown in brackets.

*pheS housekeeping gene sequence comparison*

Both, the *pheS* and the 16S rRNA gene sequence comparison, revealed that the three isolates belong to one strain and are closer related to the species *L. parabrevis* than to the *L. brevis* branch. Further analyses were performed with only one of the isolates, *L.* sp. 2301.

*rpoA housekeeping gene sequence comparison*

The comparison between the *rpoA* housekeeping gene sequences shows the distance between the unknown isolate *L.* sp. 2301 and the two other branches (Fig. 3; accession numbers in brackets).

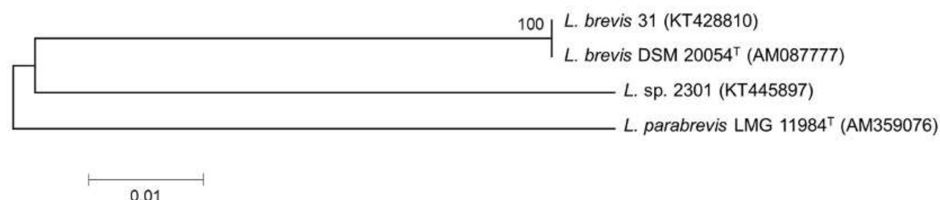



**Fig. 3 Reconstruction of a phylogenetic tree based on the neighbor-joining method of MEGA6 software [37, 53, 54]. The obtained *rpoA* housekeeping gene sequences of *L. brevis* 31 and *L.* sp. 2301 were compared to the deposited type strain sequences. A bootstrap analysis with 1000 replicates was executed [18]. Bar, 0.01 substitutions per nucleotide position**

No close relationship between the subgroups is recognizable.

### *DNA-DNA hybridization*

DNA-DNA similarity values obtained at the DSMZ are shown in table 4. The values in brackets indicate the results of the measurement repetition.

**Table 4 DNA-DNA similarity values [%]**

| | *L.* sp. 2301 |
|---|---|
| *L. parabrevis* LMG 11984$^T$ | 50.5 (56.5) |
| *L. brevis* DSM 20054$^T$ | 55.3 (58.9) |

## 3.2 Physiological characteristics

### *Carbohydrate utilization*

The main results of API CHL 50 systems applied on the four tested strains are shown in table 5. All strains were positive for D-xylose, galactose, glucose, fructose, N-acetyl glucosamine and maltose.

### *Stress tolerance*

The results of the stress tolerance tests after four weeks of anaerobic incubation at 28 ± 1°C in MRS broth are displayed in table 6 (see next page). All tested strains tolerated up to 8 % (v/v) ethanol in MRS broth and pH values between 4.0 and 7.8. At 10 and 15 °C, all strains were able to proliferate and cause a turbidity increase in the test tubes. None of the strains was able to grow at 40 °C or 45 °C in MRS broth. The differentiating stress factor seemed to be the salt concentration since the strains behaved heterogeneously if inoculated in MRS broth supplemented with different sodium chloride concentrations. *L. parabrevis* LMG 11984$^T$ tolerated NaCl concentrations of up to 8.0 % (w/w), *L. brevis* DSM 20054$^T$ up to 6.5 % (w/w). Both beer-spoilage isolates were more sensitive to salt. *L. brevis* TUM BP 111115005-2022 tolerated a NaCl concentration of 4.0 % (w/w) in MRS broth and the unknown isolate was not able to grow in any of the tested media supplemented with salt.

**Table 5 Main results of carbohydrate fermentation test system API CHL 50**

| Acid production from: | *L.* sp. 2301 | *L. brevis* DSM 20054$^T$ | *L. brevis* 31 | *L. parabrevis* LMG 11984$^T$ |
|---|---|---|---|---|
| L-Arabinose | w | + | + | + |
| Ribose | w | + | + | + |
| Methyl β-xyloside | – | – | – | w |
| Mannitol | w | – | – | w |
| Methyl α-D-glucoside | w | + | + | + |
| Lactose | – | w | – | – |
| Melibiose | w | + | w | – |
| Sucrose | – | – | + | w |
| D-Turanose | – | – | – | w |
| D-Arabitol | – | – | – | + |
| Gluconate | + | w | w | + |
| 5-Ketogluconate | – | + | – | – |

+ = positive after 48 hours of incubation; w = weakly positive after 48 h and positive after 120 h; – = negative after 120 hours of incubation

## 3.3 Presence of genes associated with hop resistance

The realtime PCR for the presence of the hop resistance genes *horA* and *horC* as well as the endpoint PCR for the detection of *hitA* resulted in the scheme in table 7 (see next page). Both type strains did not contain any of the analyzed genes associated with hop resistance, in contrast to the isolates obtained from beer environment.

## 3.4 Results of beer incubation test

The isolate *L.* sp. 2301 could proliferate in four out of five tested beer samples up to a cell concentration of at least 2 x $10^7$ cells per bottle or 40,000 cells per milliliter. The lager beer with a higher alcohol content of 5.1 % (v/v), a higher amount of hop bitter units (19.9 BU) and a pH value of 4.46 did not show any signs of microorganism growth.

## 3.5 Physico-chemical analyses of spoiled beer samples

The values of the physico-chemical analyses carried out with the four beer types that were positive for microbial growth were compared to those of the respective un-inoculated but equally treated beer samples which served as reference. The percentage deviations from the reference values were calculated for every spoiled beer type and every analyzed compound. If a compound concentration was increased or decreased by microorganism growth by more than 50 % in all analyzed samples (n = 12), it was regarded as significant. All significant changes obtained by physico-chemical analyses are shown in table 8.

# 4 Discussion

The question if the newly isolated strain *Lactobacillus* sp. 2301 belongs to any known beer-spoiling species has been the main

**Table 6 Results of stress tolerance tests**

| Strain | Alcohol [vol. %] | | | | | NaCl [% (w/w)] | | | pH value | | | | Temperature [°C] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2.5 | 3.5 | 5.0 | 6.5 | 8.0 | 4.0 | 6.5 | 8.0 | 4.0 | 5.0 | 7.2 | 7.8 | 10 | 15 | 40 | 45 |
| *L.* sp. 2301 | + | + | + | + | + | – | – | – | + | + | + | + | + | + | – | – |
| *L. parabrevis* LMG 11984$^T$ | + | + | + | + | + | + | + | + | + | + | + | + | + | + | – | – |
| *L. brevis* DSM 20054$^T$ | + | + | + | + | + | + | + | – | + | + | + | + | + | + | – | – |
| *L. brevis* 31 | + | + | + | + | + | + | – | – | + | + | + | + | + | + | – | – |

+ = growth (turbidity/sediments) visible after 4 weeks of incubation; – = no growth (turbidity/sediments) visible after 4 weeks of incubation; experiments were carried out in triplicate, anaerobically, in 10 ml MRS broth supplemented with the particular stress factor at 28 ± 1°C or at different temperatures, respectively

objective of this study. Regarding the postulation that two bacteria belong to the same species if the 16S rRNA gene homology displays values ≥ 97 %, *L.* sp. 2301 belongs to either the species *L. parabrevis* or *L. brevis*. In contrast, the sequences of the two analyzed housekeeping genes *pheS* and *rpoA* suggest that the unknown beer-spoiler does not belong to one of these species. Also, the DNA-DNA similarity values were far below the threshold of 70 % which would be necessary to assign this bacterium to a certain species according to Wayne et al. [59].

The pattern of carbohydrate utilization does not provide any relevant insights into the crucial question as the newly isolated bacterium has commonalities with both species it was compared to. The stress tolerance tests indicated that *L.* sp. 2301 is not affected by alcohol concentrations up to 8 % (v/v) or by deviations in pH value (4.0–7.8) in MRS broth. But it is influenced by temperature since growth was observable at low (10 °C and 15 °C), but not at higher temperatures (40 °C or 45 °C). It is noticeable that the unknown brewery isolate seemed to be highly salt-sensitive, which is probably the result of an adaptation to the low-salt medium beer. Another factor that confirms the adaptation to the brewing environment is the presence of two genes that are discussed to confer hop resistance to bacterial cells (*horA* and *horC*).

**Table 7 Results of the screening for genes *horA*, *horC* and *hitA***

| Strain | *horA* | *horC* | *hitA* |
|---|---|---|---|
| *L.* sp. 2301 | + | + | – |
| *L. parabrevis* LMG 11984$^T$ | – | – | – |
| *L. brevis* DSM 20054$^T$ | – | – | – |
| *L. brevis* 31 | + | + | + |

+ = presence of selected gene; – = absence of selected gene; analyses carried out by realtime PCR (for *horA* and *horC*) or endpoint PCR and subsequent gelelectrophoresis (for *hitA*)

**Table 8 Significant concentration changes (≥ ± 50 %) in beer induced by contamination with *L.* sp. 2301 compared to the un-inoculated beer samples**

| Compound | Group | *L.* sp. 2301 |
|---|---|---|
| | | Increase/Decrease [%] |
| Alanine | Amino acids | – 90.6 ± 2.73 |
| Acetaldehyde | Fermentation by-product | – 62.8 ± 41.08 |
| Pyruvate | Organic acid | – 97.8 ± 11.30 |
| Acetic acid | Organic acid | 90.1 ± 33.60 |
| Lactic acid | Organic acid | 133.5 ± 26.57 |
| Citric acid | Organic acid | – 97.7 ± 1.07 |

Growth of *L.* sp. 2301 was detected in four of the five tested beer types. Only the lager beer with normal alcohol (5.1 % (v/v)) and hop bitter acid concentrations (19.9 BU) was negative for microorganism growth after six weeks of incubation at 28 ± 1 °C. The remaining beer types are classified as more sensitive to microorganism spoilage based on the reduction in alcohol and/or hop content resulting in the spoilage by the recently isolated beer-spoiler. According to the classes established by *Prof. Back*, *L.* sp. 2301 has to be classified as a potential beer-spoiler [2].

The growth of *L.* sp. 2301 resulted in a significant decrease of the amino acid alanine and of the fermentation by-product acetaldehyde. The two organic acids pyruvate and citric acid were reduced and the levels of acetic acid and lactic acid were raised. This indicates a heterofermentative metabolism of the unknown brewery isolate resulting in the production of lactic acid, acetic acid and $CO_2$ as main products from glucose. Diacetyl production was not observed.

In conclusion, it could be determined that the unknown brewery isolate did not belong to L. brevis or *L. parabrevis* which were the two most probable species from the brewing microbiology perspective. Further analyses confirmed that a new species within the genus *Lactobacillus* has to be described. In parallel, a species description is submitted to the International Journal of Systematic and Evolutionary Microbiology. Therein, further discrimination of the isolated bacterium from genetically related species (*L. yonginensis*, *L. koreensis*, *L. hammesii*) with no relation to the brewing sector is shown.

## 5 Literature


1. Anger, H.-M.: MEBAK Brautechnische Analysenmethoden In: Rohstoffe, Selbstverlag der MEBAK, Freising-Weihenstephan, 2006.
2. Back, W.: Farbatlas und Handbuch der Getränkebiologie Band 1, Hans Carl Verlag, 1994.
3. Back, W.: Sekundärkontaminationen im Abfüllbereich, BRAUWELT **134** (1994), no. 16, pp. 686-695.
4. Back, W.: Biofilme in der Brauerei und Getränkeindustrie – 15 Jahre Praxiserfahrung, BRAUWELT **143** (2003), no. 24/25, pp. 1-5.

5. Back, W.; Breu, S. and Weigand, C.: Infektionsursachen im Jahre 1987, BRAUWELT **128** (1988), no. 31/32, pp. 1358-1362.
6. Behr, J.: Mechanisms of hop inhibition, tolerance and adaptation in *Lactobacillus brevis,* Wissenschaftszentrum Weihenstephan, Technische Universität München, 2008.
7. Behr, J.; Israel, L.; Gaenzle, M. G. and Vogel, R. F.: Proteomic approach for characterization of hop-inducible proteins in *Lactobacillus brevis*, Applied and Environmental Microbiology **73** (2007), no. 10, pp. 3300-3306.
8. Bohak, I.; Thelen, K. and Beimfohr, C.: Description of *Lactobacillus backi* sp. nov., an Obligate Beer-Spoiling Bacterium, BrewingScience – Monatsschrift fur Brauwissenschaft **59** (2006), no. 3/4, pp. 78-82.
9. Brandl, A.: Entwicklung und Optimierung von PCR-Methoden zur Detektion und Identifizierung von brauereirelevanten Mikroorganismen zur Routine-Anwendung in Brauereien, Lehrstuhl für Technologie der Brauerei II, TU München, Dissertation, 2006.
10. Cashion, P.; Holderfranklin, M. A.; McCully, J. and Franklin, M.: Rapid method for base ratio determination of bacterial DNA, Analytical Biochemistry **81** (1977), no. 2, pp. 461-466.
11. Corsetti, A.; Settanni, L.; van Sinderen, D.; Felis, G. E.; Dellaglio, F. and Gobbetti, M.: *Lactobacillus rossii* sp nov., isolated from wheat sourdough, International Journal of Systematic and Evolutionary Microbiology **55** (2005), no. 1, pp. 35-40.
12. De Ley, J.; Cattoir, H. and Reynaert.A: Quantitative measurement of DNA hybridization from renaturation rates, European Journal of Biochemistry **12** (1970), no. 1, pp. 133-142.
13. De Man, J. C.; Rogosa, M. and Sharpe, M. E.: A medium for the cultivation of *lactobacilli*, Journal of Applied Bacteriology **23** (1960), no. 1, pp. 130-135.
14. Donhauser, S. and Wagner, D.: Determination of sugar anf attenuation limit using HPLC, Monatsschrift für Brauwissenschaft **43** (1990), no. 9, pp. 306-309.
15. Ehrmann, M. A.; Muller, M. R. A. and Vogel, R. F.: Molecular analysis of sourdough reveals *Lactobacillus* mindensis sp. nov., International Journal of Systematic and Evolutionary Microbiology **53** (2003), no. 1, pp. 7-13.
16. Ehrmann, M. A.; Preissler, P.; Danne, M. and Vogel, R. F.: *Lactobacillus paucivorans* sp. nov., isolated from a brewery environment, International Journal of Systematic and Evolutionary Microbiology **60** (2010), no. 10, pp. 2353-2357.
17. Entani, E.; Masai, H. and Suzuki, K. I.: *Lactobacillus acetotolerans,* anew species from fermented vinegar broth, International Journal of Systematic Bacteriology **36** (1986), no. 4, pp. 544-549.
18. Felsenstein, J.: Confidence-limitas on phylogenies – an approach using the bootstrap, Evolution **39** (1985), no. 4, pp. 783-791.
19. Fujii, T.; Nakashima, K. and Hayashi, N.: Random amplified polymorphic DNA-PCR based cloning of markers to identify the beer-spoilage strains of *Lactobacillus brevis*, *Pediococcus damnosus, Lactobacillus collinoides* and *Lactobacillus coryniformis*, Journal of Applied Microbiology **98** (2005), no. 5, pp. 1209-1220.
20. Haakensen, M.: Genetic markers for beer-spoilage by lactobacilli and pediococci, Pathology and Laboratory Medicine, University of Saskatchewan, 2009.
21. Haakensen, M.; Pittet, V.; Morrow, K.; Schubert, A.; Ferguson, J. and Ziola, B.: Ability of Novel ATP-binding Cassette Multidrug Resistance Genes to Predict Growth of Pediococcus Isolates in Beer, Journal of the American Society of Brewing Chemists **67** (2009), no. 3, pp. 170-176.
22. Haakensen, M.; Schubert, A. and Ziola, B.: Multiplex PCR for putative *Lactobacillus* and *Pediococcus* beer-spoilage genes and ability of gene presence to predict growth in beer, Journal of the American Society of Brewing Chemists **66** (2008), no. 2, pp. 63-70.
23. Haakensen, M. C.; Butt, L.; Chaban, B.; Deneer, H.; Ziola, B. and Dowgiert, T.: *HorA*-Specific real-time PCR for detection of beer-spoilage lactic acid bacteria, Journal of the American Society of Brewing Chemists **65** (2007), no. 3, pp. 157-165.
24. Hayashi, N.; Ito, M.; Horiike, S. and Taguchi, H.: Molecular cloning of a putative divalent-cation transporter gene as a new genetic marker for the identification of *Lactobacillus brevis* strains capable of growing in beer, Applied Microbiology and Biotechnology **55** (2001), no. 5, pp. 596-603.
25. Huss, V. A. R.; Festl, H. and Schleifer, K. H.: Studies on the spectrophotometric determination of DNA hybridization from renaturation rates, Systematic and Applied Microbiology **4** (1983), no. 2, pp. 184-192.
26. Hutzler, M.; Koob, J.; Grammer, M.; Riedl, R. and Jacob, F.: Statistische Auswertung der PCR-Analyse bierschädlicher Bakterien in den Jahren 2010 und 2011, BRAUWELT **152** (2012), no. 18-19, pp. 546-547.
27. Iijima, K.; Suzuki, K.; Asano, S.; Kuriyama, H. and Kitagawa, Y.: Isolation and identification of potential beer-spoilage *Pediococcus inopinatus* and beer-spoilage *Lactobacillus backi* strains carrying the *horA* and *horC* gene clusters, Journal of the Institute of Brewing **113** (2007), no. 1, pp. 96-101.
28. Iijima, K.; Suzuki, K.; Asano, S.; Ogata, T. and Kitagawa, Y.: *HorC*, a Hop-Resistance Related Protein, Presumably Functions in Homodimer Form, Bioscience Biotechnology and Biochemistry **73** (2009), no. 8, pp. 1880-1882.
29. Iijima, K.; Suzuki, K.; Ozaki, K. and Yamashita, H.: *horC* confers beer-spoilage ability on hop-sensitive *Lactobacillus brevis* ABBC45(cc), Journal of Applied Microbiology **100** (2006), no. 6, pp. 1282-1288.
30. Jacob, F.: MEBAK Brautechnische Analysenmethoden In: Würze, Bier, Biermischgetränke, Selbstverlag der MEBAK, Freising - Weihenstephan, 2012.
31. Ji, N. N.; Peng, B.; Wang, G. Z.; Wang, S. Y. and Peng, X. X.: Universal primer PCR with DGGE for rapid detection of bacterial pathogens, Journal of Microbiological Methods **57** (2004), no. 3, pp. 409-413.
32. Koob, J.; Jacob, F.; Grammer, M.; Riedl, R. and Hutzler, M.: PCR-Analysen bierschädlicher Bakterien 2012 und 2013, BRAUWELT **154** (2014), no. 10, pp. 288-290.
33. Loffler, F. E.; Sun, Q.; Li, J. R. and Tiedje, J. M.: 16S rRNA gene-based detection of tetrachloroethene-dechlorinating Desulfuromonas and Dehalococcoides species, Applied and Environmental Microbiology **66** (2000), no. 4, pp. 1369-1374.
34. Naser, S. M.; Thompson, F. L.; Hoste, B.; Gevers, D.; Dawyndt, P.; Vancanneyt, M. and Swings, J.: Application of multilocus sequence analysis (MLSA) for rapid identification of Enterococcus species based on *rpoA* and *pheS* genes, Microbiology-Sgm **151** (2005), no. pp. 2141-2150.
35. Polz, M. F. and Cavanaugh, C. M.: Bias in template-to-product ratios in multitemplate PCR, Applied and Environmental Microbiology **64** (1998), no. 10, pp. 3724-3730.
36. Einfluss der Bierzusammensetzung auf die mikrobiologische Stabilität von Reisbier 2009.
37. Saitou, N. and Nei, M.: The neigbor-joining method – a new method for reconstructing phylogenetic trees, Molecular Biology and Evolution **4** (1987), no. 4, pp. 406-425.
38. Sakamoto, K. and Konings, W. N.: Beer spoilage bacteria and hop resistance, International Journal of Food Microbiology **89** (2003), no. 2-3, pp. 105-124.

39. Sakamoto, K.; Margolles, A.; van Veen, H. W. and Konings, W. N.: Hop resistance in the beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-binding cassette multidrug transporter *horA,* Journal of Bacteriology **183** (2001), no. 18, pp. 5371-5375.
40. Sakamoto, K.; van Veen, H. W.; Saito, H.; Kobayashi, H. and Konings, W. N.: Membrane-bound ATPase contributes to hop resistance of *Lactobacillus brevis*, Applied and Environmental Microbiology **68** (2002), no. 11, pp. 5374-5378.
41. Sami, M.; Yamashita, H.; Hirono, T.; Kadokura, H.; Kitamoto, K.; Yoda, K. and Yamasaki, M.: Hop-resistant *Lactobacillus brevis* contains a novel plasmid harboring a multidrug resistance-like gene, Journal of Fermentation and Bioengineering **84** (1997), no. 1, pp. 1-6.
42. A mixture of a *Lactobacillus brevis* and a *Saccharomyces dairensis* for preparing leavening barm. US Patent -4666719. May 19, 1987.
43. Stackebrandt, E. and Goebel, B. M.: A place for DNA-DNA reassociation and 16S ribosomal rRNA sequence-analysis in the present species definition in bacteriology, International Journal of Systematic Bacteriology **44** (1994), no. 4, pp. 846-849.
44. Stackebrandt, E. and Goodfellow, M.: Nucleic acid techniques in bacterial systematics In: Lane, D. J.: 16S/23S rRNA sequencing, John Wiley & Sons, New York, 1991.
45. Suzuki, K.; Iijima, K.; Ozaki, K. and Yamashita, H.: Isolation of a hop-sensitive variant of *Lactobacillus lindneri* and identification of genetic markers for beer spoilage ability of lactic acid bacteria, Applied and Environmental Microbiology **71** (2005), no. 9, pp. 5089-5097.
46. Suzuki, K.; Iijima, K.; Sakamoto, K.; Sami, M. and Yamashita, H.: A review of hop resistance in beer spoilage lactic acid bacteria, Journal of the Institute of Brewing **112** (2006), no. 2, pp. 173-191.
47. Suzuki, K.; Koyanagi, M. and Yamashita, H.: Isolation of hop-sensitive variants from beer-spoilage *Lactobacillus brevis* strains, Journal of the American Society of Brewing Chemists **62** (2004), no. 2, pp. 71-74.
48. Suzuki, K.; Sami, M.; Iijima, K.; Ozaki, K. and Yamashita, H.: Characterization of *horA* and its flanking regions of *Pediococcus damnosus* ABBC478 and development of more specific and sensitive *horA* PCR method, Letters in Applied Microbiology **42** (2006), no. 4, pp. 392-399.
49. Suzuki, K.; Sami, M.; Kadokura, H.; Nakajima, H. and Kitamoto, K.: Biochemical characterization of *horA*-independent hop resistance mechanism in *Lactobacillus brevis*, International Journal of Food Microbiology **76** (2002), no. 3, pp. 223-230.
50. Suzuki, K.; Sami, M.; Ozaki, K. and Yamashita, H.: Nucleotide sequence identities of *horA* homologues and adjacent DNA regions identified in three species of beer-spoilage lactic acid bacteria, Journal of the Institute of Brewing **110** (2004), no. 4, pp. 276-283.
51. Suzuki, K.; Sami, M.; Ozaki, K. and Yamashita, H.: Genetic characterization of hop-sensitive variants obtained from beer-spoilage *Lactobacillus brevis*, Journal of the American Society of Brewing Chemists **63** (2005), no. 1, pp. 5-10.
52. Suzuki, K.; Sami, M.; Ozaki, K. and Yamashita, H.: Comparative study of two plasmids, pRH45 and pRH 20690, isolated from beer-spoilage *Lactobacillus brevis* ABBC45 and *L. lindneri* DSM20690, Journal of the American Society of Brewing Chemists **63** (2005), no. 1, pp. 11-16.
53. Tamura, K.; Nei, M. and Kumar, S.: Prospects for inferring very large phylogenies by using the neighbor-joining method, Proceedings of the National Academy of Sciences of the United States of America **101** (2004), no. 30, pp. 11030-11035.
54. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A. and Kumar, S.: MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0, Molecular Biology and Evolution **30** (2013), no. 12, pp. 2725-2729.
55. Tindall, B. J.; Rossello-Mora, R.; Busse, H. J.; Ludwig, W. and Kaempfer, P.: Notes on the characterization of prokaryote strains for taxonomic purposes, International Journal of Systematic and Evolutionary Microbiology **60** (2010), no. 1, pp. 249-266.
56. Tohno, M.; Kitahara, M.; Irisawa, T.; Masuda, T.; Uegaki, R.; Ohkuma, M. and Tajima, K.: Description of *Lactobacillus iwatensis* sp. nov., isolated from orchardgrass (*Dactylis glomerata L.*) silage, and *Lactobacillus backii* sp. nov., International Journal of Systematic and Evolutionary Microbiology **63** (2013), no. 10, pp. 3854-3860.
57. Vancanneyt, M.; Naser, S. M.; Engelbeen, K.; De Wachter, M.; Van der Meulen, R.; Cleenwerck, I.; Hoste, B.; De Vuyst, L. and Swings, J.: Reclassification of *Lactobacillus brevis* strains LMG 11494 and LMG 11984 as *Lactobacillus parabrevis* sp. nov., International Journal of Systematic and Evolutionary Microbiology **56** (2006), no. 7, pp. 1553-1557.
58. Vogel, R. F., Preissler, P., Behr, J.: Towards an Understanding of Hop Tolerance in Beer Spoiling *Lactobacillus brevis*, BrewingScience **63** (2010), no. 1/2, pp. 23-30.
59. Wayne, L. G.; Brenner, D. J.; Colwell, R. R.; Grimont, P. A. D.; Kandler, O.; Krichevsky, M. I.; Moore, L. H.; Moore, W. E. C.; Murray, R. G. E.; Stackebrandt, E.; Starr, M. P. and Truper, H. G.: Report of the Ad-Hoc-Committee on reconciliation of approaches to bacterial systematics, International Journal of Systematic Bacteriology **37** (1987), no. 4, pp. 463-464.


*Received 29 June 2016, accepted 2 August 2016*

## Section 2 *Lactobacillus cerevisiae* sp. nov., isolated from a spoiled brewery sample

INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION
Koob *et al.*, *Int J Syst Evol Microbiol* 2017;67:3452–3457
DOI 10.1099/ijsem.0.002139

MICROBIOLOGY SOCIETY

# *Lactobacillus cerevisiae* sp. nov., isolated from a spoiled brewery sample

Jennifer Koob,[1] Fritz Jacob,[1] Mareike Wenning[2] and Mathias Hutzler[1,*]

**Abstract**

A Gram-stain-positive, non-motile, rod-shaped bacterium, designated TUM BP 140423000-2250$^T$ (=DSM 100836$^T$=LMG 29073$^T$), was isolated from spoiled beer. This bacterium did not form spores, and was catalase-negative and facultatively anaerobic. Its taxonomic position was determined in a polyphasic study. The 16S rRNA gene sequence similarity data showed that the strain belonged to the *Lactobacillus* genus with the nearest neighbours being *Lactobacillus koreensis* DCY50$^T$ (sequence similarity 99.5 %), *Lactobacillus yonginensis* THK-V8$^T$ (99.2 %) and *Lactobacillus parabrevis* LMG 11984$^T$ (98.7 %). Sequence comparisons of additional phylogenetic markers, *pheS* and *rpoA*, confirmed the 16S rRNA gene sequence tree topology. The maximum *rpoA* sequence similarity was 92.3 % with *L. yonginensis* THK-V8$^T$. The DNA G+C content of the isolate was 50.0 mol%. The DNA–DNA relatedness showed that strain TUM BP 140423000-2250$^T$ could be clearly distinguished from *L. koreensis* DCY 50$^T$ (30.8±0.4 %) and *L. yonginensis* THK-V8$^T$ (23.6±5.9 %). The major fatty acids were $C_{18:1}\omega 9c$, summed feature 7 (comprised of $C_{19:0}$ cyclo $\omega 10c/C_{19:1}\omega 6c$) and $C_{16:0}$. Based on phenotypic and genotypic studies, the authors propose classifying the new isolate as a representative of a novel species of the genus *Lactobacillus*, *Lactobacillus cerevisiae* sp. nov. The type strain is deposited at the Research Centre Weihenstephan for Brewing and Food Quality as TUM BP 140423000-2250$^T$ (=DSM 100836$^T$=LMG 29073$^T$).

Every year, the damage caused by beer-spoilage micro-organisms results in financial losses. The consequences of the growth of these micro-organisms in beer range from slight changes in smell and taste to the product becoming complete inedible including consumer complaints and refunds [1]. The primary task of microbiological quality control in breweries is to detect even traces of these spoilage microbes and identify them if necessary. A basic requirement for successful analytics is to be aware of/knowledgeable of all microbes with spoilage potential [2].

The group of beer-spoilage micro-organisms is a limited pool of bacteria and yeast species that are able to tolerate the adverse conditions of beer. These antimicrobial beer properties include especially high levels of alcohol and hop acids, low pH and an anaerobic atmosphere as well as low concentrations of utilizable carbon sources [2–7]. The mechanisms protecting beer-spoilage micro-organisms from the hostile beer conditions are still not fully understood. The dominant genus causing beer-spoilage incidents is the genus *Lactobacillus*. Species belonging to the beer-spoiling group newly described in the last decade are *Lactobacillus backii*, *Lactobacillus rossiae* and *Lactobacillus paucivorans* [8–11].

Recently, bacterial isolates were obtained from contaminated brewery samples that were first assigned to the species *Lactobacillus parabrevis*, which was not known to be a beer-spoilage bacterium [12]. One isolate (culture collection number TUM BP 140423000-2250$^T$, working number 2301$^T$) was selected for further study. It was obtained from a bright beer tank sample exhibiting turbidity and slightly enhanced acidity, but no significant sensory changes. Phylogenetic analyses led to the conclusion that the isolate could not be assigned to *Lactobacillus brevis*, the most dominant beer-spoiling species [3, 13–16], or *L. parabrevis*. Physiological characteristics were also determined such as temperature, acid, alcohol and salt tolerance, the presence of known hop resistance genes as well as the degree of its beer-spoilage potential [12].

In this study, a polyphasic approach was used to demarcate the isolate TUM BP 140423000-2250$^T$ from related species based on the sequences of the 16S rRNA gene and two

**Author affiliations:** [1]Technische Universität München, Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität, Alte Akademie 3, 85354 Freising, Germany; [2]Department Microbiology, Technische Universität München, Zentralinstitut für Ernährungs- und Lebensmittelforschung, Weihenstephaner Berg 3, 85354 Freising, Germany.
*Correspondence: Mathias Hutzler, hutzler@wzw.tum.de
**Keywords:** *Lactobacillus cerevisiae*; beer-spoilage bacteria; spoilage; *Lactobacillus koreensis*; *Lactobacillus yonginensis*; *Lactobacillus parabrevis*.
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *pheS* and *rpoA* gene sequences of *Lactobacillus cerevisiae* TUM BP 140423000-2250$^T$ are KT445896, KT445900 and KT445897, respectively.
One supplementary table and four supplementary figures are available with the online Supplementary Material.

002139 © 2017 IUMS

Downloaded from www.microbiologyresearch.org by

housekeeping genes, *pheS* and *rpoA*. In addition, genomic relatedness, fatty acid profile and further phenotypic and physiological characteristics were determined, which resulted in the description of a novel species that belongs to the genus *Lactobacillus*. The closely related type strains *Lactobacillus koreensis* DCY50$^T$ and *Lactobacillus yonginensis* THK-V8$^T$ were obtained from the German Collection of Micro-organisms and Cell Cultures (DSMZ, Braunschweig, Germany), and *Lactobacillus parabrevis* LMG 11984$^T$ was obtained from the Belgian Co-ordinated Collections of Micro-organisms (BCCM, Gent, Belgium) [17–19].

All strains used in this study were separated on De Man, Rogosa and Sharpe agar (MRS; pH 6.2) and cultivated in an anaerobic atmosphere at 28±1 °C [20]. Ten colonies of each were picked and combined to form the initial cultures followed by storage in cryostock at −80 °C.

Cell morphology of 48-h-old broth cultures and spore-forming ability were examined by dark-field and phase-contrast microscopy [Nikon, Eclipse Ti microscope; Andor, (DIS) Zyla V - 3tap camera]. Motility was tested using the hanging-drop technique [21]. Gram staining was performed according to Buck [22]. Catalase activity was examined by bubble production in 3 % (v/v) $H_2O_2$ solution, and oxidase activity was determined using Bactident strips (Merck). To determine the fermentation type of isolate TUM BP 140423000-2250$^T$, tests for gas production from glucose and gluconate were carried out in triplicate in MRS broth with Durham tubes [3]. The production of D- and L-lactic acid was analysed using a D-lactic acid/L-lactic acid enzyme kit according to the manufacturer's instructions (R-Biopharm). The test for $NH_3$ production from arginine was carried out according to Back [23]. Carbohydrate fermentation pattern was determined using the API CHL 50 kit (Biomérieux), and enzyme activity using the API ZYM kit (Biomérieux). The growth behaviour in the presence of oxygen was determined by stab cultures in NBB agar (Döhler).

The fatty acid profile and the cell-wall composition, including the presence of *meso*-diaminopimelic acid, of strain TUM BP 140423000-2250$^T$ and the DNA–DNA relatedness values of the combinations of *Lactobacillus cerevisiae* sp. nov. TUM BP

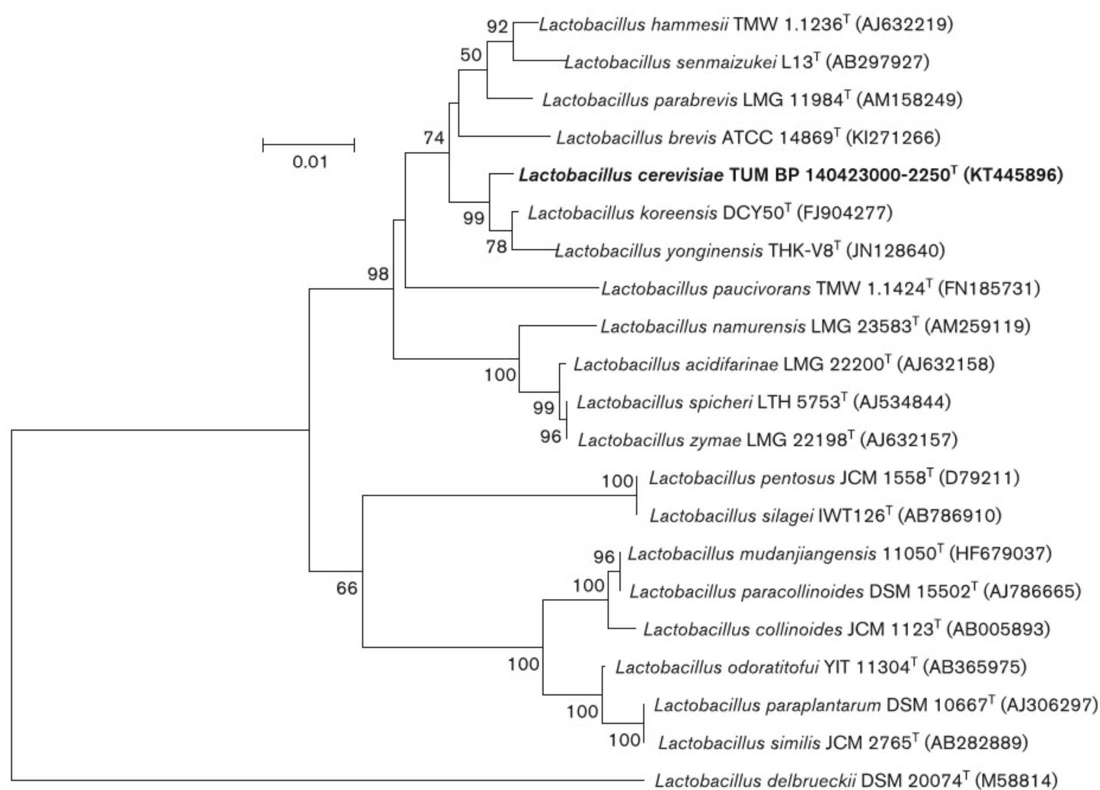



**Fig. 1.** Neighbour-joining tree based on the 16S rRNA gene sequences showing the phylogenetic relationship between *L. cerevisiae* sp. nov. TUM BP 140423000-2250$^T$ and related species of the genus *Lactobacillus*; *Lactobacillus delbrueckii* DSM 20074$^T$ was included as an outgroup species. Bootstrap values (expressed as percentages of 1000 replicates) ≥50 % are shown. Bar, 0.01 substitutions per nucleotide position.

Downloaded from www.microbiologyresearch.org by
IP: 129.187.254.46

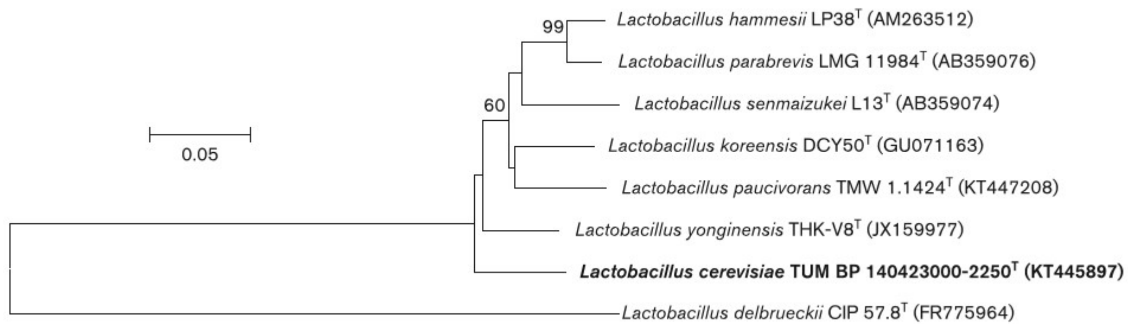



**Fig. 2.** Neighbour-joining tree based on the sequence of the *rpoA* housekeeping gene showing the phylogenetic relationship between *L. cerevisiae* sp. nov. TUM BP 140423000-2250$^T$ and closely related taxa. Bootstrap values (expressed as percentages of 1000 replicates) ≥50 % are shown. Bar, 0.05 substitutions per nucleotide position.

140423000-2250$^T$ and *L. koreensis* DCY50$^T$ as well as *L. cerevisiae* sp. nov. and *L. yonginensis* THK-V8$^T$ were analysed at the DSMZ [24–36].

The 16S rRNA gene sequence was determined using the primer pairs 27 f (5′ AGAGTTTGATCMTGGCTCAG 3′) and 1492 r (5′ TACGGYTACCTTGTTACGACTT 3′) as well as 933 f (5′ GCACAAGCGGTGGAGCATGTGG 3′) and 1541 r (5′ AAGGAGGTGATCCAGCCGCA 3′) [37–40]. Since the sequences of the RNA polymerase alpha subunit (*rpoA*) and the phenylalanyl-tRNA synthase alpha subunit (*pheS*) have a higher resolution potential with regard to the genera *Enterococcus* and *Lactobacillus* [41–43], both housekeeping gene sequences were determined from TUM BP 140423000-2250$^T$ and compared with the sequences of the nearest phylogenetic neighbours. The temperature protocol and the primer set (rpoA-21-F and rpoA-23-R) for *rpoA* PCR were adopted from the study by Naser *et al.* [44]. The temperature protocol and the primer set (pheS-forward and pheS-reverse) for the *pheS* gene PCR were adopted from the study by Ehrmann *et al.* [10]. The sequencing was performed by GATC Biotech (Constance, Germany).

The 16S rRNA gene sequence of TUM BP 140423000-2250$^T$ was compared with those of related taxa of the genus *Lactobacillus* obtained from the EzTaxon database [45]. *rpoA* and *pheS* housekeeping gene sequences of closely related species were obtained from the National Centre for Biotechnology Information (NCBI) database or determined in this study. Phylogenetic trees were reconstructed using the MEGA6 software [46–50].

The 16S rRNA gene sequence of strain TUM BP 140423000-2250$^T$ was a continuous stretch of 1477 bp. The neighbour-joining tree topology (Fig. 1) was evaluated and confirmed by the maximum-likelihood method (Fig. S1, available in the online Supplementary Material). The trees classified *L. cerevisiae* sp. nov. TUM BP 140423000-2250$^T$ as a member of the *Lactobacillus buchneri* group [51] with the two nearest neighbours, *L. koreensis* and *L. yonginensis*, showing sequence similarities of 99.5 % and 99.2 %, respectively. Due to the high 16S rRNA gene sequence similarities, alternative chronometers and DNA–DNA hybridization experiments were undertaken [25, 34–36, 52–55].

The analyses of the *rpoA* (Fig. 2) and *pheS* gene sequences (Fig. S2) had a higher resolution potential. Using continuous

**Table 1.** Comparative cellular fatty acid content (percentages) of strain TUM BP 140423000-2250$^T$ and related type strains of species of the genus *Lactobacillus*

Strains: 1, *L. cerevisiae* sp. nov. TUM BP 140423000–2250$^T$; 2, *L. yonginensis* THK-V8$^T$; 3, *L. koreensis* DCY50$^T$; 4, *L. parabrevis* LMG 11984$^T$; 5, *L. hammesii* TMW 1.1236$^T$; 6, *L. brevis* ATCC 14869$^T$. –, Not detected; all data from this study.

| Fatty acid | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Saturated | | | | | | |
| $C_{12:0}$ | 0.2 | 1.3 | – | – | – | – |
| $C_{14:0}$ | 1.2 | **16.1** | 5.8 | 7.7 | 9.9 | 7.8 |
| $C_{16:0}$ | **22.5** | **19.1** | **13.9** | **18.5** | **17.1** | **16.5** |
| $C_{18:0}$ | 3.9 | 3.0 | 2.3 | 3.1 | 2.8 | 2.7 |
| Unsaturated | | | | | | |
| $C_{18:1}\omega9c$ | **24.5** | 6.4 | **10.3** | **13.8** | **10.2** | **11.4** |
| Branched-chain fatty acid | | | | | | |
| iso-$C_{19:0}$ | 1.4 | 1.2 | 0.9 | 1.2 | – | 0.7 |
| Hydroxy fatty acid | | | | | | |
| $C_{15:0}$ 3-OH | – | – | – | – | – | 0.7 |
| Cyclo fatty acid | | | | | | |
| $C_{17:0}$ cyclo | – | 1.2 | – | – | – | 0.5 |
| $C_{19:0}$ cyclo $\omega8c$ | – | – | – | – | – | 8.2 |
| Summed features* | | | | | | |
| 3; $C_{16:1}\omega7c/C_{16:1}\omega6c$ | 0.9 | 2.5 | 1.1 | 1.5 | 1.7 | 1.4 |
| 7; $C_{19:0}$ cyclo $\omega10c/C_{19:1}\omega6c$ | **35.2** | **45.6** | **26.9** | **35.9** | **36.1** | **33.0** |
| 8; $C_{18:1}\omega7c/C_{18:1}\omega6c$ | 8.9 | 3.5 | 4.1 | 5.5 | 4.4 | 4.7 |

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system.

Downloaded from www.microbiologyresearch.org by
IP: 129.187.254.46

stretches of 801 bp for *rpoA* and 371 bp for *pheS*, both sequence comparisons indicated that the novel beer-spoilage isolate represents a novel species within the genus *Lactobacillus*. The nearest phylogenetic neighbours of *L. cerevisiae* sp. nov. TUM BP 140423000-2250$^T$ regarding the *rpoA* sequence were *L. yonginensis* THK-V8$^T$ and *L. koreensis* DCY 50$^T$, showing 92.3 % and 91.2 % similarity, respectively.

DNA–DNA relatedness values between strain TUM BP 140423000-2250$^T$ and *L. koreensis* DCY50$^T$ as well as *L. yonginensis* THK-V8$^T$ were 30.5 % (31.1) and 19.4 % (27.8), respectively (values in parentheses are results of measurements in duplicate). Both values were far below the threshold value of 70 % postulated by Wayne *et al.* for the description of a novel species [52]. The DNA G+C content of strain *L. cerevisiae* sp. nov. TUM BP 140423000-2250$^T$ was determined as 50.0 mol% [12]. The major fatty acids were determined as $C_{18:1}\omega9c$, summed feature 7 (comprised of $C_{19:0}$ cyclo $\omega10c/C_{19:1}\omega6c$) and $C_{16:0}$. The comparison between the fatty acid content of *L. cerevisiae* sp. nov. TUM BP 140423000-2250$^T$ and those of related type strains is shown in Table 1. The novel type strain can be clearly demarcated by the low level of the $C_{14:0}$ fatty acid and the high amount of the $C_{18:1}\omega9c$ unsaturated fatty acid.

The analyses for the presence of *meso*-diaminopimelic acid and the cell-wall composition of the novel isolate proved difficult. Even after repeated attempts, only small quantities of protein-contaminated peptidoglycan cell-wall compound could be isolated by the DSMZ scientists and this did not allow further structural analysis. The presence of *meso*-diaminopimelic acid was confirmed after highly sensitive gas chromatography/mass spectrometry (GC/MS) analysis [56], but it was not possible to determine the full structure of the cell-wall peptidoglycan.

Strain TUM BP 140423000-2250$^T$ produced gas from glucose and gluconate. Cells were short or long, and slender rods that occurred singly, in pairs or in short chains (Fig. S3). Beige colonies appeared in two morphological forms: circular with either smooth or fringed edges (Fig. S4). Motility and spore formation could not be observed. The D/L-lactic acid ratio for strain TUM BP 140423000-2250$^T$ was 4 : 6. Further physiological characteristics can be extracted from a previous study (e.g. alcohol, salt, acid and temperature tolerance, hop resistance genes *horA*, *horC*, *hitA*) [12].

With regard to the carbohydrate fermentation pattern, isolate TUM BP 140423000-2250$^T$ was positive for acid production from D-xylose, D-galactose, D-glucose, D-fructose, *N*-acetylglucosamine, maltose and potassium gluconate. It was weakly positive for acid production from L-arabinose, D-ribose, D-mannitol, methyl α-D-glucopyranoside and melibiose. The key difference between TUM BP 140423000-2250$^T$ and its three closest phylogenetic neighbours wass the weakly positive fermentation of D-mannitol and the non-fermentation of D-arabitol (Table 2). The enzymic profiles of TUM BP 140423000-2250$^T$ and closely related species are summed up in Table S1. Therefore, based on morphology, and physiological and phylogenetic information it is proposed that the novel organism belongs to a novel species of the genus *Lactobacillus* for which the name *Lactobacillus cerevisiae* sp. nov. is proposed.

**Table 2.** Differential characteristics between *L. cerevisiae* sp. nov. TUM BP140423000-2250$^T$ and related type strains

Strains: 1, *L. cerevisiae* sp. nov. TUM BP 140423000-2250$^T$; 2, *L. koreensis* DCY50$^T$; 3, *L. parabrevis* LMG 11984$^T$; 4, *L. yonginensis* THK-V8$^T$. +, Positive, −, negative; w, weakly positive after 48 h and positive after 120 h; v, strain-dependent

| Characteristic | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Colony colour | Beige | Beige[a]* | Beige[b] | Cream-coloured[c] |
| Catalase activity | Negative | Negative[a] | Negative[b] | Negative[c] |
| Motility | Negative | Positive[a] | Negative[b] | Negative[c] |
| Ratio of D- and L-lactic acid | 4 : 6 | 3 : 7[a] | 4 : 6[b] | 1 : 1[c] |
| DNA G+C content (mol%) | 50.0 | 49.0[a] | 49.0[b] | 47.8[c] |
| Acid production from: | | | | |
| Methyl-βD-xylopyranoside | − | − | + | − |
| D-Mannitol | w | − | − | − |
| Methyl α-D-glucopyranoside | w | − | v | − |
| *N*-Acetylglucosamine | + | − | + | + |
| Aesculin ferric citrate | − | + | − | + |
| Salicin | − | − | − | + |
| Maltose | + | − | + | + |
| Lactose | − | − | v | − |
| Melibiose | w | + | − | − |
| D-Arabitol | − | + | + | + |
| Potassium gluconate | + | + | + | + |

*Data taken from: *a*, Bui *et al.* [17]; *b*, Vancanneyt *et al.* [18]; *c*, Yi *et al.* [19].

Downloaded from www.microbiologyresearch.org by
IP: 129.187.254.46

## DESCRIPTION OF *LACTOBACILLUS CEREVISIAE* SP. NOV.

*Lactobacillus cerevisiae* (ce.re.vi'si.ae. L. fem. gen. n. *cerevisiae* of beer).

Cells are Gram-stain-positive, rod-shaped, non-motile, non-spore-forming, catalase-negative, oxidase-negative, heterofermentative and facultatively anaerobic. Colonies on MRS agar after 48 h are beige and circular, with either smooth or fringed edges. Growth can be observed at temperatures between 4 and 37 °C, but not at 40 or 45 °C. Cells can grow at pH values between pH 4.0 and 7.8. Alcohol is tolerated up to at least 8.0 vol% in MRS broth. No growth can be observed in MRS broth supplemented with 4 % (w/w) NaCl [12]. Both D- and L-lactic acid are produced (in a ratio of 4 : 6). The major fatty acids are $C_{18:1}\omega9c$, summed feature 7 (comprised of $C_{19:0}$ cyclo $\omega10c/C_{19:1}\omega6c$) and $C_{16:0}$. *meso*-Diaminopimelic acid is detected as the diagnostic diamino acid. Acid is produced from D-xylose, D-galactose, D-glucose, D-fructose, *N*-acetylglucosamine, maltose and potassium gluconate and produced weakly from L-arabinose, D-ribose, D-mannitol, methyl α-D-glucopyranoside and melibiose. No acid is produced from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, amygdalin, arbutin, aesculin ferric citrate, salicin, cellobiose, lactose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol or potassium 2- or 5-ketogluconate. Ammonia is not produced from arginine hydrolysis. Lactic acid, acetic acid and ethanol are produced from glucose.

The type strain is TUM BP 140423000-2250$^T$ (=DSM 100836$^T$=LMG 29073$^T$), which was isolated from a bright beer tank sample from a German brewery. The DNA G+C content of the type strain is 50.0 mol%.

**Funding information**
This work did not receive any specific grant from any funding agency.

**Conflicts of interest**
There does not exist any conflict of interest due to personal or financial relationship with other people or organisations.

**References**

1. **Iijima K, Suzuki K, Asano S, Kuriyama H, Kitagawa Y.** Isolation and identification of potential Beer-Spoilage *Pediococcus inopinatus* and beer-spoilage *Lactobacillus backi* strains carrying the *horA* and *horC* gene clusters. *J Inst Brew* 2007;113:96–101.
2. **Haakensen M, Pittet V, Morrow K, Schubert A, Ferguson J *et al.*** Ability of novel ATP-binding cassette multidrug resistance genes to predict growth of *Pediococcus* isolates in beer. *J Am Soc Brew Chem* 2009;67:170–176.
3. **Back W.** *Farbatlas und Handbuch der Getränkebiologie*, vol. 1. Nürnberg: Hans Carl Verlag; 1994.
4. **Suzuki K, Iijima K, Sakamoto K, Sami M, Yamashita H.** A review of hop resistance in beer spoilage lactic acid bacteria. *J Inst Brew* 2006;112:173–191.
5. **Sakamoto K, Konings WN.** Beer spoilage bacteria and hop resistance. *Int J Food Microbiol* 2003;89:105–124.
6. **Rainbow C.** Beer spoilage lactic acid bacteria. In: Carr JG, Cutting CV and Whiting GC (editors). *Lactic Acid Bacteria in Beverages and Food*. London: Academic Press; 1973.
7. **Bokulich NA, Bamforth CW.** The microbiology of malting and brewing. *Microbiol Mol Biol Rev* 2013;77:157–172.
8. **Bohak I, Thelen K, Beimfohr C.** Description of *Lactobacillus backi* sp. nov., an obligate beer-spoiling bacterium.. *Monatsschr Brauwiss* 2006:78. 82.
9. **Corsetti A, Settanni L, van Sinderen D, Felis GE, Dellaglio F *et al.*** *Lactobacillus rossii* sp. nov., isolated from wheat sourdough. *Int J Syst Evol Microbiol* 2005;55:35–40.
10. **Ehrmann MA, Preissler P, Danne M, Vogel RF.** *Lactobacillus paucivorans* sp. nov., isolated from a brewery environment. *Int J Syst Evol Microbiol* 2010;60:2353–2357.
11. **Tohno M, Kitahara M, Irisawa T, Masuda T, Uegaki R *et al.*** Description of *Lactobacillus iwatensis* sp. nov., isolated from orchardgrass (*Dactylis glomerata* L.) silage, and *Lactobacillus backii* sp. nov. *Int J Syst Evol Microbiol* 2013;63:3854–3860.
12. **Koob J, Jacob F, Methner F-J, Hutzler M.** *Lactobacillus* sp. brewery isolate: a new threat to the brewing industry? *Brewing Science* 2016;69:42–49.
13. **Back W.** Sekundärkontaminationen im Abfüllbereich. *Brauwelt* 1994;16:686–695.
14. **Koob J, Jacob F, Grammer M, Riedl R, Hutzler M.** PCR-Analysen bierschädlicher Bakterien 2012 und 2013. *Brauwelt* 2014;10:288–290.
15. **Hutzler M, Koob J, Grammer M, Riedl R, Jacob F.** Statistische auswertung der PCR-analyse bierschädlicher Bakterien in den Jahren 2010 und 2011. *Brauwelt* 2012;152:546–547.
16. **Back W, Breu S, Weigand C.** Infektionsursachen Im Jahre 1987. *Brauwelt* 1988;31/32:1358–1362.
17. **Bui TP, Kim YJ, In JG, Yang DC.** *Lactobacillus koreensis* sp. nov., isolated from the traditional Korean food kimchi. *Int J Syst Evol Microbiol* 2011;61:772–776.
18. **Vancanneyt M, Naser SM, Engelbeen K, de Wachter M, van der Meulen R *et al.*** Reclassification of *Lactobacillus brevis* strains LMG 11494 and LMG 11984 as *Lactobacillus parabrevis* sp. nov. *Int J Syst Evol Microbiol* 2006;56:1553–1557.
19. **Yi EJ, Yang JE, Lee JM, Park Y, Park SY *et al.*** *Lactobacillus yonginensis* sp. nov., a lactic acid bacterium with ginsenoside converting activity isolated from Kimchi. *Int J Syst Evol Microbiol* 2013;63:3274–3279.
20. **de Man JC, Rogosa M, Sharpe ME.** A medium for the cultivation of lactobacilli. *J Appl Bacteriol* 1960;23:130–135.
21. **Harrigan WF.** *Laboratory Methods in Food Microbiology*, 3rd ed. London: Academic Press; 1998.
22. **Buck JD.** Nonstaining (KOH) method for determination of gram reactions of marine bacteria. *Appl Environ Microbiol* 1982;44:992–993.
23. **Back W.** *Farbatlas und Handbuch der Getränkebiologie*, vol. 2. Nürnberg: Hans Carl Verlag; 2000.
24. **Cashion P, Holder-Franklin MA, Mccully J, Franklin M.** A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* 1977;81:461–466.
25. **de Ley J, Cattoir H, Reynaerts A.** The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* 1970;12:133–142.
26. **Huss VA, Festl H, Schleifer KH.** Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* 1983;4:184–192.
27. **Kämpfer P, Kroppenstedt RM.** Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* 1996;42:989–1005.

Downloaded from www.microbiologyresearch.org by
IP: 129.187.254.46

28. **Kuykendall LD, Roy MA, O'Neill JJ, Devine TE.** Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. *Int J Syst Bacteriol* 1988;38:358–361.
29. **Miller LT.** Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. *J Clin Microbiol* 1982;16:584–586.
30. **Rhuland LE, Work E, Denman RF, Hoare DS.** The behavior of the isomers of $\alpha,\beta$-diaminopimelic acid on paper chromatograms. *J Am Chem Soc* 1955;77:4844–4846.
31. **Schleifer KH, Seidl PH.** Chemical composition and structure of murein. In: Goodfellow M and Minnikin DE (editors). *Chemical Methods in Bacterial Systematics*. London: Academic Press; 1985. pp. 201–220.
32. **Schleifer KH, Kandler O.** Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* 1972;36: 407–477.
33. **Schumann P.** Peptidoglycan structure. In: Rainey F and Oren A (editors). *Methods in Microbiology*, vol. 38. London: Taxonomy of Prokaryotes. Academic Press; 2011. pp. 101–129.
34. **Tindall BJ, Rosselló-Móra R, Busse HJ, Ludwig W, Kämpfer P.** Notes on the characterization of prokaryote strains for taxonomic purposes. *Int J Syst Evol Microbiol* 2010;60:249–266.
35. **Stackebrandt E, Goebel BM.** Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Evol Microbiol* 1994;44:846–849.
36. **Stackebrandt E, Ebers J.** Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* 2006;33:152–155.
37. **Stackebrandt E, Goodfellow M (editors).** *Nucleic acid techniques in bacterial systematics*. New York: John Wiley & Sons; 1991.
38. **Ji N, Peng B, Wang G, Wang S, Peng X.** Universal primer PCR with DGGE for rapid detection of bacterial pathogens. *J Microbiol Methods* 2004;57:409–413.
39. **Polz MF, Cavanaugh CM.** Bias in template-to-product ratios in multitemplate PCR. *Appl Environ Microbiol* 1998;64:3724–3730.
40. **Löffler FE, Sun Q, Li J, Tiedje JM.** 16S rRNA gene-based detection of tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* species. *Appl Environ Microbiol* 2000;66:1369–1374.
41. **Naser SM, Dawyndt P, Hoste B, Gevers D, Vandemeulebroecke K *et al*.** Identification of lactobacilli by pheS and rpoA gene sequence analyses. *Int J Syst Evol Microbiol* 2007;57:2777–2789.
42. **Svec P, Vancanneyt M, Koort J, Naser SM, Hoste B *et al*.** *Enterococcus devriesei* sp. nov., associated with animal sources. *Int J Syst Evol Microbiol* 2005;55:2479–2484.
43. **Svec P, Vancanneyt M, Devriese LA, Naser SM, Snauwaert C *et al*.** *Enterococcus aquimarinus* sp. nov., isolated from sea water. *Int J Syst Evol Microbiol* 2005;55:2183–2187.
44. **Naser SM, Thompson FL, Hoste B, Gevers D, Dawyndt P *et al*.** Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on rpoA and pheS genes. *Microbiology* 2005;151:2141–2150.
45. **Kim OS, Cho YJ, Lee K, Yoon SH, Kim M *et al*.** Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* 2012;62:716–721.
46. **Tamura K, Stecher G, Peterson D, Filipski A, Kumar S.** MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 2013;30:2725–2729.
47. **Tamura K, Nei M, Kumar S.** Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Natl Acad Sci USA* 2004;101:11030–11035.
48. **Saitou N, Nei M.** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406–425.
49. **Kumar S, Nei M, Dudley J, Tamura K.** MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* 2008;9:299–306.
50. **Felsenstein J.** Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985;39:783–791.
51. **Schleifer KH, Ludwig W.** Phylogeny of the Genus *Lactobacillus* and related genera. *Syst Appl Microbiol* 1995;18:461–467.
52. **Wayne LG, Moore WEC, Stackebrandt E, Kandler O, Colwell RR *et al*.** Report of the ad Hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Evol Microbiol* 1987;37:463–464.
53. **Yarza P, Yilmaz P, Pruesse E, Glöckner FO, Ludwig W *et al*.** Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nat Rev Microbiol* 2014; 12:635–645.
54. **Kim M, Oh HS, Park SC, Chun J.** Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *Int J Syst Evol Microbiol* 2014;64:346–351.
55. **Kim M, Hs O, Park SC, Chun J.** Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes (vol 64, pg 346, 2014). *Int J Syst Evol Microbiol* 1825;2014:64.
56. **Jeske O, Schüler M, Schumann P, Schneider A, Boedeker C *et al*.** Planctomycetes do possess a peptidoglycan cell wall. *Nat Commun* 2015;6:7116.


**Five reasons to publish your next article with a Microbiology Society journal**

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as 'excellent' or 'very good'.
5. Your article will be published on an interactive journal platform with advanced metrics.

**Find out more and submit your article at microbiologyresearch.org.**

Downloaded from www.microbiologyresearch.org by
IP: 129.254.46

## CHAPTER C – Section 2 Supplementary Material

# *Lactobacillus cerevisiae* sp. nov., isolated from a spoiled brewery sample

Jennifer Koob[1], Fritz Jacob[1], Mareike Wenning[2], Mathias Hutzler[1]

International Journal of Systematic and Evolutionary Microbiology

[1] Technische Universität München, Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität, Alte Akademie 3, 85354 Freising, Germany

[2] Technische Universität München, Zentralinstitut für Ernährungs- und Lebensmittelforschung, Department Microbiology, Weihenstephaner Berg 3, 85354 Freising, Germany

*Corresponding author:*

*Email:* hutzler@wzw.tum.de*, Phone: +49 8161 713100, Address: Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität, Department Microbiology, Alte Akademie 3, 85354 Freising, Germany*

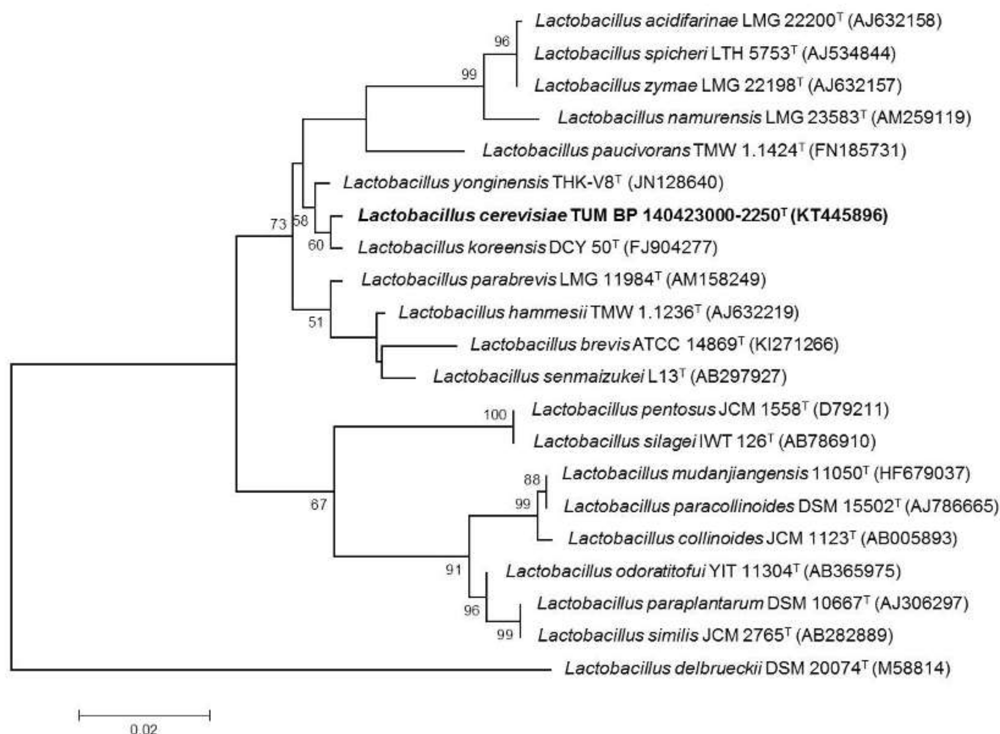



**Fig. S1.** Maximum likelihood phylogenetic tree reconstructed from a comparative analysis of 16S rRNA gene sequences of *L. cerevisiae* TUM BP 140423000-2250$^T$ and related type strains. Bootstrap values ≥ 50 % are shown (1000 replicates). Bar, 0.02 substitutions per nucleotide position.

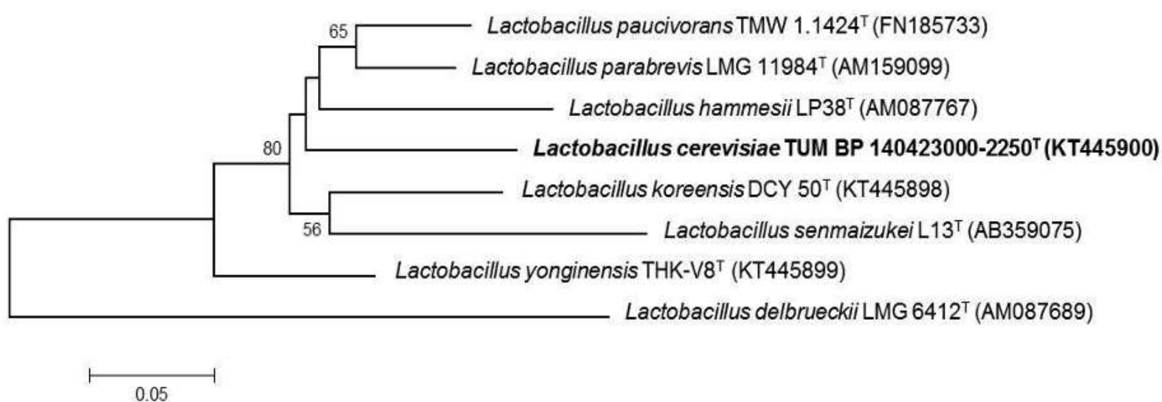



**Fig. S2.** Neighbor-joining tree based on the sequence of the *pheS* housekeeping gene showing the phylogenetic relationship between *L. cerevisiae* TUM BP 140423000-2250$^T$ and closely related taxa. Bootstrap values (expressed as percentages of 1000 replicates) ≥ 50 % are shown. Bar, 0.05 substitutions per nucleotide position.

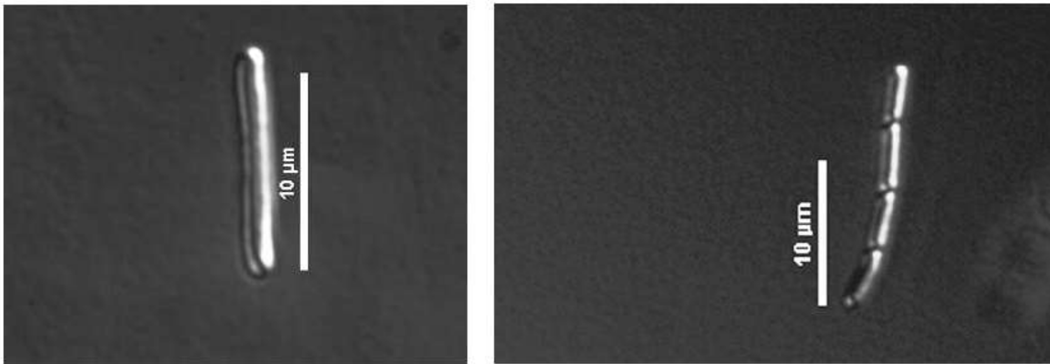



**Fig. S3.** 100x DIC oil immersion microscopic pictures of bacterium *L. cerevisiae* TUM BP 140423000-2250$^{T}$ after 48 h in MRS broth; recorded with a Nikon Eclipse Ti microscope and (DIS)Zyla V - 3tap camera

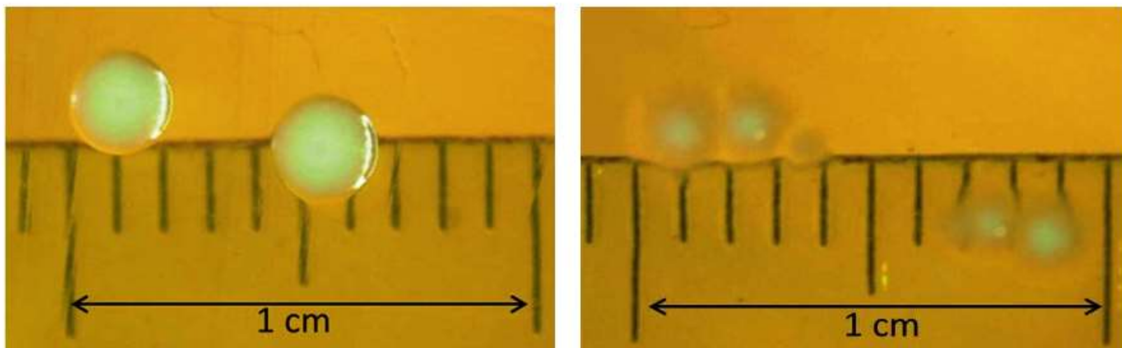


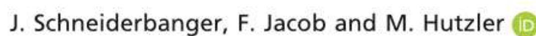

**Fig. S4.** Colony morphology of a) colonies with smooth edges and b) colonies with fringed edges on MRS agar after 48 h of cultivation; recorded with a Nikon D5100 camera

**Table S1.** Enzymatic activities of *L. cerevisiae* TUM BP 140423000-2250$^T$ and closely related taxa

Strains*:* 1, *Lactobacillus cerevisiae* TUM BP 140423000-2250$^T$; 2, *L. yonginensis* THK-V8$^T$; 3, *L. koreensis* DCY50$^T$; 4, *L. parabrevis* LMG 11984$^T$*;* 5, *L. hammesii* TMW 1.1236$^T$; 6, *L. brevis* ATCC 14687$^T$; 0, no activity; 1, low activity; 2, 3, intermediate activity; 4, 5, high activity ; all data from this study.

| **Enzyme** | **1** | **2** | **3** | **4** | **5** | **6** |
|---|---|---|---|---|---|---|
| Alkaline phosphatase | 0 | 2 | 1 | 0 | 1 | 1 |
| Esterase | 2 | 2 | 2 | 3 | 3 | 1 |
| Esterase lipase | 1 | 3 | 2 | 1 | 1 | 0 |
| Lipase | 0 | 0 | 0 | 0 | 0 | 0 |
| Leucine arylamidase | 2 | 1 | 5 | 4 | 4 | 4 |
| Valine arylamidase | 1 | 0 | 4 | 3 | 4 | 3 |
| Cystine arylamidase | 0 | 0 | 0 | 0 | 0 | 0 |
| Trypsin | 0 | 0 | 0 | 0 | 0 | 0 |
| α-Chymotrypsin | 0 | 0 | 3 | 0 | 0 | 0 |
| Acid phosphatase | 2 | 2 | 5 | 3 | 3 | 4 |
| Naphtol-AS-BI-phosphohydrolase | 1 | 1 | 1 | 2 | 2 | 1 |
| α-Galactosidase | 3 | 3 | 5 | 0 | 0 | 4 |
| β-Galactosidase | 3 | 4 | 5 | 0 | 5 | 5 |
| β-Glucuronidase | 5 | 2 | 0 | 4 | 5 | 4 |
| α-Glucosidase | 4 | 4 | 4 | 5 | 0 | 4 |
| β-Glucosidase | 4 | 3 | 4 | 4 | 5 | 5 |
| N-acetyl-β-glucosaminidase | 0 | 0 | 0 | 0 | 0 | 0 |
| α-Mannosidase | 0 | 0 | 0 | 0 | 0 | 0 |
| α-Fucosidase | 0 | 0 | 0 | 0 | 0 | 0 |

## 3.5 CHAPTER D – Genotypic and phenotypic diversity of *Lactobacillus rossiae* from beer



Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

# Genotypic and phenotypic diversity of *Lactobacillus rossiae* isolated from beer

J. Schneiderbanger, F. Jacob and M. Hutzler

Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität, Technische Universität München, Freising, Germany

**Keywords**
beer-spoilage bacteria, brewing microbiology, *Lactobacillus rossiae*, polyphasic characterization, rep-PCR genomic fingerprinting, sourdough microbiota.

**Correspondence**
Mathias Hutzler, Department Microbiology, Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität, Technische Universität München, Alte Akademie 3, 85354 Freising, Germany.
E-mail: hutzler@wzw.tum.de

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rpoA* and *pheS* gene sequences of beer-related *Lactobacillus rossiae* isolates are MG674703–MG674713, MG755218–MG755228 and MG755229–MG755239 respectively.

2018/2000: received 19 October 2018, revised 13 December 2018 and accepted 4 January 2019

doi:10.1111/jam.14202

## Abstract

**Aims:** Over the past few years, the lactic acid bacteria (LAB) species *Lactobacillus rossiae* has appeared on occasion as a beer spoiler, in addition to its role as an inhabitant of sourdough and other foods. Many authors have described the *L. rossiae* sourdough isolates as phenotypically and genotypically extremely versatile. This characterization was confirmed in a comprehensive genotypic and phenotypic study based on 11 beer-related *L. rossiae* isolates.
**Materials and methods:** The beer-related isolates and the *L. rossiae* type strain were classified in a polyphasic approach applying 16S rRNA, *rpoA* and *pheS* housekeeping gene sequence comparisons, DNA–DNA hybridization and rep-PCR technique. Additionally, carbohydrate fermentation and amino-acid metabolism were examined. In terms of the beer-spoilage ability, the growth in two different beer types was examined and the presence of three prominent hop resistance genes (*horA*, *horC* and *hitA*) and of one gene presumably responsible for the production of exopolysaccharides (*gtf*) was checked.
**Conclusion:** The carbohydrate fermentation pattern $(GTG)_5$ rep-PCR and the *pheS* gene sequence comparison showed deviations between sourdough and beer-related isolates. DNA–DNA hybridization values and the *pheS* gene sequence comparison between beer-related isolates point towards the need for expansion of the limits for species description.
**Significance and Impact of the Study:** *Lactobacillus rossiae* shows great phenotypic and genotypic variability stretching the limits of species description. The correlation between *pheS* gene sequence and the presence of the *horC* gene is important for brewing microbiologists and the search for beer-spoilage prediction methods.

## Introduction

*Lactobacillus rossiae* (formerly *rossii*) was primarily isolated from Italian sourdough that contains many homo- and heterofermentative lactic acid bacteria (LAB) species (Wood and Holzapfel 1995; Corsetti *et al.* 2005; Ehrmann and Vogel 2005; De Vuyst and Vancanneyt 2007). *Lactobacillus rossiae* was thought to be an autochthonous species whose habitat was exclusively sourdough (Di Cagno *et al.* 2007). But further *L. rossiae* isolates were obtained from pig faeces (De Angelis *et al.* 2006), human faeces (Di Cagno *et al.* 2009), spelt flour (Coda *et al.* 2010), pineapples (Di Cagno *et al.* 2010) and fermented meat (Doan Thi Lam *et al.* 2013). *Lactobacillus rossiae* was also found several times in the brewing environment (Thelen *et al.* 2006; Taskila *et al.* 2011; Hutzler *et al.* 2012; Riedl *et al.* 2017). *Lactobacillus rossiae*'s occupation of the beer habitat is not surprising, since sourdough and beer resemble one another, with maltose being the most abundant fermentable carbohydrate.

The remarkable genotypic and phenotypic diversity of *L. rossiae* strains isolated from sourdough was examined by Di Cagno *et al.* (2007) and Scheirlinck *et al.* (2009). No correlation between genotypic and physiological analyses could be observed (Di Cagno *et al.* 2007). Furthermore, *L. rossiae* isolates from sourdough

could not be assigned to a certain species by the API 50 CHL system (Corsetti *et al.* 2005; Scheirlinck *et al.* 2009).

Polyphasic approaches are used to validly classify bacterial strains that have high values of 16S rRNA gene sequence homology (Vandamme *et al.* 1996; Stackebrandt *et al.* 2002). Random Amplification of Polymorphic DNA-PCR, fermentation profile by Analytical Profile Index (API) and Biolog systems, acidification kinetics, proteinase and peptidase activities (Di Cagno *et al.* 2007), rep-PCR genomic fingerprinting, DNA–DNA hybridization, *pheS* and *rpoA* housekeeping gene sequence comparisons (Scheirlinck *et al.* 2007) as well as multilocus sequencing (De Angelis *et al.* 2014) were employed to reveal inter- and intra-species differences between sourdough LAB including *L. rossiae* isolates.

At the Research Center for Brewing and Food Quality (BLQ) of the Technical University of Munich (TUM), the microbiology laboratory staff analyses thousands of beer samples every year for the presence of beer-spoiling bacteria. In a comprehensive study of the real-time PCR results obtained from routine analysis samples, the percentages of the most abundant beer-spoiling bacteria were evaluated, resulting in a low 7-year mean percentage for *L. rossiae* of 1·52% of all identified LAB (Schneiderbanger *et al.* 2018).

*Lactobacillus rossiae* belongs to the *Lactobacillus reuteri* group (De Vuyst and Vancanneyt 2007) which is closely related to the *Lactobacillus brevis* and *Lactobacillus plantarum* groups, indicating that these species could have been following the same adaptation strategies (De Angelis *et al.* 2014). *Lactobacillus rossiae* cells are Gram-positive, catalase-negative rods of $0{\cdot}5 \times 1{\cdot}0\text{–}1{\cdot}5\ \mu m$. They are microaerophilic and obligately heterofermentative cells that are able to grow at 15°C but not at 45°C. Arginine hydrolysis is positive and DL-lactic acid is produced. The peptidoglycan structure is of the A3$\alpha$ (L-Lys-L-Ser-L-$Ala_2$) type and the G+C content is 44·6 mol.% (Corsetti *et al.* 2005). Based on the 16S rRNA gene sequence, the nearest neighbours are *Lactobacillus siliginis* (98·6%; primarily isolated from the Korean wheat sourdough) and the recently described *Lactobacillus curtus* (98·2%; primarily isolated from Finnish beer) (Aslam *et al.* 2006; Asakawa *et al.* 2017).

In this study, selected strains of *L. rossiae* previously isolated from contaminated beer were examined for their genotypic and phenotypic diversity and compared with the results of sourdough isolates. Since *L. rossiae* has not yet been described as a beer spoiler, different beer-related characteristics were assessed. It is of practical relevance for brewing microbiologists to determine the range of genotypic and phenotypic properties of spoilage species.

## Materials and methods

### Strains

Eleven different strains, isolated from contaminated beer samples of seven different breweries, were chosen to uncover the phenotypic and genotypic diversity of beer-related *L. rossiae* strains (Table 1). The *L. rossiae* type strain $CS1^T$ (=DSM $15814^T$) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). One beer-spoiling *L. plantarum* strain was included in the phylogenetic analyses as an outside species of the group of beer-spoiling bacteria. For phylogenetic analyses, gene sequences of *L. rossiae* sourdough isolates were included as well, if available.

### Growth and cultivation conditions

All strains used in this study were routinely examined by the laboratory staff of the Research Center Weihenstephan for Brewing and Food Quality and identified by the real-time PCR method using the foodproof® beer screening kit (Biotecon Diagnostics, Potsdam, Germany). The strains were then separated on de Man, Rogosa and Sharpe agar (MRS; pH 6·2) under anaerobic conditions at 28 ± 1°C (De Man *et al.* 1960). Ten colonies of each strain were combined to form the initial cultures followed by storage at −80°C in cryostock. The purity of active strains was controlled microscopically and by preparing streak cultures.

### Phenotypic characterization methods

Gram staining was performed according to Buck (1982). Catalase activity was determined by transferring fresh

**Table 1** List of *Lactobacillus rossiae* species isolated from spoiled beer samples; the *L. rossiae* type strain DSM $15814^T$ (all analyses) as well as one beer-spoilage *Lactobacillus plantarum* strain (phylogenetic analyses) were included

| Species | Internal number | Culture collection number BLQ | Brewery |
|---|---|---|---|
| *L. rossiae* | 84 | TUM BP 111219031-2086 | Brewery A |
| *L. rossiae* | 396 | TUM BP 120525004-2385 | Brewery B |
| *L. rossiae* | 1495 | TUM BP 121217008-2365 | Brewery A |
| *L. rossiae* | 1702 | TUM BP 130607017-2573 | Brewery B |
| *L. rossiae* | 1769 | TUM BP 130612024-2555 | Brewery C |
| *L. rossiae* | 1775 | TUM BP 130717030-2662 | Brewery A |
| *L. rossiae* | 1918 | TUM BP 131011001-2846 | Brewery D |
| *L. rossiae* | 1922 | TUM BP 131022000-2858 | Brewery E |
| *L. rossiae* | 1950 | TUM BP 131022011-2866 | Brewery E |
| *L. rossiae* | 2202 | TUM BP 140129030-2132 | Brewery B |
| *L. rossiae* | 2403 | TUM BP 000-2360 | Brewery F |
| *L. rossiae* | $DSM15814^T$ | TUM BP 000-2358 | |
| *L. plantarum* | 1173 | TUM BP 120919039-3095 | |

colonies from MRS agar to a glass slide and adding 3% (volume per volume) $H_2O_2$ solution. Oxidase activity was determined using Bactident oxidase strips (Merck, Darmstadt, Germany). The test for gas production from glucose and gluconate was carried out in triplicate in MRS broth with Durham tubes (Back 1994). The test for $NH_3$ production from arginine was carried out according to Back (2000). The carbohydrate fermentation patterns were analysed using the API 50 CHL medium kit (bioMérieux, Nürtingen, Germany). The utilization of the sugar compounds contained in beer as well as the utilization of free amino acids was checked by HPLC at the Research Center Weihenstephan for Brewing and Food Quality (MEBAK chapters 2.7.1 and 2.7.2 for carbohydrates and 2.6.4.1.2 for amino acids; (Jacob 2012)). Therefore, all strains were incubated anaerobically in a wheat beer medium (Riedl *et al.* 2017) for 8 days at 28 ± 1°C in duplicate and, after microscopic confirmation of sufficient cell proliferation (end cell count $\geq 1 \times 10^6$ cells per ml), analysed for the amount of residual sugars and residual amino-acid content.

Additional physiological characteristics were analysed in triplicate in MRS broth inoculated with 100 $\mu$l of overnight bacterial suspension. Temperature tolerance (10, 15, 40 and 45°C), NaCl tolerance (4·0, 6·5 and 8·0% (weight per weight) NaCl), alcohol tolerance (2·5–8·0% (volume per volume) undenatured ethanol) and acid tolerance (pH values 4·0, 5·0, 6·0, 7·0 and 8·0, adjusted with lactic acid or sodium hydroxide respectively) were evaluated by visual control of turbidity and sediment formation in the test tubes over a period of 4 weeks. Incubation was carried out anaerobically using the Anaerocult® system (Merck, Darmstadt, Germany) at 28 ± 1°C, if not indicated otherwise (i.e. temperature tolerance). Since at least one of the *L. rossiae* isolates (TUM BP 131022000-2858) caused ropiness in beer, the strains were checked by glycosyltransferase (*gtf*) gene PCR. The gene encodes for the potential production of

**Table 2** Discriminating physiological characteristics between the *Lactobacillus rossiae* beer-spoiling isolates and the type strain DSM 15814ᵀ

| Strain | Gas production from gluconate | $NH_3$ from arginine | Temperature tolerance 45°C | Salt tolerance 6·5% NaCl | Salt tolerance 8·0% NaCl |
|---|---|---|---|---|---|
| *L. rossiae* 84 | − | − | − | + | − |
| *L. rossiae* 396 | + | + | − | + | − |
| *L. rossiae* 1495 | − | − | − | − | − |
| *L. rossiae* 1702 | + | + | + | + | − |
| *L. rossiae* 1769 | − | − | − | − | − |
| *L. rossiae* 1775 | − | − | − | − | − |
| *L. rossiae* 1918 | + | + | + | + | + |
| *L. rossiae* 1922 | − | − | − | − | − |
| *L. rossiae* 1950 | + | + | + | + | − |
| *L. rossiae* 2202 | + | + | + | + | + |
| *L. rossiae* 2403 | + | + | − | + | − |
| *L. rossiae* 15814ᵀ | + | + | − | + | − |

**Table 3** Differential characteristics of carbohydrate fermentation via API CHL 50 system between the beer-spoiling *Lactobacillus rossiae* strains and the type strain DSM 15814ᵀ

| | L-arabinose | D-xylose | D-galactose | D-fructose | D-mannose | Methyl-αD-glucosepyranosid | D-melibiose | D-lyxose | Potassium gluconate |
|---|---|---|---|---|---|---|---|---|---|
| *L. rossiae* 84 | − | − | − | + | + | − | − | − | − |
| *L. rossiae* 396 | + | + | + | + | + | + | + | + | + |
| *L. rossiae* 1495 | − | − | − | − | − | w | − | − | − |
| *L. rossiae* 1702 | + | + | + | + | + | − | + | + | + |
| *L. rossiae* 1769 | + | − | − | + | w | − | − | − | − |
| *L. rossiae* 1775 | − | − | − | − | − | + | − | − | − |
| *L. rossiae* 1918 | + | + | + | + | w | + | − | + | w |
| *L. rossiae* 1922 | + | + | − | + | + | + | − | − | + |
| *L. rossiae* 1950 | + | + | w | + | w | + | − | − | w |
| *L. rossiae* 2202 | + | + | + | + | w | + | − | + | w |
| *L. rossiae* 2403 | + | + | + | + | w | + | − | + | w |
| *L. rossiae* DSM 15814ᵀ | w | + | w | + | w | w | − | − | w |

+, positive; −, negative; w, weakly positive after 48 h and positive after 120 h.

**Table 4** Sugar utilization of *Lactobacillus rossiae* after 8 days in wheat beer medium analysed by HPLC

| | 84 | 396 | 1495 | 1702 | 1769 | 1775 | 1918 | 1922 | 1950 | 2202 | 15814$^T$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Fructose | +/– | + | – | + | – | +/– | + | – | + | + | + |
| Glucose | + | + | + | + | + | + | + | + | + | + | + |
| Sucrose | – | + | +/– | +/– | – | – | – | – | – | – | – |
| Maltose | + | + | + | + | + | + | + | + | + | + | + |
| Maltotriose | +/– | – | + | + | +/– | + | + | +/– | + | + | + |

+, positive (≥50% of original sugar content degraded); –, negative (≤10% of original sugar content degraded), +/–, weak fermentation (>10 and <50% of original sugar content degraded).

exopolysaccharides. The primer pair GTFF and GTFR as well as the temperature protocol and PCR conditions were adopted from Werning *et al.* (2006). The presence of resulting PCR products was checked by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

### Genotypic characterization methods

Bacterial DNA was extracted and prepared as described before (Koob *et al.* 2016). One hundred and fifty nanograms of bacterial DNA and the $(GTG)_5$ primer (5′-GTGGTGGTGGTGGTG-3′) was used to create rep-PCR fingerprints of all isolates under the PCR conditions described previously (Versalovic *et al.* 1994) using an Eppendorf Mastercycler gradient (Eppendorf, Wesseling-Berzdorf, Germany). The fingerprints were visualized using an Agilent 2100 Bioanalyzer. The resulting profiles were analysed using the BioNumerics ver. 7.5 software package (Applied-Maths, Sint-Martens-Latem, Belgium). The similarity between the profiles was calculated by the Pearson correlation and a dendrogram was created by the UPGMA (unweighted pair group method with arithmetic mean) method. In this study, $(GTG)_5$ clusters were specified by 50 % Pearson correlation.

The 16S rRNA gene sequences were determined using the primer pairs 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) as well as 933f (5′-GCACAAGCGGTGGAGCATGTGG-3′) and 1541r (5′-AAGGAGGTGATCCAGCCGCA-3′) (Stackebrandt and Goodfellow 1991; Polz and Cavanaugh 1998; Löffler *et al.* 2000; Ji *et al.* 2004).

A higher resolution potential with regard to the genera *Lactobacillus* and *Enterococcus* can be achieved by comparing the sequences of the phenylalanyl-tRNA synthetase alpha subunit (*pheS*) or the RNA polymerase alpha subunit (*rpoA*) housekeeping genes (Cooper and Feil 2004; Svec *et al.* 2005a, 2005b; Naser *et al.* 2007). The primer set (rpoA-21-F and rpoA-23-R) as well as the temperature protocol for *rpoA* PCR was adopted from Naser *et al.* (2005). The primer set (pheS-forward and pheS-reverse) as well as the temperature for *pheS* PCR was adopted from Ehrmann *et al.* (2010). The sequencing was performed by GATC Biotech (Konstanz, Germany).

**Table 5** List of beer-spoilage *Lactobacillus rossiae* strains studied, applied phylogenetic techniques and clustering results

| | | | Techniques applied | | | | |
|---|---|---|---|---|---|---|---|
| Isolate | Brewery | Country | a | b | c | d | e |
| *L. rossiae* 84 | A | Austria | I | 1 | • | I | |
| *L. rossiae* 396 | B | Germany | III | 1 | •• | I | |
| *L. rossiae* 1495 | A | Austria | I | 1 | • | I | + |
| *L. rossiae* 1702 | B | Germany | III | 1 | •• | II | |
| *L. rossiae* 1769 | C | Croatia | I | 1 | • | I | |
| *L. rossiae* 1775 | A | Austria | I | 1 | • | I | |
| *L. rossiae* 1918 | D | Germany | III | 1 | • | III | + |
| *L. rossiae* 1922 | E | Germany | I | 1 | • | I | |
| *L. rossiae* 1950 | E | Germany | II | 1 | •• | II | + |
| *L. rossiae* 2202 | B | Germany | III | 1 | •• | II | |
| *L. rossiae* 2403 | F | Germany | III | 1 | •• | II | |
| *L. rossiae* DSM 15814$^T$ | | | II | 1 | •• | II | |

Comments: A–F, original isolation site and country; I–III, $(GTG)_5$ group I–III; 1, 16S rRNA group 1; •/••, *rpoA* group • and ••; I–III, *pheS* group I–III. Applied techniques: a, $(GTG)_5$ Rep-PCR fingerprinting; b, 16S rRNA gene sequencing; c, *rpoA* gene sequencing; d, *pheS* gene sequencing; e, DNA–DNA hybridization.

The sequences of 16S rRNA, *pheS* and *rpoA* housekeeping genes were compared with each other and phylogenetic trees were constructed using the MEGA6 software (Felsenstein 1985; Saitou and Nei 1987; Kumar *et al.* 2001, 2008; Tamura *et al.* 2004, 2013).

DNA–DNA hybridization values between three different isolates (*L. rossiae* 1495, 1918 and 1950) were determined at the DSMZ (De Ley *et al.* 1970; Cashion *et al.* 1977; Huss *et al.* 1983; Stackebrandt and Goebel 1994; Stackebrandt and Ebers 2006; Tindall *et al.* 2010).

### Hop resistance genes

The tolerance of micro-organisms to hop iso-α-acids is a crucial characteristic for the ability to grow in and spoil beer. Different genes have been described to confer hop resistance and were applied to differentiate beer spoilers from nonspoilers (Sami *et al.* 1997; Hayashi *et al.* 2001;

Sakamoto *et al.* 2001, 2002; Suzuki *et al.* 2002, 2004a, 2004b, 2004c, 2004d, 2006a, 2006b; Sakamoto and Konings 2003; Fujii *et al.* 2005; Iijima *et al.* 2006, 2007, 2009; Behr *et al.* 2007; Haakensen *et al.* 2007, 2008; Behr 2008; Haakensen 2009; Vogel *et al.* 2010; Geißler 2016). The presence of the genes *horA* and *horC* was analysed by a real-time PCR method developed by Koob *et al.* (2016) to be compatible with a PCR system established by Brandl (2006). The endpoint PCR method for the *hitA* hop resistance gene was adopted from Haakensen and modified (Haakensen 2009; Koob *et al.* 2016). The resulting PCR products were detected by capillary electrophoresis as mentioned above.

### Growth in beer

To determine the ability of the *L. rossiae* isolates to grow in beer, two different beer types (filtered (=crystal) wheat beer and wheat beer) were inoculated in duplicate with cell concentrations of 1000 cells per ml for all analysed isolates and incubated for 6 weeks at 28 ± 1°C. The end cell concentrations were determined by dilution series poured into Petri dishes with MRS agar (De Man *et al.* 1960).

## Results

All tested strains were Gram-positive, catalase-negative, oxidase-negative and form gas from glucose. All isolates tolerated ethanol up to a concentration of 8·0% (volume per volume) and pH values from 4·0 to 8·0. Seven of the 12 tested strains were positive for the production of gas from gluconate and simultaneously positive for $NH_3$ production from arginine. Only four of the examined strains were able to grow at 45°C while tolerating 10, 15 and 40°C. Although all strains demonstrated tolerance to 4·0% (weight per weight) NaCl, only eight strains tolerated 6·5% (weight per weight) and two were able to grow in MRS broth supplemented with 8·0% (weight per weight) NaCl (Table 2).

All strains as well as the type strain were checked for their carbohydrate fermentation pattern via API CHL 50 system. All tested strains were positive for D-ribose, D-

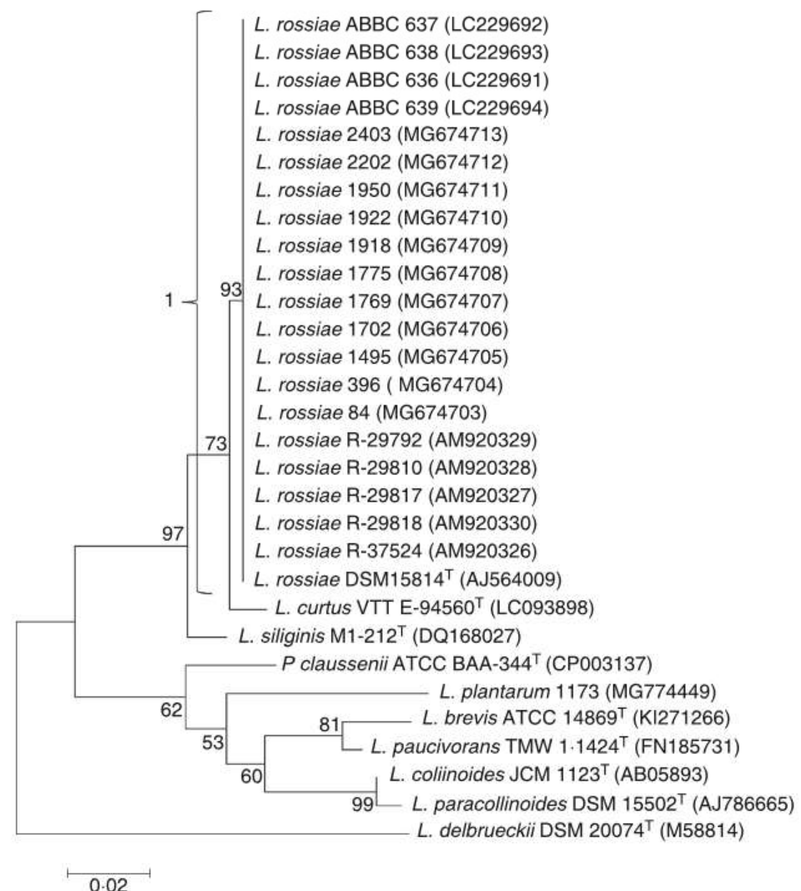



**Figure 1** Dendrogram of maximum-likelihood phylogenetic tree of 16S rRNA gene sequences of *Lactobacillus rossiae* beer-spoilage (bold letters) and sourdough isolates with *L. rossiae* and phylogenetically related type strains as well as the beer-spoilage isolate *Lactobacillus plantarum* 1173 using MEGA6 software. *Lactobacillus delbrueckii* DSM 20074$^T$ was used as an outside species. Bootstrap values ≥50% were included (1000 replicates); bar = number of substitutions per site; 1 = 16S rRNA gene cluster 1.

glucose, N-acetylglucosamine and D-maltose. The differing carbohydrates are listed in Table 3.

The results of HPLC sugar and amino-acid analyses are shown in Table 4 and Table S1. Glucose and maltose are metabolized by all strains. The trisaccharide maltotriose was utilized by all strains except one (isolate 396). Sucrose was metabolized by only three strains. The usage of fructose as an energy supplier varies again between strains, although the results were incongruent with the fructose utilization analysed by the API 50 CHL medium kit (compare with Table 3).

A summary of conducted phylogenetic analyses and their results is shown in Table 5.

The $(GTG)_5$ rep-PCR genomic fingerprinting including all beer-spoiling strains as well as the *L. rossiae* type strain DSM 15814$^T$ would result in three distinctive clusters based on 50 % Pearson correlation (Fig. S1).

All beer-spoilage isolates show ≥99·5% 16S rRNA gene homology with each other including the type strain DSM 15814$^T$ (Fig. 1; see Fig. S2 for the UPGMA phylogenetic tree). Beer-spoilage and sourdough strains are not discriminated by 16S rRNA analysis.

The comparison of the partial *rpoA* gene sequences revealed a great homology between the *L. rossiae* isolates (Fig. 2; for the *rpoA* gene UPGMA analysis, see Fig. S3). Clusters '•' and '••' show ≥98·5% *rpoA* sequence homology. The two originating *L. rossiae* main clusters do not differentiate between beer-spoilage and sourdough isolates.

Based upon the *pheS* gene sequence comparison, the beer-spoilage strains are classified into three different clusters, with cluster I containing exclusively beer-spoilage isolates and clusters II and III also containing sourdough *L. rossiae* isolates (Fig. 3; for the *pheS* UPGMA tree, see Fig. S4). *Lactobacillus rossiae* 1918 (cluster III) shared ≥94·6% *pheS* gene sequence homology with cluster I and ≥88·4% with cluster III. The delineation level between cluster I and cluster II is ≥88·3%.

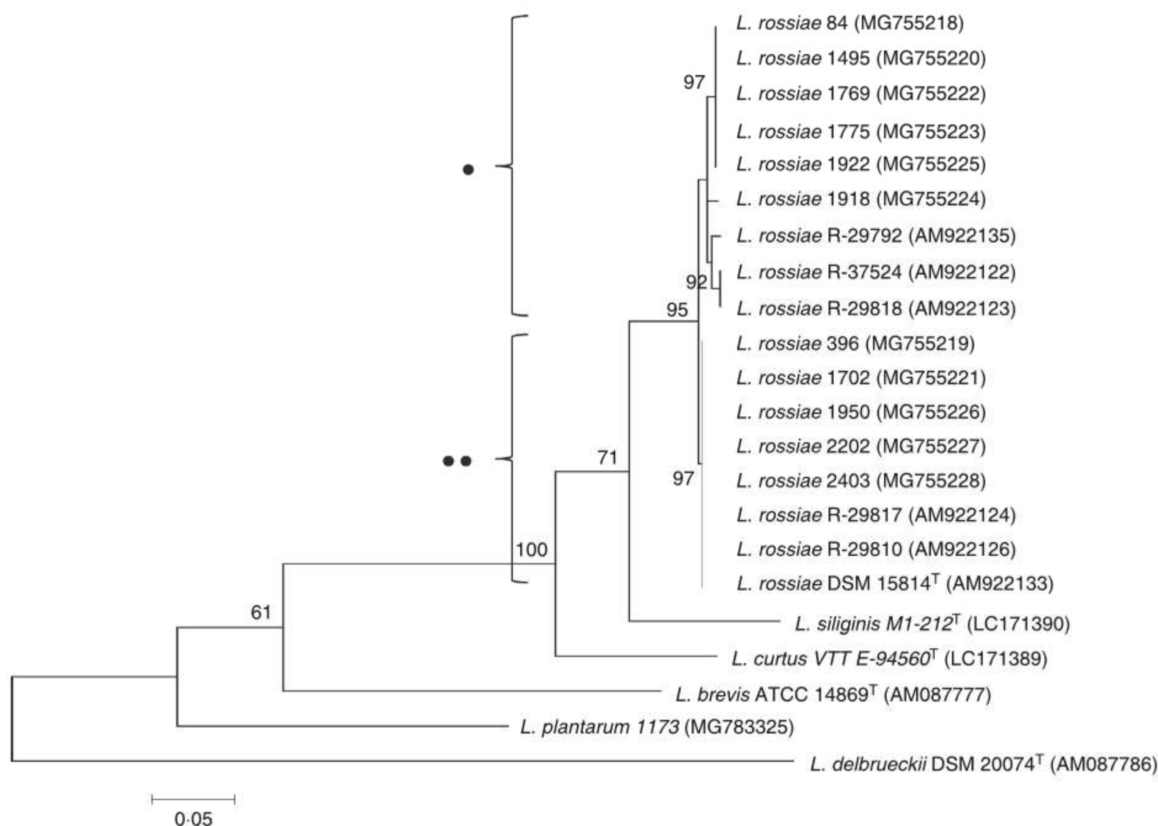



**Figure 2** Dendrogram of maximum-likelihood phylogenetic tree of *rpoA* gene sequences of *Lactobacillus rossiae* beer-spoilage (bold letters) and sourdough isolates with *L. rossiae* and phylogenetically related type strains as well as the beer-spoilage isolate *Lactobacillus plantarum* 1173. *Lactobacillus delbrueckii* DSM 20074$^T$ was used as an outside species. Bootstrap values ≥50% were included (1000 replicates); bar = number of substitutions per site; •/•• = *rpoA* gene clusters '•' and '••'.

The DNA–DNA hybridization values between three members of different *pheS* gene sequence clusters (isolate 1495, *pheS* gene cluster I; isolate 1950, *pheS* cluster II; isolate 1918, *pheS* gene cluster III) were determined to conclusively assign the beer-spoilage isolates to the species *L. rossiae*. The DNA–DNA relatedness values between *L. rossiae* 1950 (II) and 1495 (I) were 64·6% (62·3%) and between *L. rossiae* 1950 (II) and 1918 (III) 67·4% (58·1%) (values in parentheses are results of measurements in duplicate).

The inoculation of wheat beer resulted in the growth of four *L. rossiae* isolates and inoculating filtered wheat beer resulted in two (Table 6). The hop resistance gene *horA*, which is widespread within the group of beer-spoiling bacteria, was not found in any of the tested strains. *HorC* was found in four, *hitA* in another two *L. rossiae* isolates. The *gtf* gene, which is related to the exopolysaccharide production of bacteria, was found in two isolates (*L. rossiae* 1922 and 2403), which also displayed ropiness when a colony was carefully drawn upwards with an inoculation loop. Only *L. rossiae* 1922 was able to increase the viscosity in the cultivation medium.

## Discussion

All conducted phenotypic analyses are in concordance with the *L. rossiae* type strain description (Corsetti *et al.* 2005). In comparison with the description of the *L. rossiae* type strain, $NH_3$ production from arginine (55% positive) and growth at 45°C (36% positive) are variable properties.

As proposed for *L. rossiae* sourdough isolates, the examined beer-spoilage isolates showed a broad range of carbohydrate fermentation capability. In contrast to the sourdough isolates examined by Di Cagno *et al.* (2007), not all beer-spoilage isolates were able to use fructose

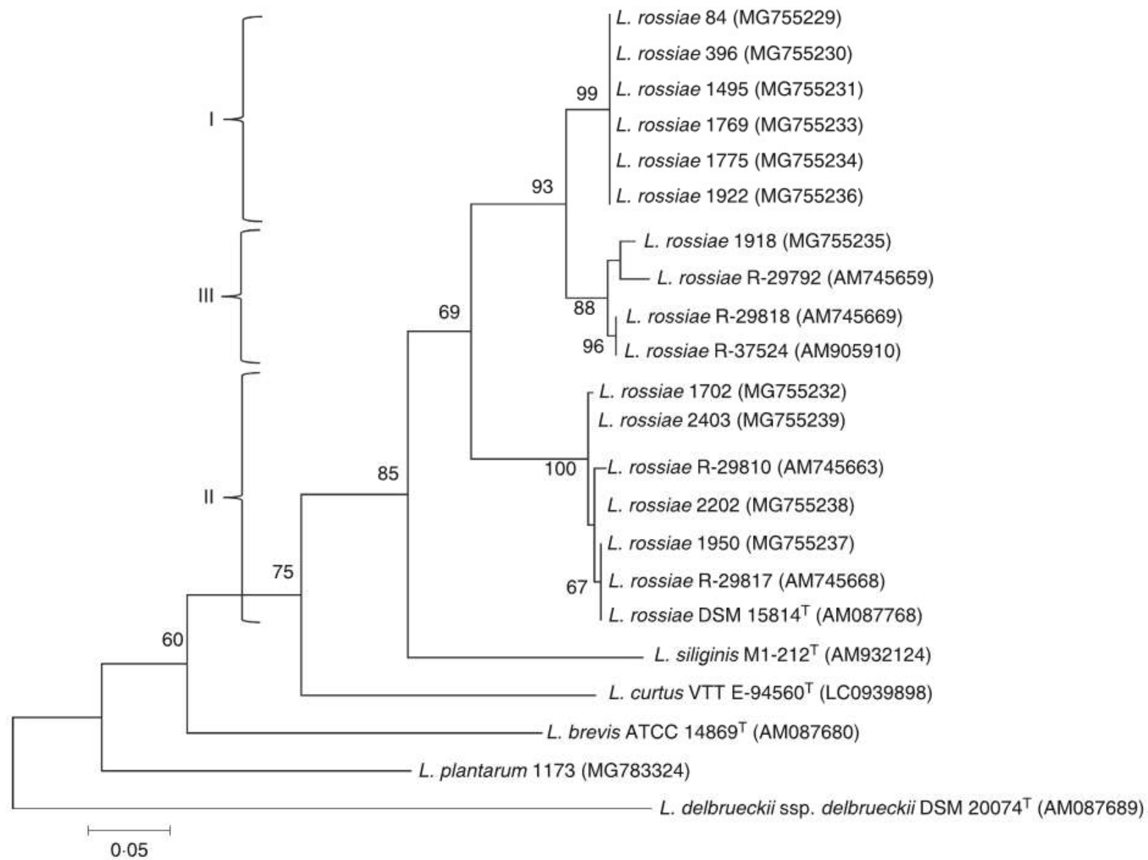



**Figure 3** Maximum-likelihood phylogenetic tree of *pheS* gene sequences of *Lactobacillus rossiae* beer-spoilage (bold letters) and sourdough isolates with *L. rossiae* and phylogenetically related type strains as well as the beer-spoilage isolate *Lactobacillus plantarum* 1173 using the MEGA6 software. *Lactobacillus delbrueckii* DSM 20074$^T$ was used as an outside species. Bootstrap values ≥50% were included (1000 replicates); bar = number of substitutions per site; I–III: *pheS* gene clusters I–III.

**Table 6** Growth of *Lactobacillus rossiae* isolates in two different beer types and presence of *horA*, *horC*, *hitA* and *gtf* genes; +, positive growth (end cell concentration ≥1 × $10^6$ cells per ml)/presence of examined gene; −, negative growth/absence of examined gene

| Isolate | Growth in wheat beer | Growth in filtered wheat beer | *horA* | *horC* | *hitA* | *gtf* |
|---|---|---|---|---|---|---|
| *L. rossiae* 84 | + | − | − | + | − | − |
| *L. rossiae* 396 | − | − | − | − | − | − |
| *L. rossiae* 1495 | − | − | − | − | − | − |
| *L. rossiae* 1702 | − | − | − | − | + | − |
| *L. rossiae* 1769 | + | + | − | + | − | − |
| *L. rossiae* 1775 | + | + | − | + | − | − |
| *L. rossiae* 1918 | − | − | − | − | − | − |
| *L. rossiae* 1922 | + | − | − | + | − | + |
| *L. rossiae* 1950 | − | − | − | − | + | − |
| *L. rossiae* 2202 | − | − | − | − | − | − |
| *L. rossiae* 2403 | − | − | − | − | − | + |
| *L. rossiae* DSM 15814$^T$ | − | − | − | − | − | − |

(82% positive), but all could use ribose as a carbon source and none of the examined strains used D-lactose. The suggestion that the sugar fermentation pattern was an unreliable method for identifying sourdough-related LAB (De Vuyst and Vancanneyt 2007) was confirmed in this study for beer-related *L. rossiae* isolates. The usage of ribose and the nonusage of lactose separated beer-spoilage and sourdough *L. rossiae* isolates.

With regard to the composition and degradation of free amino acids, the tested strains showed no distinct pattern (Table S1). This analysis is not suitable for assigning isolates to the species *L. rossiae* or assigning *L. rossiae* isolates to a certain cluster. The $(GTG)_5$ PCR clusters below 50% are considered to be separate species (Scheirlinck *et al.* 2007, 2008). This would permit the conclusion that the examined beer-spoilage isolates can be classified into at least two species or subspecies.

Based on a *rpoA* gene sequence intraspecies variation <1·5%, all beer-spoilage isolates are considered to be members of *L. rossiae* (variations of up to 2% possible within the *Lactobacillus* species; Naser *et al.* 2007).

The analysis of partial *pheS* gene sequences revealed a higher differentiation potential between the *L. rossiae* beer-spoilage and sourdough isolates. The proposed interspecies cut-off value for this analysis is ≤10% (Naser *et al.* 2007), which implies that only the four isolates *L. rossiae* 1702, 1950, 2202 and 2403, which are clustered together with the type strain DSM 15814$^T$, can unambiguously be assigned to the species *L. rossiae*. Based exclusively on this analysis, the examined set of isolates has to be classified into three separate species.

According to Wayne *et al.* (1987), DNA–DNA hybridization values ≤70% indicate an assignment to different species. Based upon the results of the often-discussed DNA–DNA hybridization method, the three analysed isolates belong to three different species. Based on the cut-off DNA–DNA values below 50% proposed by Gevers *et al.* (2005), all beer-related isolates belong to one species.

The presence of *horC* correlates with the ability of *L. rossiae* isolates to grow in wheat beer (in contrast to the hop resistance gene *hitA*), which indicates at least weak beer-spoilage potential. All isolates harbouring *horC* are members of the $(GTG)_5$ rep-PCR cluster I (Fig. S1), *rpoA* gene cluster '•' (Fig. 2) and *pheS* gene cluster I (Fig. 3). Especially, the distinction of one *pheS* gene cluster (I) containing only beer-related *L. rossiae* isolates—and, most importantly, all isolates showing beer-spoilage potential (compare Fig. 3 and Table 6)—is an extraordinary result since modern brewing microbiology focuses on the link between certain genes and the ability to spoil beer to develop fast spoilage-prediction methods.

16S rRNA and *rpoA* housekeeping gene sequences of all examined isolates as well as the results of phenotypic analyses pointed towards the affiliation to one LAB species – to *L. rossiae*. In contrast, $(GTG)_5$ rep-PCR (min. two species), *pheS* gene sequences (three species) and DNA–DNA hybridization values (one or three species, depending on the critical values of different authors) indicated membership to different species. None of the applied phylogenetic techniques was able to clearly differentiate between sourdough and beer-spoilage isolates.

The screening of a greater pool of beer-related *L. rossiae* isolates and the additional evaluation of whole genome sequence data could confirm the results and may result either in the definition of new interspecies cut-off values for $(GTG)_5$ rep-PCR and *pheS* gene sequence comparisons or in the definition of *L. rossiae* subspecies.

## Acknowledgements

We thank the microbiology laboratory staff for their help in micro-organism handling and Sarah Silva (MA BSc) for her English editing service. The research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

## Conflict of Interest

No conflict of interest declared.

## References

Asakawa, Y., Takesue, N., Asano, S., Shimotsu, S., Iijima, K., Suzuki, K., Motoyama, Y. and Aizawa, M. (2017) *Lactobacillus curtus* sp. nov., isolated from beer in Finland. *Int J Syst Evol Microbiol* **67**, 3899–3906.

Aslam, Z., Im, W.T., Ten, L.N., Lee, M.J., Kim, K.H. and Lee, S.T. (2006) *Lactobacillus siliginis* sp nov., isolated from wheat sourdough in South Korea. *Int J Syst Evol Microbiol* **56**, 2209–2213.

Back, W. (1994) *Farbatlas und Handbuch der Getränkebiologie Volume 1*. Nürnberg: Hans Karl Verlag.

Back, W. (2000) *Farbatlas und Handbuch der Getränkebiologie Volume 2*. Nürnberg: Hans Karl Verlag.

Behr, J. (2008). Mechanisms of hop inhibition, tolerance and adaptation in *Lactobacillus brevis*. PhD thesis, Wissenschaftszentrum Weihenstephan, Technische Universität München, Freising.

Behr, J. Israel, L., Gaenzle, M.G. and Vogel, R.F. (2007) Proteomic approach for characterization of hop-inducible proteins in *Lactobacillus brevis*. *Appl Environ Microbiol* **73**, 3300–3306.

Brandl, A. (2006) Entwicklung und Optimierung von PCR-Methoden zur Detektion und Identifizierung von brauereirelevanten Mikroorganismen zur Routine-Anwendung in Brauereien. PhD thesis, Lehrstuhl für Technologie der Brauerei II, Technische Universität München, Freising.

Buck, J.D. (1982) Non-staining (KOH) method for determination of gram reactions of marine-bacteria. *Appl Environ Microbiol* **44**, 992–993.

Cashion, P., Holderfranklin, M.A., McCully, J. and Franklin, M. (1977) Rapid method for base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.

Coda, R., Nionelli, L., Rizzello, C.G., De Angelis, M., Tossut, P. and Gobbetti, M. (2010) Spelt and emmer flours: characterization of the lactic acid bacteria microbiota and selection of mixed starters for bread making. *J Appl Microbiol* **108**, 925–935.

Cooper, J.E. and Feil, E.J. (2004) Multilocus sequence typing – what is resolved? *Trends Microbiol* **12**, 373–377.

Corsetti, A., Settanni, L., van Sinderen, D., Felis, G.E., Dellaglio, F. and Gobbetti, M. (2005) *Lactobacillus rossii* sp nov., isolated from wheat sourdough. *Int J Syst Evol Microbiol* **55**, 35–40.

De Angelis, M., Siragusa, S., Berloco, M., Caputo, L., Settanni, L., Alfonsi, G., Amerio, M., Grandi, A. *et al.* (2006) Selection of potential probiotic lactobacilli from pig feces to be used as additives in pelleted feeding. *Res Microbiol* **157**, 792–801.

De Angelis, M., Bottacini, F., Fosso, B., Kelleher, P., Calasso, M., Di Cagno, R., Ventura, M., Picardi, E. *et al.* (2014) *Lactobacillus rossiae*, a vitamin B-12 producer, represents a metabolically versatile species within the genus *Lactobacillus*. *PLoS ONE* **9**, 1–11.

De Ley, J., Cattoir, H. and Reynaert, A. (1970) Quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

De Man, J.C., Rogosa, M. and Sharpe, M.E. (1960) A medium for the cultivation of lactobacilli. *J Appl Bacteriol* **23**, 130–135.

De Vuyst, L. and Vancanneyt, M. (2007) Biodiversity and identification of sourdough lactic acid bacteria. *Food Microbiol* **24**, 120–127.

Di Cagno, R., De Angelis, M., Gallo, G., Settanni, L., Berloco, M.G., Siragusa, S., Parente, E., Corsetti, A. *et al.* (2007) Genotypic and phenotypic diversity of *Lactobacillus rossiae* strains isolated from sourdough. *J Appl Microbiol* **103**, 821–835.

Di Cagno, R., Rizzello, C.G., Gagliardi, F., Ricciuti, P., Ndagijimana, M., Francavilla, R., Guerzoni, M.E., Crecchio, C. *et al.* (2009) Different fecal microbiotas and volatile organic compounds in treated and untreated children with celiac disease. *Appl Environ Microbiol* **75**, 3963–3971.

Di Cagno, R., Cardinali, G., Minervini, G., Antonielli, L., Rizzello, C.G., Ricciuti, P. and Gobbetti, M. (2010) Taxonomic structure of the yeasts and lactic acid bacteria microbiota of pineapple (*Ananas comosus* L. Merr.) and use of autochthonous starters for minimally processing. *Food Microbiol* **27**, 381–389.

Doan Thi Lam, N., Van Hoorde, K., Cnockaert, M., De Brandt, E., De Bruyne, K., Binh Thanh, L. and Vandamme, P. (2013). A culture-dependent and -independent approach for the identification of lactic acid bacteria associated with the production of nem chua, a Vietnamese fermented meat product. *Food Res Int* **50**, 232–240.

Ehrmann, M.A. and Vogel, R.F. (2005) Molecular taxonomy and genetics of sourdough lactic acid bacteria. *Trends Food Sci Technol* **16**, 31–42.

Ehrmann, M.A., Preissler, P., Danne, M. and Vogel, R.F. (2010) *Lactobacillus paucivorans* sp. nov., isolated from a brewery environment. *Int J Syst Evol Microbiol* **60**, 2353–2357.

Felsenstein, J. (1985) Confidence-limits on phylogenies – an approach using the bootstrap. *Evolution* **39**, 783–791.

Fujii, T., Nakashima, K. and Hayashi, N. (2005) Random amplified polymorphic DNA-PCR based cloning of markers to identify the beer-spoilage strains of *Lactobacillus brevis*, *Pediococcus damnosus*, *Lactobacillus collinoides* and *Lactobacillus coryniformis*. *J Appl Microbiol* **98**, 1209–1220.

Geißler, A.. (2016) Lifestyle of beer spoiling lactic acid bacteria. PhD thesis, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Freising.

Gevers, D., Cohan, F.M., Lawrence, J.G., Spratt, B.G., Coenye, T., Feil, E.J., Stackebrandt, E., Van de Peer, Y. *et al.* (2005). Re-evaluating prokaryotic species. *Nat Rev Microbiol* **3**, 733–739.

Haakensen, M.. (2009). Genetic markers for beer-spoilage by lactobacilli and pediococci. PhD thesis, Pathology and Laboratory Medicine, University of Saskatchewan, Saskatoon.

Haakensen, M.C., Butt, L., Chaban, B., Deneer, H., Ziola, B. and Dowgiert, T. (2007) horA-Specific real-time PCR for detection of beer-spoilage lactic acid bacteria. *J Am Soc Brew Chem* **65**, 157–165.

Haakensen, M., Schubert, A. and Ziola, B. (2008) Multiplex PCR for putative *Lactobacillus* and *Pediococcus* beer-spoilage genes and ability of gene presence to predict growth in beer. *J Am Soc Brew Chem* **66**, 63–70.

Hayashi, N., Ito, M., Horiike, S. and Taguchi, H. (2001) Molecular cloning of a putative divalent-cation transporter gene as a new genetic marker for the identification of *Lactobacillus brevis* strains capable of growing in beer. *Appl Microbiol Biotechnol* **55**, 596–603.

Huss, V.A.R., Festl, H. and Schleifer, K.H. (1983) Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.

Hutzler, M., Koob, J., Grammer, M., Riedl, R. and Jacob, F. (2012) Statistische Auswertung der PCR-Analyse bierschädlicher Bakterien in den Jahren 2010 und 2011. *Brauwelt* **152**, 546–547.

Iijima, K., Suzuki, K., Ozaki, K. and Yamashita, H. (2006) HorC confers beer-spoilage ability on hop-sensitive *Lactobacillus brevis* ABBC45(cc). *J Appl Microbiol* **100**, 1282–1288.

Iijima, K., Suzuki, K., Asano, S., Kuriyama, H. and Kitagawa, Y. (2007) Isolation and identification of potential beer-spoilage *Pediococcus inopinatus* and beer-spoilage *Lactobacillus backi* strains carrying the horA and horC gene clusters. *J Inst Brew* **113**, 96–101.

Iijima, K., Suzuki, K., Asano, S., Ogata, T. and Kitagawa, Y. (2009) HorC, a hop-resistance related protein, presumably functions in homodimer form. *Biosci Biotechnol Biochem* **73**, 1880–1882.

Jacob, F. (2012) MEBAK Brautechnische Analysenmethoden, Würze, Bier, Biermischgetränke. Selbstverlag der MEBAK, Freising - Weihenstephan.

Ji, N.N., Peng, B., Wang, G.Z., Wang, S.Y. and Peng, X.X. (2004) Universal primer PCR with DGGE for rapid detection of bacterial pathogens. *J Microbiol Methods* **57**, 409–413.

Koob, J., Jacob, F., Methner, F.-J. and Hutzler, M. (2016) *Lactobacillus* sp. brewery isolate: A new threat to the brewing industry? *Brew Sci* **69**, 42–49.

Kumar, S., Tamura, K., Jakobsen, I.B. and Nei, M. (2001) MEGA2: molecular evolutionary genetics analysis software. *Bioinform* **17**, 1244–1245.

Kumar, S., Nei, M., Dudley, J. and Tamura, K. (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* **9**, 299–306.

Löffler, F.E., Sun, Q., Li, J.R. and Tiedje, J.M. (2000) 16S rRNA gene-based detection of tetrachloroethene-dechlorinating Desulfuromonas and Dehalococcoides species. *Appl Environ Microbiol* **66**, 1369–1374.

Naser, S.M., Thompson, F.L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M. and Swings, J. (2005) Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on rpoA and pheS genes. *Microbiol Sgm* **151**, 2141–2150.

Naser, S.M., Dawyndt, P., Hoste, B., Gevers, D., Vandemeulebroecke, K., Cleenwerck, I., Vancanneyt, M. and Swings, J. (2007) Identification of lactobacilli by pheS and rpoA gene sequence analyses. *Int J Syst Evol Microbiol* **57**, 2777–2789.

Polz, M.F. and Cavanaugh, C.M. (1998) Bias in template-to-product ratios in multitemplate PCR. *Appl Environ Microbiol* **64**, 3724–3730.

Riedl, R., Goderbauer, P., Brandl, A., Jacob, F. and Hutzler, M. (2017) Bavarian wheat Beer, an example of a special microbe habitat – cultivation, detection, biofilm formation, characterization of selected lactic acid bacteria hygiene indicators and spoilers. *Brew Sci* **70**, 39–50.

Saitou, N. and Nei, M. (1987) The neighbor-joining method – a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sakamoto, K. and Konings, W.N. (2003) Beer spoilage bacteria and hop resistance. *Int J Food Microbiol* **89**, 105–124.

Sakamoto, K., Margolles, A., van Veen, H.W. and Konings, W.N. (2001) Hop resistance in the beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-binding cassette multidrug transporter HorA. *J Bacteriol* **183**, 5371–5375.

Sakamoto, K., van Veen, H.W., Saito, H., Kobayashi, H. and Konings, W.N. (2002) Membrane-bound ATPase contributes to hop resistance of *Lactobacillus brevis*. *Appl Environ Microbiol* **68**, 5374–5378.

Sami, M., Yamashita, H., Hirono, T., Kadokura, H., Kitamoto, K., Yoda, K. and Yamasaki, M. (1997) Hop-resistant *Lactobacillus brevis* contains a novel plasmid harboring a multidrug resistance-like gene. *J Ferentm Bioeng* **84**, 1–6.

Scheirlinck, I., Van der Meulen, R., Van Schoor, A., Vancanneyt, M., De Vuyst, L., Vandamme, P. and Huys, G. (2007) Influence of geographical origin and flour type on diversity of lactic acid bacteria in traditional Belgian sourdoughs. *Appl Environ Microbiol* **73**, 6262–6269.

Scheirlinck, I., Van der Meulen, R., Van Schoor, A., Vancanneyt, M., De Vuyst, L., Vandamme, P. and Huys, G. (2008) Taxonomic structure and stability of the bacterial community in Belgian sourdough ecosystems as assessed by culture and population fingerprinting. *Appl Environ Microbiol* **74**, 2414–2423.

Scheirlinck, I., Van der Meulen, R., Vrancken, G., De Vuyst, L., Settanni, L., Vandamme, P. and Huys, G. (2009) Polyphasic taxonomic characterization of *Lactobacillus rossiae* isolates from Belgian and Italian sourdoughs reveals intraspecific heterogeneity. *Syst Appl Microbiol* **32**, 151–156.

Schneiderbanger, J., Grammer, M., Jacob, F. and Hutzler, M. (2018) Statistical evaluation of beer spoilage bacteria by real-time PCR analyses from 2010 to 2016. *J Inst Brew* **124**, 173–181.

Stackebrandt, E. and Ebers, J. (2006) Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* **33**, 152–155.

Stackebrandt, E. and Goebel, B.M. (1994) A place for DNA-DNA reassociation and 16S ribosomal RNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Stackebrandt, E. and Goodfellow, M. (1991) Nucleic acid techniques in bacterial systematics. In *16S/23S rRNA sequencing* ed. Lane, D.J.. pp. 115–147. New York, NY: John Wiley & Sons.

Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A.D., Kampfer, P., Maiden, M.C.J., Nesme, X., Rossello-Mora, R. *et al.* (2002) Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.

Suzuki, K., Sami, M., Kadokura, H., Nakajima, H. and Kitamoto, K. (2002) Biochemical characterization of horA-independent hop resistance mechanism in *Lactobacillus brevis*. *Int J Food Microbiol* **76**, 223–230.

Suzuki, K., Koyanagi, M. and Yamashita, H. (2004a) Isolation of hop-sensitive variants from beer-spoilage *Lactobacillus brevis* strains. *J Am Soc Brew Chem* **62**, 71–74.

Suzuki, K., Sami, M., Ozaki, K. and Yamashita, H. (2004b) Nucleotide sequence identities of horA homologues and adjacent DNA regions identified in three species of beer-spoilage lactic acid bacteria. *J Inst Brew* **110**, 276–283.

Suzuki, K., Koyanagi, M. and Yamashita, H. (2004c) Genetic characterization of non-spoilage variant isolated from beer-spoilage *Lactobacillus brevis* ABBC45C. *J Appl Microbiol* **96**, 946–953.

Suzuki, K., Ozaki, K. and Yamashita, H. (2004d) Comparative analysis of conserved genetic markers and adjacent DNA regions identified in beer-spoilage lactic acid bacteria. *Lett Appl Microbiol* **39**, 240–245.

Suzuki, K., Iijima, K., Sakamoto, K., Sami, M. and Yamashita, H. (2006a) A review of hop resistance in beer spoilage lactic acid bacteria. *J Inst Brew* **112**, 173–191.

Suzuki, K., Sami, M., Iijima, K., Ozaki, K. and Yamashita, H. (2006b) Characterization of horA and its flanking regions of *Pediococcus damnosus* ABBC478 and development of more specific and sensitive horA PCR method. *Lett Appl Microbiol* **42**, 392–399.

Svec, P., Vancanneyt, M., Devriese, L.A., Naser, S.M., Snauwaert, C., Lefebvre, K., Hoste, B. and Swings, J. (2005a) *Enterococcus aquimarinus* sp nov., isolated from sea water. *Int J Syst Evol Microbiol* **55**, 2183–2187.

Svec, P., Vancanneyt, M., Koort, J., Naser, S.M., Hoste, B., Vihavainen, E., Vandamme, P., Swings, J. *et al.* (2005b) *Enterococcus devriesei* sp nov., associated with animal sources. *Int J Syst Evol Microbiol* **55**, 2479–2484.

Tamura, K., Nei, M. and Kumar, S. (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Nat Acad Sci USA* **101**, 11030–11035.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* **30**, 2725–2729.

Taskila, S., Kronlof, J. and Ojamo, H. (2011) Enrichment cultivation of beer-spoiling lactic acid bacteria. *J Inst Brew* **117**, 285–294.

Thelen, K., Beimfohr, C. and Snaidr, J. (2006) Evaluation study of the frequency of different beer-spoiling bacteria using the VIT analysis. *Tech Q Master Brew Assoc* **43**, 31–35.

Tindall, B.J., Rossello-Mora, R., Busse, H.J., Ludwig, W. and Kaempfer, P. (2010) Notes on the characterization of prokaryote strains for taxonomic purposes. *Int J Syst Evol Microbiol* **60**, 249–266.

Vandamme, P., Pot, B., Gillis, M., DeVos, P., Kersters, K. and Swings, J. (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol Rev* **60**, 407–438.

Versalovic, J., Schneider, M., De Bruijn, F.J. and Lupski, J.R. (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* **5**, 25–40.

Vogel, R.F., Preissler, P. and Behr, J. (2010) Towards an understanding of hop tolerance in beer spoiling *Lactobacillus brevis*. *Brew Sci* **63**, 23–30.

Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, L.H., Moore, W.E.C. *et al.* (1987) Report of the ad-hoc-committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Werning, M.L., Ibarburu, I., Duenas, M.T., Irastorza, A., Navas, J. and Lopez, P. (2006) *Pediococcus parvulus* gtf gene encoding the GTF glycosyltransferase and its application for specific PCR detection of $\beta$-D-Glucan-producing bacteria in foods and beverages. *J Food Prot* **69**, 161–169.

Wood, B.J.B. and Holzapfel, W. (1995) The lactic acid bacteria. In *The Genera of Lactic Acid Bacteria* ed. Hammes, W.P. and Vogel, R.F.. pp. 19–54. London: Blackie Academic & Professional.

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

**Table S1.** Amino-acid increase (+, ≥50% of original amino-acid content increased) and decrease (−, ≥ −50% of original amino-acid content decreased) of *Lactobacillus rossiae* after 8 days of incubation in wheat beer medium; percentage increase and decrease compared with the values of the inoculated wheat beer medium in parentheses; GABA = gamma-amino-butyric-acid.

**Figure S1.** $(GTG)_5$ rep-PCR fingerprint pattern of the *Lactobacillus rossiae* beer-spoilage isolates and the *L.*

*rossiae* type strain DSM 15814$^T$ created with BIONUMERICS 7.5 software using the Pearson correlation and the UPGMA (unweighted pair group method with arithmetic mean) method; I–III = clusters I–III.

**Figure S2.** Dendrogram of UPGMA (unweighted pair group method with arithmetic mean) phylogenetic tree of 16S rRNA gene sequences of *Lactobacillus rossiae* beer-spoilage and sourdough isolates with *L. rossiae* and phylogenetically related type strains as well as the beer-spoilage isolate *Lactobacillus plantarum* 1173 using MEGA6 software. *Lactobacillus delbrueckii* DSM 20074$^T$ was used as an outside species. Bootstrap values ≥50% were included (1000 replicates); bar = number of base substitutions per site.

**Figure S3.** Dendrogram of UPGMA (unweighted pair group method with arithmetic mean) phylogenetic tree of *rpoA* gene sequences of *Lactobacillus rossiae* beer-spoilage and sourdough isolates with *L. rossiae* and phylogenetically related type strains as well as the beer-spoilage isolate *Lactobacillus plantarum* 1173 using MEGA6 software. *Lactobacillus delbrueckii* DSM 20074$^T$ was used as an outside species. Bootstrap values ≥50% were included (1000 replicates); bar = number of base substitutions per site.

**Figure S4.** Dendrogram of UPGMA (unweighted pair group method with arithmetic mean) phylogenetic tree of *pheS* gene sequences of *Lactobacillus rossiae* beer-spoilage and sourdough isolates with *L. rossiae* and phylogenetically related type strains as well as the beer-spoilage isolate *Lactobacillus plantarum* 1173 using MEGA6 software. *Lactobacillus delbrueckii* DSM 20074$^T$ was used as an outside species. Bootstrap values ≥50% were included (1000 replicates); bar = number of base substitutions per site.

# CHAPTER D – Supplementary Material

## Genotypic and phenotypic diversity of *Lactobacillus rossiae* isolated from beer

Running title: Diversity of beer-related *L. rossiae* isolates

Jennifer Schneiderbanger [a], Fritz Jacob [a], Mathias Hutzler [a]*

[a] Technische Universität München, Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität, Alte Akademie 3, 85354 Freising, Germany

*Corresponding author:*

*Email adress: hutzler@wzw.tum.de, phone: +49 8161 713100, address: Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität, Department Microbiology, Alte Akademie 3, 85354 Freising, Germany*

**Table S1** Amino acid increase (**+**, ≥ 50 % of original amino acid content increased) and decrease (**−**, ≥ − 50 % of original amino acid content decreased) of *L. rossiae* after eight days of incubation in wheat beer medium; percentage increase and decrease compared with the values of the inoculated wheat beer medium in parentheses; GABA = gamma-amino-butyric-acid

| | 84 | 396 | 1495 | 1702 | 1769 | 1775 | 1918 | 1922 | 1950 | 2202 | DSM15814$^T$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Glutamic acid | | − (90) | | | | | | | | − (94) | − (95) |
| Histidine | | | + (78) | + (50) | | | | | | | |
| Glutamine | + (200) | | | | | | | | | | |
| Alanine | | − (93) | | − (91) | | | − (88) | | − (87) | − (90) | − (88) |
| Tyrosine | | | | | | | | | − (98) | | − (64) |
| GABA | + (74) | + (669) | | | | | + (109) | | | + (590) | + (549) |

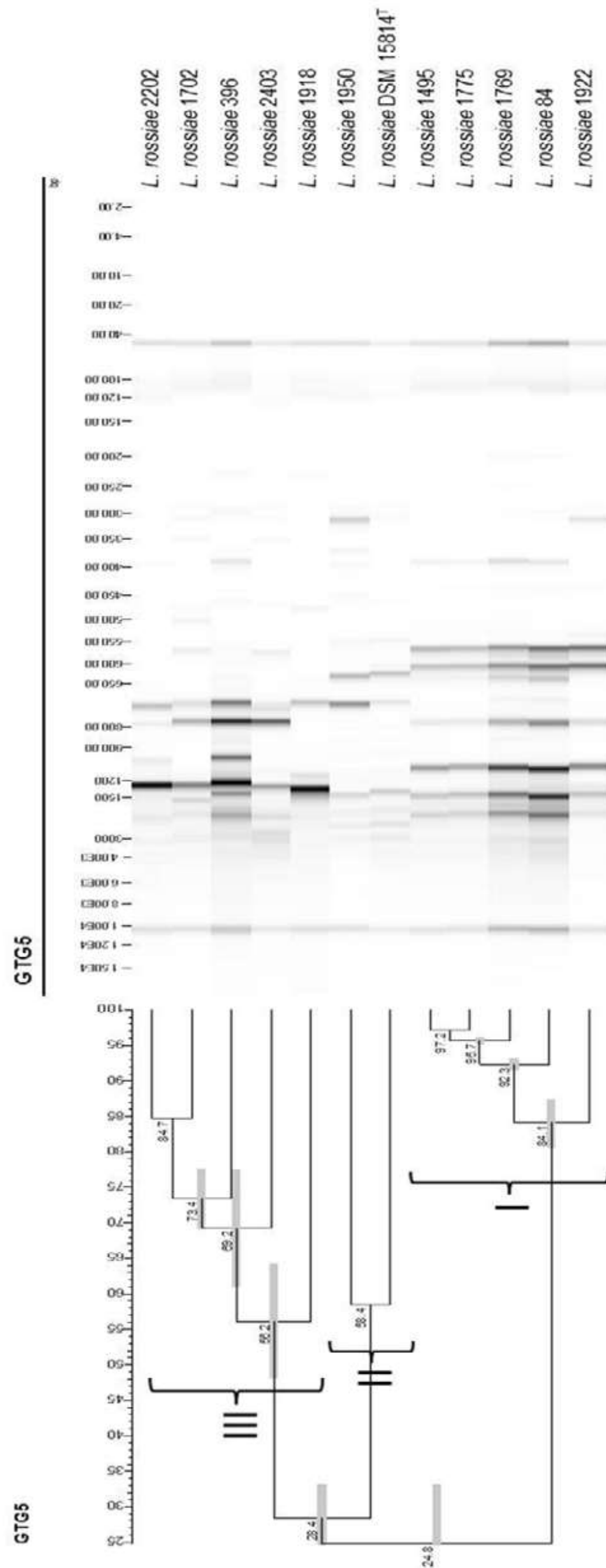



**Figure S1** (GTG)$_5$ rep-PCR fingerprint pattern of the *L. rossiae* beer-spoilage isolates and the *L. rossiae* type strain DSM 15814$^T$ created with Bionumerics 7.5 software using the Pearson correlation and the UPGMA (unweighted pair group method with arithmetic mean) method; I − III = clusters I – III

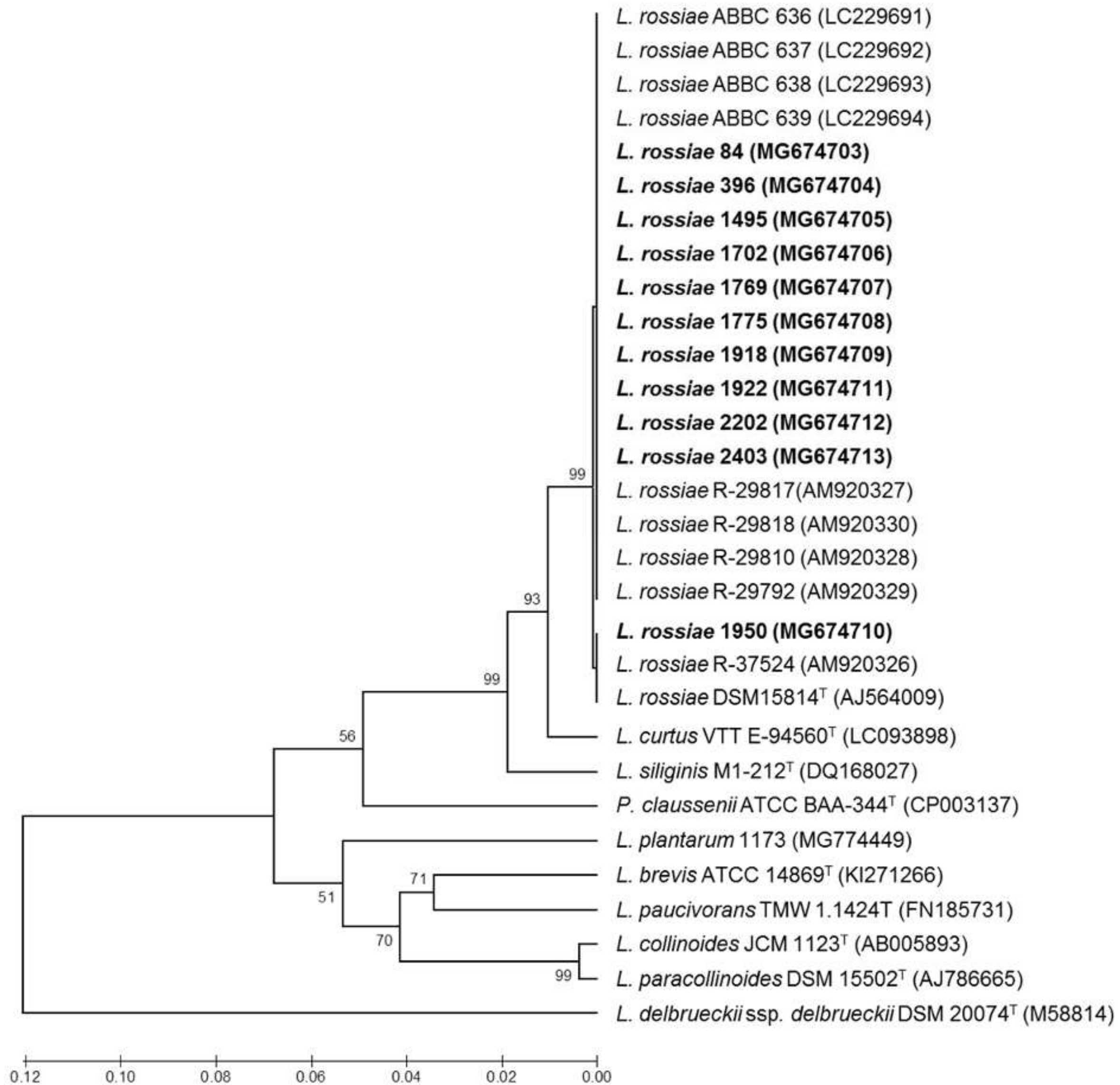



**Figure S2** Dendrogram of UPGMA phylogenetic tree of 16S rRNA gene sequences of *L. rossiae* beer-spoilage and sourdough isolates with *L. rossiae* and phylogenetically related type strains as well as the beer-spoilage isolate *L. plantarum* 1173 using MEGA6 software. *L. delbrueckii* DSM 20074$^{T}$ was used as an outside species. Bootstrap values ≥ 50 % were included (1000 replicates); bar = number of base substitutions per site.

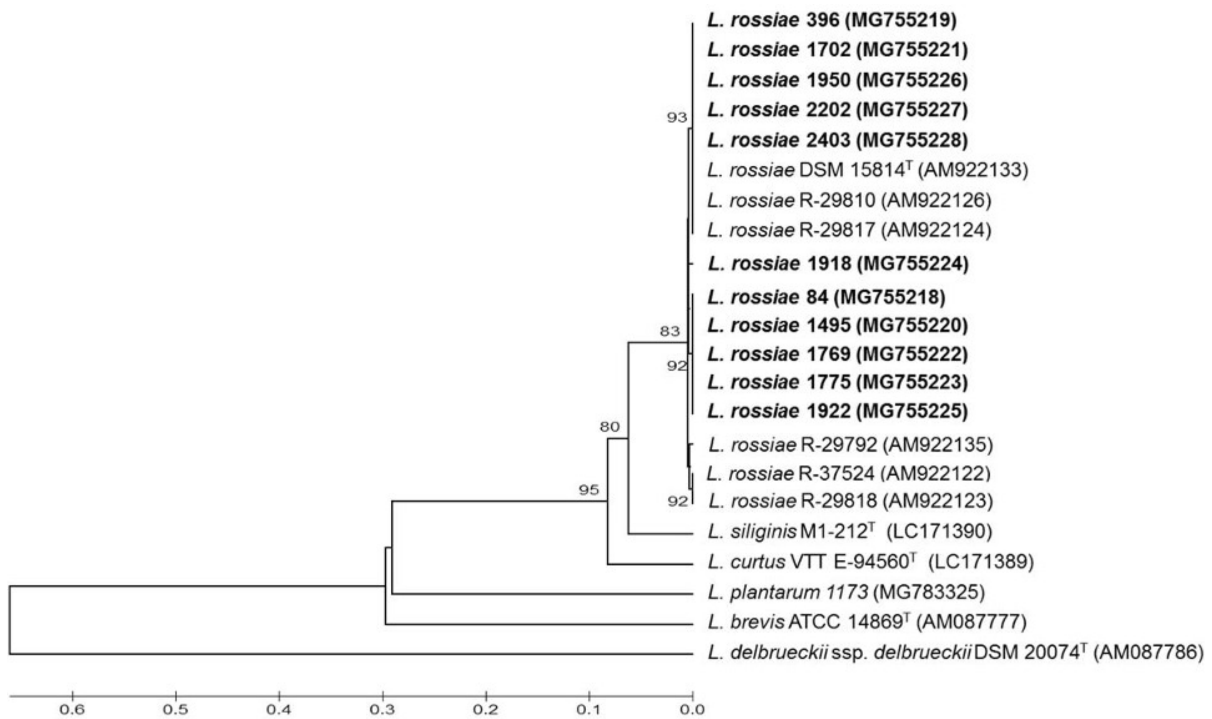



**Figure S3** Dendrogram of UPGMA phylogenetic tree of *rpoA* gene sequences of *L. rossiae* beer-spoilage and sourdough isolates with *L. rossiae* and phylogenetically related type strains as well as the beer-spoilage isolate *L. plantarum* 1173 using MEGA6 software. *L. delbrueckii* DSM 20074$^T$ was used as an outside species. Bootstrap values $\geq$ 50 % were included (1000 replicates); bar = number of base substitutions per site.

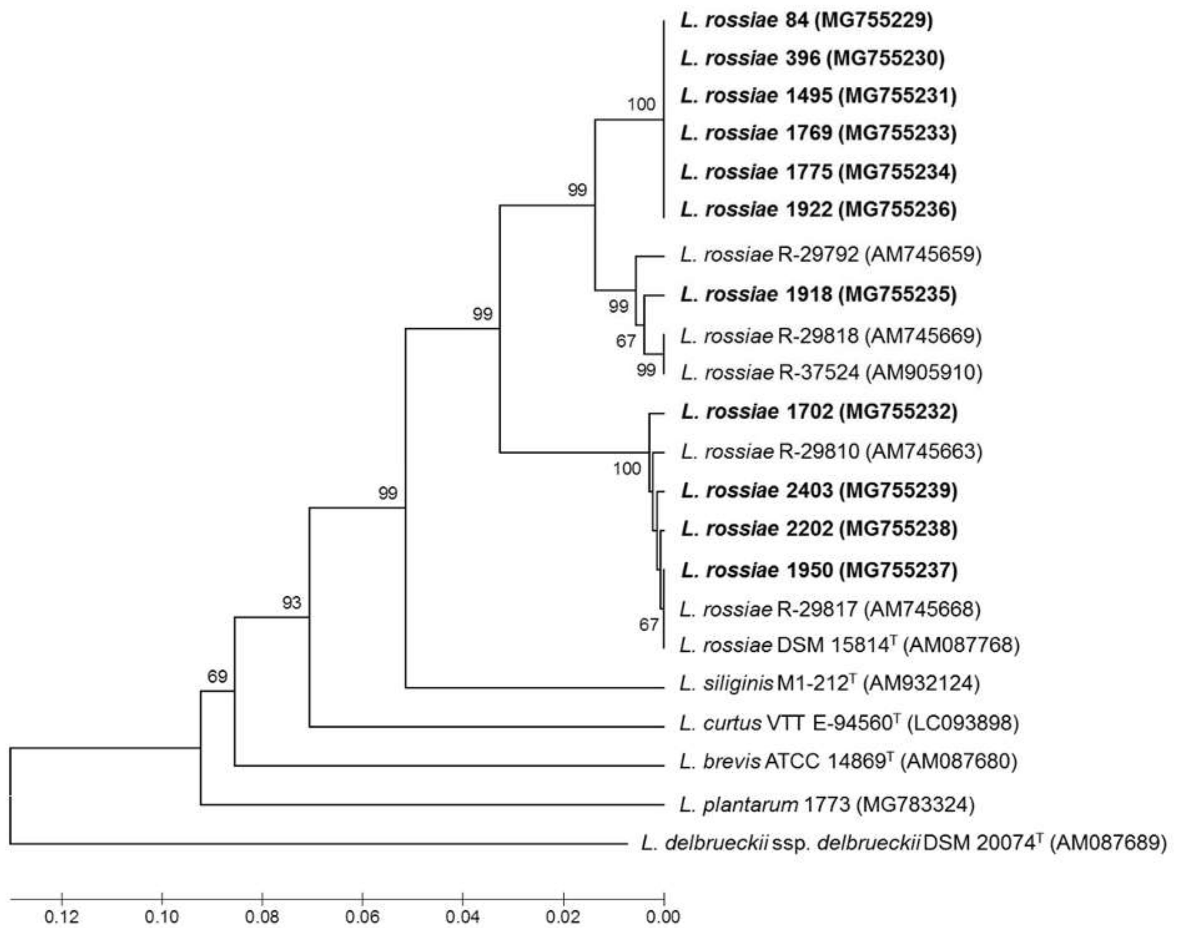



**Figure S4** Dendrogram of UPGMA phylogenetic tree of *pheS* gene sequences of *L. rossiae* beer-spoilage and sourdough isolates with *L. rossiae* and phylogenetically related type strains as well as the beer-spoilage isolate *L. plantarum* 1173 using MEGA6 software. *L. delbrueckii* DSM 20074$^{T}$ was used as an outside species. Bootstrap values ≥ 50 % were included (1000 replicates); bar = number of base substitutions per site.

# 4. DISCUSSION

Microbiological QC is a very important part of a brewery in terms of quality maintenance. Beer as a niche environment is rather hostile to microorganisms. But a few bacteria and yeast species have adapted to it and have gained the ability to spoil beer to some extent. LAB, which are ubiquitous in food production and mostly considered to be positive microorganisms (van de Guchte et al., 2002, Suzuki, 2011), constitute the largest group within beer-spoiling bacteria. To overcome the antimicrobial hurdles present in beer, a multifactorial stress response is required (Suzuki, 2009). An important prerequisite for growth in beer is the microorganism's tolerance to hop acids (Fernandez and Simpson, 1993, Suzuki et al., 2006b, Preissler, 2011), which correlates highly with BSP (see Table 1) for BS species. GEIßLER described that the characteristics necessary to spoil the hostile niche environment of beer comprise species-specific, chromosomally encoded traits and a species-independent mobile genetic pool, which encodes hop tolerance and different traits such as oxidative stress response or metabolism (Geißler, 2016).

The term 'beer-spoilage potential' is not clearly defined. In some cases brewing microbiologists refer to BSP as a species-specific property that characterizes a species that includes BS strains. In other cases, BSP is described by the effective ability of a single strain to grow in beer types with specified microbiological hurdles such as lager beer or Pilsener beer (see Section 1.5.3). Both approaches deserve attention as any information about the possible damages BSB could cause is helpful. In order to provide practical assistance for brewing microbiologists and information that can be directly implemented in their daily routine, a species-specific approach was pursued in this work. Thus, it is important to note that, in some cases, identification at the species level provides a precise statement about BSP, e.g. if a certain species has been exclusively isolated from the brewing environment and no strains without BSP have been found. In other cases, as with *L. brevis*, a species comprises beer-spoiling and non-spoiling strains. But again, for practical purposes, it was considered more important to establish all the endangering species and to be able to react accordingly, if such isolates are obtained from beer, instead of clarifying the effective strain-specific BSP and, more importantly, to rely on this analysis and resulting consequences. Differentiation at the strain level can be a useful tool, for example, to track down contamination routes in a brewery (as conducted by RIEDL ET AL. (2019)) or in other food industries (Hyytia-Trees et al., 2007), but this should not be the first measurement in case of contamination.

The group of BSB is likely to continue to change. Technological advances in beer production, changes to the raw materials and additives used and novel beer types (e.g. low-alcohol beers, non-alcohol beers, beers containing other herbs and spices in addition to or instead of hops) generate varying selective beer properties and, consequently, produce a slightly varying microorganism spectrum. Furthermore, the possible uptake of mobile genetic elements by HGT may cause species that are foreign to the brewery environment to transform into beer-spoiling ones (Suzuki, 2015). In this context, biofilms play an important role as many different species (i.e. AAB, *Enterobacteriaceae*, brewing yeasts, wild yeasts, lactobacilli, anaerobic bacteria) participate in its establishment and the pool of plasmids that impart tolerance to overcome the beer hurdles are present in high amounts. Finally, it should be noted that over time, a brewery and its specific microflora may 'evolve' its own species that is perfectly adapted

to the present conditions. One newly described species that is likely to fit this scheme is *L. paucivorans*. This species was described in 2010 and has not yet been found in any other sample outside the brewery from which it was isolated (Ehrmann et al., 2010).

Routine analysis in the brewing microbiological lab has changed in recent decades by the increasing use of molecular biological methods. One reason for this is that these methods are getting more affordable over time. Another reason is the ever-increasing quality requirements sought after by many breweries and also implemented in microbiological QC. It is easy for experienced staff to determine the genus of a bacterial contaminant using selective enrichment methods and microscopic analysis, but it is the species determination that helps to track contamination routes within a brewery and, ideally, provides information on the 'in-house' flora and the possible expected damage. It is very difficult to determine the exact species by cell morphology, even for technicians with many years of experience.

To provide brewing microbiologists with the knowledge they need for success, literature, empirical and statistical data must be combined. Table 1, which is an excerpt from HUTZLER ET AL. (2012b, 2013), gives practical information on the spectrum of BSB, physiological characteristics that are useful to narrow down present contaminants, the BSP related to the whole species (not related to single strains), the degree of hop tolerance, the literature and empirical data about the preferred growth of individual species in the primary or secondary area of the brewing process, and the ability to form exopolysaccharides. In the following sections this overview is supported with data acquired in the past few years and altered in several ways to meet modern brewing microbiology requirements.

The 'Rods / Cocci' and 'Gram reaction' columns were deleted as they do not provide information about the hazard potential. The species *Lac. lactis*, *Leuc. (para-)mesenteroides*, *Micrococcus kristinae* (now: *Kocuria kristinae*), and *Zymomonas mobilis* were not considered further as their weak BSP means they pose little threat to the brewing industry. *L. perolens* was isolated from soft drinks and the brewing environment, but did not show any considerable beer-spoilage potential (Back et al., 1999). It is likely, due to its close genotypic relationship to *L. harbinensis* with notedly higher BSP, that there were many misidentifications in the past. Therefore, *L. perolens* was also removed and *L. harbinensis* was included.

Two newly described species were additionally included in the overview in Table 3: *L. cerevisiae* and *L. curtus* (Koob et al., 2017, Asakawa et al., 2017). Both have been to date exclusively isolated from the brewing environment. *L. cerevisiae* shows only moderate BSP and it was most recently found outside of the brewery it was originally isolated from for the first time (unpublished data FZW BLQ). The *L. curtus* type strain was stored as *L. rossiae* in a culture collection, before it was genotypically re-classified and newly described as *L. curtus*. Only the presence of *horA* and *horC* indicates a possible potential to spoil beer, but there is no practical evidence to date. Further studies need to be conducted to reveal the extent of its BSP.

From Table 1, it is remarkable that the degree of BSP and hop tolerance correlates with nearly all listed bacterial species. There are only three cases where hop tolerance exceeds BSP: *M. sueciensis*, *Kocuria kristinae* (previously: *Micrococcus kristinae*) and *Zymomonas mobilis*. All three species have little impact on brewing microbiology. Thus, the 'hop tolerance' column was eliminated in Table 3. As previously described, the 'beer-spoilage potential' characteristic relates to the ability of a species to spoil beer and to the probability of an isolate to be beer spoiling. In other words, this column combines information on the effects that can occur if the

specified species is isolated from beer and information on the probability of a strain to be beer spoiling. For example, *L. backii* was exclusively isolated from the brewing environment. This means that all *L. backii* isolates are considered to be beer spoilers. Combined with the effects caused during its growth in beer (slight acidity, turbidity, sediment formation, slight changes in smell and taste), *L. backii* has a high BSP ('++', see Table 1) (Bohak et al., 2006, Tohno et al., 2013).

The trend of identifying BSM allows external laboratories like the lab at the FZW BLQ to establish large databases of useful information for brewing microbiologists. A large volume of identified isolates is essential, especially in terms of determining actual frequencies of individual BS species. As mentioned earlier, studies on the frequencies of individual BS species were mainly conducted by BACK (Back, 1988, Back, 1994a, Back, 1994b, Back, 2003, Back and Bohak, 2005). A detailed overview about BACK's findings was provided by SUZUKI (Suzuki, 2011) (see Table 2). The first studies were conducted in the early 1980s and even at that time, *L. brevis* was already the most frequent BS species by far. Contaminations with *L. lindneri*, *P. damnosus* and *Pectinatus* also occurred frequently.

Table 2: Percentages of beer-spoiling microorganisms in incident reports during the period 1980 – 2002 [a] (Table adopted from (Suzuki, 2011))

| Genus/species[b] | 1980–1990 | 1992[c] | 1993[c] | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 |
|---|---|---|---|---|---|---|---|---|---|
| *L. brevis* | 40 | 39 | 49 | 38 | 43 | 41 | 51 | 42 | 51 |
| *L. lindneri* | 25 | 12 | 15 | 5 | 4 | 10 | 6 | 13 | 11 |
| *L. plantarum* | 1 | | | 1 | 4 | 2 | 1 | 1 | 2 |
| *L. casei/paracasei* | 2 | 3 | 2 | 6 | 9 | 5 | 8 | 4 | 4 |
| *L.coryniformis* | 3 | | | 4 | 11 | 4 | 1 | 3 | 6 |
| *Ped. damnosus* | 17 | 4 | 3 | 31 | 14 | 12 | 14 | 21 | 12 |
| *Pectinatus* | 4 | 28 | 21 | 6 | 3 | 6 | 5 | 10 | 7 |
| *Megasphaera* | 2 | 7 | 3 | 2 | 2 | 4 | 4 | 4 | 2 |
| *Saccharomyces* wild yeasts | N.A.[d] | 5 | 5 | 7 | 6 | 11 | 5 | 2 | 3 |
| Non-*Saccharomyces* wild yeasts | N.A. | 0 | 0 | 0 | 3 | 4 | 5 | 0 | 2 |
| Others | N.A. | 2 | 2 | 0 | 1 | 1 | 0 | 0 | 0 |

[a] This table is adapted from the studies conducted by Back during the 1980–2002 period[5–7].
[b] *L. brevis* includes *L. brevisimilis* that exhibits phenotypical and morphological similarities to *L. brevis*. According to Back, *L. brevis* in this table consists of several types on the basis of carbohydrate fermentation profiles, arginine utilization pattern and morphological features, suggesting that this group of LAB can be further divided into separate species or subspecies.
[c] In 1992 and 1993 studies, *L. plantarum, L. casei, L. paracasei* and *L. coryniformis* were put together into one group.
[d] Not available.

The percentage of incidents caused by the individual BS species from 2010 – 2016 were included in Table 3. The percentage graduation was selected as follows:

↑↑↑ = ≥ 15 % of bacterial incidents

↑↑ = 5 - 15 % of bacterial incidents

↑ = 1 - 5 % of bacterial incidents

→ = 0 - 1 % of bacterial incidents

In addition to the percentage frequency, it is also important to understand the tendency of a spoilage species to grow in the primary or secondary area of the brewing process and its tendency to grow in bottom-fermented or top-fermented beer types. When the $\chi^2$ test showed

high (significance level of 0.1) or very high (significance level of 0.05) probability with respect to the two specified brewing-related issues, it was included in Table 3. As some species were not included in the underlying study (this study, Chapter A), literature and empirical data were consulted and labelled as such (•). Bold letters indicate the characteristics of individual species that disagree with literature and outdated empirical values.

A rather small change was made for the 'Potential for slime formation (brewery)' characteristic. Table 1 described three BS species as positive for slime formation and one species, *Pd. claussenii*, as variable. 'Variable' implies that some strains of a species produce slime and some do not, which is the case for all the listed species. In Table 3, all four species are labelled as positive for slime formation, though it should be noted that the production of exopolysaccharides is a strain-specific trait that was proven for individual BS species in several studies (Fraunhofer, 2018, Riedl et al., 2019, Pittet et al., 2011, Schneiderbanger et al., 2019).

*L. lindneri* is a frequent contaminant of the primary area, but it is difficult to detect with routine enrichment media due to its special nutrient requirements and its oxygen sensitivity (Back, 1994a). These facts and the tendency of *L. lindneri* to slip through filtration mean that it is often only discovered in the finished product, which is confirmed by statistical analysis. Enrichment methods and media adapted to this sensitive species would adjust its growth preferences.

Table 2: Revised overview of beer-spoiling species and brewery-specific properties (compare to Table 1, Section 1.4.4)

| Species name | Beer-spoilage potential • | Occurrence frequency | Primary / secondary contamination | Bottom- / top-fermented beer types | Potential for slime formation (brewery) isolates) • |
|---|---|---|---|---|---|
| *L. acetotolerans* | + | ND | s • | TF • | − |
| *L. backii* | ++ | ↑↑ | p * | BF/TF | − |
| *L. brevis* | ++ | ↑↑↑ | **p/s** | TF ** | + |
| *L. (para-)buchneri* | + | ↑↑ | **s** | BF * | + |
| *L. (para-)casei* | + | ↑↑ | **p **** | BF * | − |
| *L. cerevisiae* | + | ND | p • | BF • | − |
| *L. (para-)collinoides* | ++ | ↑ | **p *** | BF | − |
| *L. coryniformis* [a] | + | ↑↑ | s ** | TF ** | − |
| *L. curtus* | ND | ND | ND | ND | − |
| *L. harbinensis* | + | ↑↑ | **p/s** | BF/TF | − |
| *L. lindneri* | ++ | ↑↑ | **s **** | BF ** | − |
| *L. paucivorans* | ++ | ND | p • | ND | − |
| *L. plantarum* [a] | + | ↑↑ | s ** | TF ** | − |
| *L. rossiae* | + | ↑ | **p/s** | BF/TF | + |
| *M. cerevisiae* | ++ | ↑ | s ** | BF ** | − |
| *M. paucivorans* | ++ | ND | s • | ND | − |
| *M. sueciensis* | + | ND | s • | ND | − |
| *Pd. damnosus* | ++ | ↑↑ | p | BF ** | − |
| *Pd. claussenii* [b] | + | → | p | TF * | + |
| *Pd. inopinatus* [b] | + | → | p | TF * | − |
| *P. cerevisiiphilus* [c] | ++ | ↑ | s ** | BF ** | − |
| *P. frisingensis* [c] | ++ | ↑ | s ** | BF ** | − |
| *P. haikarae* [c] | ++ | ↑ | s ** | BF ** | − |

[a] Species combined in a group by real-time PCR method (*L.* group)

[b] Species combined in a group by real-time PCR method (*Pd.* group)

[c] Species combined in a group by real-time PCR method (*P.* group)

++= very high / strong, += high / strong or positive, − = negative

ND = not determined

↑↑↑ = mean percentage of incidents ≥ 15 %; ↑↑ = mean percentage of incidents 5 - 15 %, ↑ = mean percentage of incidents 1 – 5 %, → = mean percentage of incidents ≤ 1 %

p = more primary contamination observed, s= more secondary contamination observed, p/s=nearly identical (≤ 5 % deviation from mean value) distribution between primary and secondary contamination

BF = Preferred growth in bottom-fermented beer, TF = Preferred growth in top-fermented beer, BT/TF= nearly identical (≤ 5 % deviation from mean value) distribution between bottom and top-fermented beer types

* = based on significance level of 0.1, ** = based on significance level of 0.05

• based on literature and empirical values

The factors of beer-spoilage potential and occurrence frequency determine the effective hazard potential a species poses for the brewing environment. From Table 3 it can be seen that the combination of BSP and occurrence frequency attributes a very high beer-spoilage hazard (++/↑↑↑) to

> *L. brevis.*

A high hazard potential (++/↑↑) is attributed to the species

> *L. backii*

> *L. lindneri*

> *Pd. damnosus*.

A moderate hazard potential (++/↑ and +/↑↑) is attributed to the species

> *L. (para-)buchneri*

> *L. (para-)casei*

> *L. (para-)collinoides*

> *L.* group *(L. coryniformis, L. plantarum)*

> *L. harbinensis*

> *M. cerevisiae*

> *P.* group *(P. cerevisiiphilus, P. frisingensis, P. haikarae).*

All other species listed in Table 3 pose no acute danger for the brewing industry to date due to low BSP, rare occurrence or a lack of empirical data. As the percentage of BSB incidents are subject to annual fluctuations (see, for example, Figure 2), the individual hazard potential has to be revised periodically. Reasons for the annual fluctuations are difficult to find as the underlying data is based on beer-related samples that are sent to the FZW BLQ for analysis, mostly with little background information. Evaluating incidents instead of findings, assessing a large volume of samples and recording incidents of consecutive years as well as the mean values for each individual species are all valuable tools to diminish this handicap.

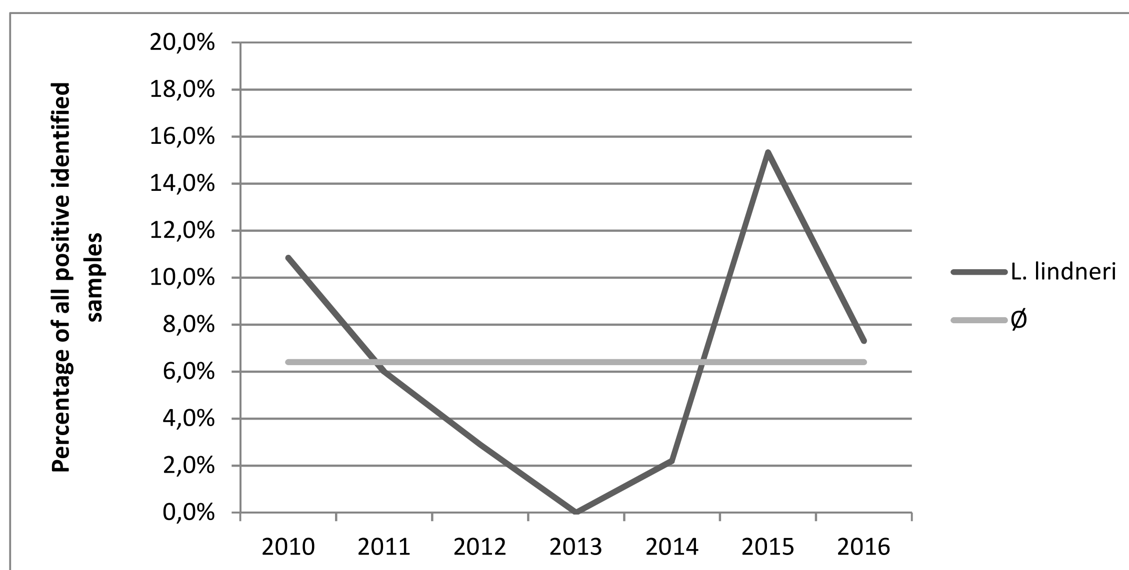



Figure 2: Percentage of samples found to be positive for *L. lindneri* from 2010 – 2016 including 7-year mean value (see CHAPTER A, Supplementary Material Figure S2)

Very useful information could be obtained from contaminated samples concerning the effects generated by the contaminating species, especially if the spoiling germ is still in its original substrate instead of the nutrient medium. The individual spoiling species may therefore be characterized by recording the degrees of turbidity, of sediment formation, of pH decrease, and aroma deviation in relation to the effective cell count per mL and in relation to chemical beer parameters such as pH, alcohol content, bitter units and residual carbohydrate quantities. This could reveal intraspecies differences in BSP and hazard potential.

One main problem of microbiological QC is the need to detect a small number of contaminants in a large volume (Suzuki, 2011, Back, 2019, Bohak, 2015, Bohak et al., 2012). Many strategies and several enrichment media have been developed to address this problem and to reliably detect spoilage germs in the finished beer and samples from the production process. NOVY ET AL. conducted a survey in 2013 of the routine analysis methods used by 32 German breweries with varying production quantities (Novy et al., 2013). By far the most breweries (94 %) relied on the incubation of product samples on selective nutrient media and the mechanical concentration of large beer and bright intermediate product volumes using the membrane filtration technique. The next most frequent microbiological routine analysis methods were optical ones, i.e. the examination of samples by microscope. These methods are still indispensable in the brewing microbiological laboratory. Enrichment is also necessary to achieve detection limits for rapid molecular biological methods (Taskila et al., 2010, Kruska and Schneegans, 2010). The advantages and disadvantages of culture-dependent methods were discussed earlier (see Section 1.5.1).

The most problematic sample types in a brewery are those that contain yeast and are therefore unsuitable for mechanical concentration. The possibility that a few bacteria are 'hiding' within a large volume of yeast cells and the suppressing force of the yeast render it almost impossible to reliably detect contaminating bacteria. Thus, detecting those contaminants, for example, in a propagation tank often provides very useful information for QC. The currently used method of adding concentrated nutrient broth to the sample and repeatedly incubating it for up to several weeks, is unfavorable due to its duration. As brewing microbiologists demand reduced

analysis time and increased detection certainty (Novy et al., 2013), it seemed reasonable to improve this method.
The method was improved by adding Natamax®, which kills yeast cells and leaves bacterial cells unaffected. The decreased suppressing force of the dead yeast cells increases the bacterial cell quantity and, consequently, the decreased time to detection. These positive effects were observed using the culture-dependent and the real-time PCR method.
Further studies using more BS species, several strains of individual species and varying common yeast species (e.g. *Saccharomyces* (*S.*) *cerevisiae*, *S. pastorianus* var. *carlsbergensis*) and strains (e.g. *S. cerevisiae* TUM 68, *S. pastorianus* TUM 66/70) may strengthen the results obtained in this study. As the developed method is low priced, simple and does not require specially trained staff, it is expected to be easy to integrate in daily analysis where routine samples could be prepared in parallel, with and without Natamax® addition, generating a lot of data for evaluation.

Modern biological methods are characterized by their rapidness and by their ability to assign contaminating germs to a specified range of organisms, in most cases by probes matching the target 16S rRNA gene. One disadvantage of these methods is that new species or subspecies are not identified and are recorded as false-negative, if there are no culture-dependent or microscopic results (Temmerman et al., 2004). But the detection of new beer spoilers is vital for brewing microbiology to be up to date and provide useful advice for affected customers or QC staff.
Identification using 16S rRNA sequencing and comparison with a suitable database are necessary in the event that culture-dependent or microscopic analyses and PCR method deviate from each other. In 2013 and 2014, three isolates from a German brewery were processed this way yielding inconclusive results. The three isolates obtained from bottom-fermented samples from different process steps were characterized by their turbidity and slight changes in acidity and aroma. After demarcation from beer-related neighbor species (Koob et al., 2016) and from genotypically related species (Koob et al., 2017) a new species, *L. cerevisiae*, was described. *L. cerevisiae* could grow in alcohol-free wheat and lager beer as well as in wheat beer and filtered wheat beer with normal alcohol contents (5.64 and 5.53 % v/v, respectively), but not in lager beer with normal alcohol content (5.10 % v/v) and 19.9 bitter units. Using the classification established by BACK (Back, 1994a) (see Section 1.4.4), *L. cerevisiae* is a potential BS species. Its sensitivity to salt (≤ 4.0 % w/w NaCl) could be an indicator of its high adaptation to the low-salt medium of beer. All three obtained *L. cerevisiae* isolates possess two genes related to hop resistance, *horA* and *horC*. During its growth in beer, the organic acids pyruvate and citric acid are degraded and acetic and lactic acid are produced, which results in a slightly acidic, but balanced beer character suitable for the production of special sour beer types such as Berliner Weisse (personal correspondence M. Hutzler). Diacetyl was not recorded above the odor and taste threshold.
According to a study conducted by SCHLEIFER AND LUDWIG (1996), *L. cerevisiae* phylogenetically belongs to the *L. buchneri* group. But a more recent study characterizes *L. cerevisiae* as a member of the *L. brevis* group (see Figure 1) (Salvetti et al., 2012). The carbohydrate fermentation pattern determined using an API CHL 50 test kit and the respective software (https://apiweb.biomerieux.com/) gave the species *L. brevis* as its nearest neighbor (99 %, data not shown). Until recently, the three obtained isolates were the only known isolates of the newly described species, which was therefore assumed to be a contaminant specifically for the brewery it was originally isolated from and therefore locally limited. In 2019, another brewery with no relationship of any kind to the brewery of origin, reported a contamination

incident with *L. cerevisiae* in collective samples from different beer types (personal correspondence, current lab results FZW BLQ). This lead to the assumptions that (1) the new species may be more wider distributed than originally thought, (2) *L. cerevisiae* did not 'evolve' within the brewery it was isolated from, and (3) the assumed hazard potential for *L. cerevisiae* is increasing.

It was assumed that no correlation could be observed between phylogenetic relationship and physiological properties within the *Lactobacillus* genus (Vandamme et al., 1996, Canchaya et al., 2006). The taxonomy of this genus is confusing due to its exceptional size and diversity (Holzapfel and Wood, 2014). In earlier times, physiological characteristics such as the carbohydrate fermentation pattern were used to discriminate the individual species (Orla-Jensen, 1919). Recently, it was proposed by ZHENG ET AL. (2015) that metabolic properties are, after all, associated with ecotype and phylogenetic position. An ecotype is defined by COHAN as a population of cells that share an ecological niche and species-independent properties (Cohan, 2001). The adaptation to niche environments is accompanied by gene loss and genome size reduction (van de Guchte et al., 2006), while harboring plasmids, which is common for many LAB, is essential for growth in specific (mostly adverse) environments (McKay and Baldwin, 1990).
The species *L. rossiae*, a common participant in sourdough fermentations, is known for its broad genotypic and phenotypic diversity (Di Cagno et al., 2007, Scheirlinck et al., 2009) which was also determined previously for *L. plantarum* (Siezen et al., 2010, Siezen and Vlieg, 2011). Since the first incidents in 2010, *L. rossiae* has occasionally occured as a beer spoiler. Its most noticeable property is the potential to form exopolysaccharides as exhibited by some strains (Dertli et al., 2016, Hutzler et al., 2012a, Hutzler et al., 2013, Fraunhofer, 2018). The mean percentage from 2010 to 2016 was determined as 1.52 % of all samples that tested positive for BSB with increasing tendency (see Figure 3) (this study, Chapter A). The species *L. rossiae* was classified as a member of the *L. reuteri* group (De Vuyst and Vancanneyt, 2007), but a more recent study determined that *L. rossiae* forms a couple with *L. siliginis* outside the *L. reuteri* group (Salvetti et al., 2012). The newly described beer spoiler *L. curtus* is supposed to be the third species of this exceptional group from a phylogenetical perspective (Asakawa et al., 2017).

The most abundant carbohydrate in sourdough and beer is maltose and the occupation of both ecological niches is therefore not surprising. The following questions arose:
(1) What is the BSP of *L. rossiae* beer isolates? (2) Was the proposed genotypic and phenotypic diversity also apparent in *L. rossiae* beer isolates? (3) Are there any genotypic or phenotypic differences between sourdough and beer isolates?

The degree of BSP was determined by beer passage and by determining three genes related to hop resistance, *horA*, *horC* and *hitA*. The BSP of *L. rossiae* was determined as weak as only four strains were able to grow in wheat beer. The role of *horC* as a prominent marker gene for the ability to spoil beer was confirmed as those four strains were the only ones to harbor it.

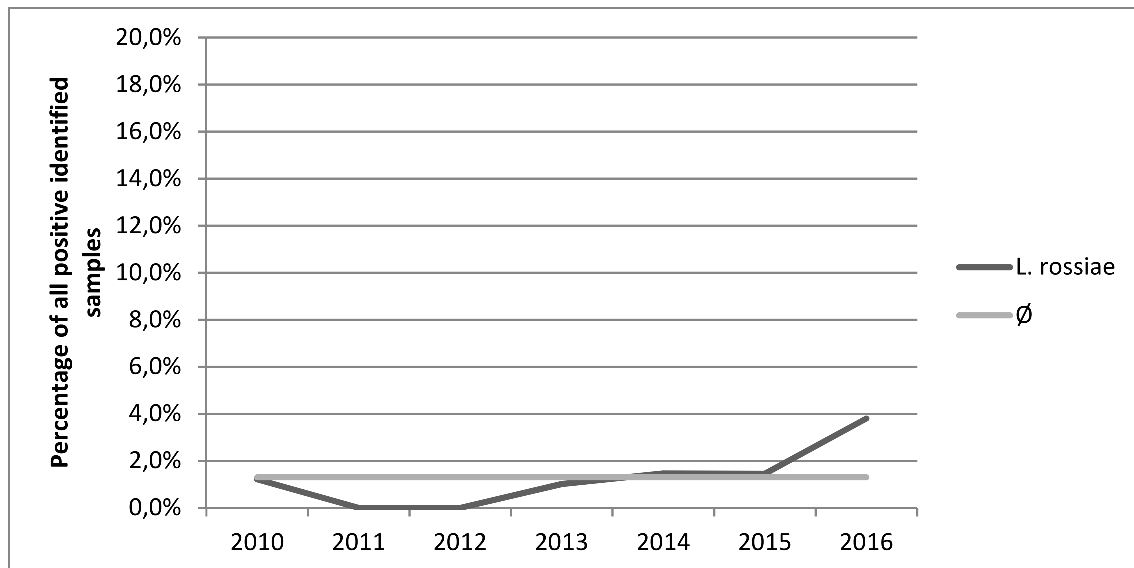



Figure 3: Percentage of samples positive for *L. rossiae* from 2010 – 2016 including 7-year mean value (see CHAPTER A, Supplementary Material Figure S8)

The proposed phenotypic variability was apparent in *L. rossiae*, especially considering carbohydrate fermentation pattern, gas production from gluconate which was accompanied by $NH_3$ production from arginine, and temperature and salt tolerance. The genotypic variability of this species was confirmed by $(GTG)_5$ rep-PCR, *pheS* housekeeping gene sequence comparisons including sourdough isolates, and DNA-DNA hybridization method. Interestingly, the *L. rossiae* isolates stretched the threshold values for species delineation of all three analyses (specified as 50 % for $(GTG)_5$ rep-PCR (Scheirlinck et al., 2007, Scheirlinck et al., 2008), ≤ 10 % for *pheS* gene sequence (Naser et al., 2007) and ≤ 70 % for DNA-DNA hybridization (Wayne et al., 1987)). In our view, the broad genotypic variety of *L. rossiae* isolates must result in the description of different subspecies or in the extension of threshold values for species delineation. It is worth noting that the *pheS* housekeeping gene comparison resulted in the demarcation of three distinct clusters with one cluster harboring only beer isolates, including the four isolates showing (weak) beer-spoilage potential. This relationship needs to be confirmed by examining a larger number of isolates from a beer and sourdough environment. Additionally, all *L. rossiae* beer isolates need to be checked for growth in lager and Pilsener beer to determine strains with a higher BSP.
The only differential phenotypic characteristic between beer and sourdough isolates was the utilization of ribose and the non-usage of lactose by beer-related isolates. The *pheS* gene sequence comparison was successful in demarcating isolates with BSP from isolates without BSP, but was unable to provide ecotype-specific clusters.

Brewing microbiology is currently focused on a few microorganisms with compositions that is still changing. The new description of *L. cerevisiae* and *L. curtus* and the determination of *L. acetotolerans* and *L. rossiae* as beer spoilers support this hypothesis. Different strategies should be considered in future to keep microbiological QC up to date and provide helpful information.
(1) The search for DMGs has proven to be of great interest for brewing microbiology. DMGs are genes that are proposed to be shared by all bacteria with the ability to grow in beer as a consequence of their adaption to this environment (Geißler, 2016, Geißler et al., 2017, Behr et al., 2015, Behr et al., 2016). The determination of DMGs that have the potential to

differentiate between BS and non-BS strains could be useful for microbiological QC as a selective, reliable and fast detection method for LAB with BSP. As no genes were detected in the core genome of BS strains that were unique to this group of bacteria and that could be the source of the beer-spoiling ability, the search for DMGs focused on the mobile genetic pool, i.e. on the plasmidome (Geißler et al., 2017, Bergsveinson et al., 2015a). The comparison of brewery-specific plasmidomes revealed the following relevant functions: cation homeostasis, oxidative stress tolerance, cell envelope mechanisms and acid reduction by producing non-acidic end products (Geißler et al., 2017). The ultimate solution would therefore be to establish a species-independent method to quickly and reliably identify LAB strains with beer-spoilage potential, but until that is possible, it is important to ensure identification at the species level (Geißler et al., 2017).
(2) The phenotypic and physiological variability of *L. plantarum* (Siezen et al., 2010, Siezen and Vlieg, 2011), *L. brevis* (Riedl et al., 2019) and *L. rossiae* (see Chapter D) indicate that BS species are versatile microorganisms, probably due to their adaptation to the adverse beer environment. To explore the specific core characteristics of all species with moderate to very high hazard potential, more studies are needed on the indicated variability. It may become necessary to re-evaluate the taxonomic position of deviating subgroups, as was needed for *L. rossiae*.
(3) As mentioned earlier, the identification of BSB at the species level is not always sufficient. Strain differentiation can be useful if there are variations in the effective spoilage potential within a species (as determined for *L. brevis* and *Pd. damnosus* (Geißler, 2016)) or if strain-specific contamination routes need to be reconstructed within a brewery that harbor several contaminating germs (Riedl et al., 2019). Many studies have been conducted to discover the strain and reveal intra-species variations. The differentiation between beer-spoiling and non-spoiling strains has been of special interest and the main focus has been on the most dangerous species of *L. brevis* (Kern et al., 2014a, Nakakita et al., 2003, Preissler, 2011, Riedl et al., 2019, Takahashi et al., 1999, Behr et al., 2015, Zhao et al., 2017). One modern method which is gaining in importance in the brewing-microbiological laboratory is the MALDI-TOF MS method (Ghyselinck et al., 2011, Homann and Kühle, 2017, Kern et al., 2014a, Kern et al., 2014b, Lay, 2001, Sandrin et al., 2013, Wenning et al., 2014, Wieme et al., 2014). This method is said to be fast, the sample preparation to be easy and the through-put capacity to be high (Kern et al., 2014a). Identification at the species level is unaffected by changes in the culture conditions of media, stable spectra are created as the majority of spectrum peaks are generated from ribosomal and cell structure proteins and all bacterial isolates are identifiable without the need to pre-select the targeted organism group (Kern et al., 2013, Welker and Moore, 2011, Kruska and Schneegans, 2010). The final goal in implementing this method in routine brewing-microbiological laboratory is the establishment of a database based on a multitude of generated spectra linked to information about species, strain, isolation source, brewery and hazard potential.
(4) The determination of the percentage distribution of spoilage bacteria species from routine microbiological analyses and the statistical evaluation thereof needs to be continued as the multitude of BS isolates can only be provided by external laboratories. This way, changes in the spectrum of BSB are recorded over a period of years which is exceptionally useful for all microbiologists and microbiological staff in the brewing industry.

## 5. REFERENCES


ABOELNAGA, I. G. & KANDLER, O. 1965. Zur Charakterisierung der in Milch verbreiteten Staphylokokken. *Zentralblatt Fur Bakteriologie Parasitenkunde Infektionskranheiten und Hygiene* 196**,** 438 ff.

AHN, H., KIM, J. & KIM, W. J. 2017. Isolation and characterization of bacteriocin-producing Pediococcus acidilactici HW01 from malt and its potential to control beer spoilage lactic acid bacteria. *Food Control,* 80**,** 59 - 66.

AMANN, R. I., LUDWIG, W. & SCHLEIFER, K. H. 1995. Phylogenetic identification and in-situ detection of individual microbial cells without cultivation. *Microbiological Reviews,* 59**,** 143 -169.

AREVALO-VILLENA, M., BARTOWSKY, E. J., CAPONE, D. & SEFTON, M. A. 2010. Production of indole by wine-associated microorganisms under oenological conditions. *Food Microbiology,* 27**,** 685 - 690.

ASAKAWA, Y., TAKESUE, N., ASANO, S., SHIMOTSU, S., IIJIMA, K., SUZUKI, K., MOTOYAMA, Y. & AIZAWA, M. 2017. Lactobacillus curtus sp. nov., isolated from beer in Finland. *International Journal of Systematic and Evolutionary Microbiology,* 67**,** 3899 - 3906.

ASANO, S., IIJIMA, K., SUZUKI, K., MOTOYAMA, Y., OGATA, T. & KITAGAWA, Y. 2009. Rapid detection and identification of beer-spoilage lactic acid bacteria by microcolony method. *Journal of Bioscience and Bioengineering,* 108**,** 124 - 129.

ASANO, S., SUZUKI, K., IIJIMA, K., MOTOYAMA, Y., KURIYAMA, H. & KITAGAWA, Y. 2007. Effects of morphological changes in beer-spoilage lactic acid bacteria on membrane filtration in breweries. *Journal of Bioscience and Bioengineering,* 104**,** 334 - 338.

AULT, R. G. 1965. Spoilage bacteria in brewing - a review. *Journal of the Institute of Brewing,* 71**,** 376 - 391.

BACK, W. 1978a. Zur Taxonomie der Gattung Pediococcus. Phänotypische und genotypische Abgrenzung der bisher bekannten Arten sowie Beschreibung einer neuen bierschädlichen Art: Pediococcus inopinatus. Teil 1. *Brauwissenschaft,* 31**,** 237 - 250.

BACK, W. 1978b. Zur Taxonomie der Gattung Pediococcus. Phänotypische und genotypische Abgrenzung der bisher bekannten Arten sowie Beschreibung einer neuen bierschädlichen Art: Pediococcus inopinatus. Teil 2. *Brauwissenschaft,* 31**,** 312 - 320.

BACK, W. 1978c. Zur Taxonomie der Gattung Pediococcus. Phänotypische und genotypische Abgrenzung der bisher bekannten Arten sowie Beschreibung einer neuen bierschädlichen Art: Pediococcus inopinatus. Teil 3. *Brauwissenschaft,* 31**,** 336 - 343.

BACK, W. 1978d. Elevation of Pediococcus cerevisiae subsp. dextrinicus Coster and White to species status Pediococcus dextrinicus (Coster and White) comb. nov. *International Journal of Systematic Bacteriology,* 28**,** 523 - 527.

BACK, W. 1980. Bierschädliche Bakterien. *Brauwelt,* 43**,** 1562 - 1569.

BACK, W. 1981. Bacteria noxious for the beer. *Monatsschrift Fur Brauwissenschaft,* 34**,** 267 - 276.

BACK, W. 1982. Bierschädliche Bakterien. Spurennachweis und Beurteilung der Bierschädlichkeit mit Hilfe geeigneter Verfahren. *Brauwelt,* 45**,** 2090 - 2102.

BACK, W. 1987. New description of a type of Lactobacillus harmful to beer - Lactobacillus brevisimilis spec. nov. *Monatsschrift Fur Brauwissenschaft,* 40**,** 484 - 488.

BACK, W. 1994a. *Farbatlas und Handbuch der Getränkebiologie Band 1,* Nürnberg, Hans Carl Verlag.

BACK, W. 1994b. Sekundärkontaminationen im Abfüllbereich. *Brauwelt,* 16**,** 686 - 695.

BACK, W. 2000. *Farbatlas und Handbuch der Getränkebiologie Band 2,* Nürnberg, Hans Carl Verlag.

BACK, W. 2003. Biofilme in der Brauerei und Getränkeindustrie - 15 Jahre Praxiserfahrung. *Brauwelt,* 143**,** 1 - 5.

BACK, W. 2005. *Colour Atlas and Handbook of Beverage Biology* Nürnberg, Hans Carl Verlag.

BACK, W. 2019. Zuverlässige Nachweisstrategien von Bier- und Getränkeschädlingen. *7. Seminar Hefe und Mikrobiologie.* Freising/Weihenstephan.

BACK, W. & BIENDL, M. 2017a. Mikrobiologisch bedeutsame Inhaltsstoffe in hopfenintensiven Bieren (Teil 1). *Brauwelt,* 11**,** 310 - 313.

BACK, W. & BIENDL, M. 2017b. Mikrobiologisch bedeutsame Inhaltsstoffe in hopfenintensiven Bieren (Teil 2). *Brauwelt,* 12-13**,** 362 - 365.

BACK, W. & BOHAK, I. 2005. *Ausgewählte Kapitel der Brauereitechnologie,* Nürnberg, Hans Carl Verlag.

BACK, W., BOHAK, I., EHRMANN, M., LUDWIG, T., POT, B. & SCHLEIFER, K. H. 1999. Lactobacillus perolens sp nov., a soft drink spoilage bacterium. *Systematic and Applied Microbiology,* 22**,** 354 - 359.

BACK, W., BOHAK, I., EHRMANN, M., LUDWIG, W. & SCHLEIFER, K. H. 1996. Revival of the species Lactobacillus lindneri and the design of a species specific oligonucleotide probe. *Systematic and Applied Microbiology,* 19**,** 322 - 325.

BACK, W., BREU, S., WEIGAND, C. 1988. Infektionsursachen im Jahre 1987. *Brauwelt,* 31/32**,** 1358 - 1362.

BACK, W., LEIBHARD, M. & BOHAK, I. 1992. Flash pasteurization - membrane filtration. Comparative biological safety. *Brauwelt international,* 1**,** 42 - 49.

BACK, W. & PITTNER, H. 1993. Kontinuierliche Herstellung gesäuerter Würze mit Hilfe immobilisierter Milchsäurebakterien. *Monatsschrift für Brauwissenschaft,* 46**,** 364 - 371.

BACK, W., WEIß, N., SEIDEL, H. 1979. Isolierung und systematische Zuordnung bierschädlicher gramnegativer Bakterien - II.: Gramnegative anaerobe Stäbchen. *Brauwissenschaft,* 32**,** 233 - 238.

BARKER, C. & PARK, S. F. 2001. Sensitization of Listeria monocytogenes to low pH, organic acids, and osmotic stress by ethanol. *Applied and Environmental Microbiology,* 67**,** 1594 - 1600.

BARNEY, M., VOLGYI, A., NAVARRO, A. & RYDER, D. 2001. Riboprinting and 16S rRNA gene sequencing for identification of brewery Pediococcus isolates. *Applied and Environmental Microbiology,* 67**,** 553 - 560.

BARNEY, M. C., KOT, E. J. & CHICOYE, E. 1990. *Culture medium for detection of beer spoilage microorganisms*.

BEALES, N. 2004. Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: A review. *Comprehensive Reviews in Food Science and Food Safety,* 3**,** 1 - 20.

BEHR, J., GAENZLE, M. G. & VOGEL, R. F. 2006. Characterization of a highly hop-resistant Lactobacillus brevis strain lacking hop transport. *Applied and Environmental Microbiology,* 72**,** 6483 - 6492.

BEHR, J., GEISSLER, A. J., PREISSLER, P., EHRENREICH, A., ANGELOV, A. & VOGEL, R. F. 2015. Identification of ecotype-specific marker genes for categorization of beer-spoiling Lactobacillus brevis. *Food Microbiology,* 51**,** 130 - 138.

BEHR, J., GEISSLER, A. J., SCHMID, J., ZEHE, A. & VOGEL, R. F. 2016. The identification of novel diagnostic marker genes for the detection of beer spoiling Pediococcus damnosus strains using the BlAst Diagnostic Gene findEr. *Plos One,* 11.

BEHR, J., ISRAEL, L., GAENZLE, M. G. & VOGEL, R. F. 2007. Proteomic approach for characterization of hop-inducible proteins in Lactobacillus brevis. *Applied and Environmental Microbiology,* 73**,** 3300 - 3306.

BEHR, J. & VOGEL, R. F. 2009. Mechanisms of hop inhibition: Hop ionophores. *Journal of Agricultural and Food Chemistry,* 57**,** 6074 - 6081.

BEHR, J. & VOGEL, R. F. 2010. Mechanisms of hop inhibition include the transmembrane redox reaction. *Applied and Environmental Microbiology,* 76**,** 142 - 149.

BEIJERINCK, M. W. 1901. Sur les ferments lactiques de l'industrie. *Archives Nederlandaises des Sciences Exactes et Naturelles (Section 2),* 6**,** 212 – 243.

BERGEY, D. H., HARRISON, D. C., BREED, R. S., HAMMER, B. W. & HUNTOON, F. M. 1923. *Bergey's Manual of Determinative Bacteriology,* Baltimore, Williams & Wilkins.

BERGSVEINSON, J., BAECKER, N., PITTET, V. & ZIOLA, B. 2015a. Role of plasmids in Lactobacillus brevis BSO 464 hop tolerance and beer spoilage. *Applied and Environmental Microbiology,* 81**,** 1234 - 1241.

BERGSVEINSON, J., REDEKOP, A., ZOERB, S. & ZIOLA, B. 2015b. Dissolved carbon dioxide selects for lactic acid bacteria able to grow in and spoil packaged beer. *Journal of the American Society of Brewing Chemists,* 73**,** 331 - 338.

BERGSVEINSON, J. & ZIOLA, B. 2017. Investigation of beer spoilage lactic acid bacteria using omic approaches. *In:* BOKULICH, N. A. & BAMFORTH, C. W. (eds.) *Brewing Microbiology - Current research, omics and microbial ecology.* Norfolk, UK: Caister Academic Press.

BEVERLY, M. B., BASILE, F. & VOORHEES, K. J. 1997. Fatty acid analysis of beer spoiling microorganisms using pyrolysis mass spectrometry. *Journal of the American Society of Brewing Chemists,* 55**,** 79 - 82.

BHANDARI, R. R., RUSSELL, C. & WALKER, T. K. 1954. Studies of lactic acid bacteria associated with brewery products. 1. Identification of types isolated from beer and from yeast. *Journal of the Science of Food and Agriculture,* 5**,** 27 - 31.

BHANDARI, R. R. & WALKER, T. K. 1953. Lactobacillus frigidus n. sp. isolated from brewery yeast. *Journal of General Microbiology,* 8**,** 330 - 332.

BOHAK, I. 2015. *Merkmale und Taxonomie brauereispezifischer Laktobazillen.* PhD, Technische Universität München.

BOHAK, I., BACK, W., RICHTER, L., EHRMANN, M., LUDWIG, W. & SCHLEIFER, K. H. 1998. Lactobacillus amylolyticus sp. nov., isolated from beer malt and beer wort. *Systematic and Applied Microbiology,* 21**,** 360 - 364.

BOHAK, I., QIAN, F. & ENGELMANN, J. 2012. Gängige Nachweismedien für bierschädliche Bakterien. *Brauwelt,* 14-15**,** 384 - 393.

BOHAK, I., THELEN, K. & BEIMFOHR, C. 2006. Description of Lactobacillus backi sp. nov., an obligate beer-spoiling bacterium. *Monatsschrift Fur Brauwissenschaft***,** 78 - 82.

BOHM, M. E., HUPTAS, C., KREY, V. M. & SCHERER, S. 2015. Massive horizontal gene transfer, strictly vertical inheritance and ancient duplications differentially shape the evolution of Bacillus cereus enterotoxin operons hbl, cytK and nhe. *Bmc Evolutionary Biology,* 15.

BOKULICH, N. A. & BAMFORTH, C. W. 2013. The microbiology of malting and brewing. *Microbiology and Molecular Biology Reviews,* 77**,** 157 - 172.

BOKULICH, N. A., BAMFORTH, C. W. & MILLS, D. A. 2012. A review of molecular methods for microbial community profiling of beer and wine. *Journal of the American Society of Brewing Chemists,* 70**,** 150 - 162.

BOKULICH, N. A. & MILLS, D. A. 2012. Differentiation of mixed lactic acid bacteria communities in beverage fermentations using targeted terminal restriction fragment length polymorphism. *Food Microbiology,* 31**,** 126 - 132.

BOOTH, I. R. 1985. Rrgulation of cytoplasmic pH in bacteria. *Microbiological Reviews,* 49**,** 359 - 378.

BOOTH, I. R. & KROLL, R. G. 1989. The preservation of foods by low pH. *In:* GOULD, G. W. (ed.) *Mechanisms of action of food preservation procedures.* London: Elsevier Applied Science.

BOTTARI, B., ERCOLINI, D., GATTI, M. & NEVIANI, E. 2006. Application of FISH technology for microbiological analysis: current state and prospects. *Applied Microbiology and Biotechnology,* 73**,** 485 - 494.

BREITBACH, A., JACOB, F., HUTZLER, M. & KOOB, J. 2015. Einfacher Schnelltest zum Nachweis bierschädigender Organismen. *Brauwelt,* 33**,** 961 - 966.

BRIGGS, D. E., BOULTON, C. A., BROOKES, P. A. & STEVENS, R. 2004. Microbiology. *In:* BRIGGS, D. E., BOULTON, C. A., BROOKES, P. A. & STEVENS, R. (eds.) *Brewing science and practice.* Cambridge: Woodhead Publishing Limited.

BRINGEL, F., CURK, M. C. & HUBERT, J. C. 1996. Characterization of lactobacilli by southern-type hybridization with a Lactobacillus plantarum pyrDFE probe. *International Journal of Systematic Bacteriology,* 46**,** 588 - 594.

BRINGEL, F., QUENEE, P. & TAILLIEZ, P. 2001. Polyphasic investigation of the diversity within Lactobacillus plantarum related strains revealed two L-plantarum subgroups. *Systematic and Applied Microbiology,* 24**,** 561 - 571.

BROCK, T. D. & MADIGAN, M. T. 1991. *Biology of microorganisms,* New Jersey, Prentice-Hall Inc.

BROOIJMANS, R. J. W., DE VOS, W. M. & HUGENHOLTZ, J. 2009. Lactobacillus plantarum WCFS1 electron transport chains. *Applied and Environmental Microbiology,* 75**,** 3580 - 3585.

BUNKER, H. J. The survival of pathogenic bacteria in beer. Proc. Eur. Brew. Conv. Congr. Baden-Baden, 1955 Amsterdam. Elsevier.

CAMPBELL, I. 2003a. Chapter 1: Microbiological aspects of brewing. *In:* PRIEST, F. G., CAMPBELL, I. (ed.) *Brewing Microbiology.* Ney York: Kluwer Academic/Plenum Publishers.

CAMPBELL, I. 2003b. Chapter 7: Wild yeasts in brewing and identification of microbial spoilage. *Brewing Microbiology.* New York: Kluwer Academic/Plenum Publishers.

CANCHAYA, C., CLAESSON, M. J., FITZGERALD, G. F., VAN SINDEREN, D. & O'TOOLE, P. W. 2006. Diversity of the genus Lactobacillus revealed by comparative genomics of five species. *Microbiology-Sgm,* 152**,** 3185 - 3196.

CARR, J. G. & DAVIES, P. A. 1972. Ecology and classification of strains of Lactobacillus collinoides nov. spec. - bacterium commonly found in fermenting apple juice. *Journal of Applied Bacteriology,* 35**,** 463 - 471.

CARRIERE, A. 1959. *Über die Biologie und Systematik der bierschädigenden stäbchenförmigen Bakterienarten.* PhD, Humboldt-Universität.

CASEY, G. P. & INGLEDEW, W. M. 1981. The use and understanding of media used in brewing bacteriology. 2. Selective media for the isolation of lactic acid bacteria. *Brewers Digest,* 56**,** 38 ff.

CASEY, G. P. & INGLEDEW, W. M. M. 1986. Ethanol tolerance in yeasts. *Crc Critical Reviews in Microbiology,* 13**,** 219 - 280.

CHABAN, B., DENEER, H., DOWGIERT, T., HYMERS, J. & ZIOLA, B. 2005. The flagellin gene and protein from the brewing spoilage bacteria Pectinatus cerevisiiphilus and Pectinatus frisingensis. *Canadian Journal of Microbiology,* 51**,** 863 - 874.

CHASSY, B. M., GIBSON, E. & GIUFFRIDA, A. 1976. Evidence for extrachromosomal elements in Lactobacillus. *Journal of Bacteriology,* 127**,** 1576 - 1578.

CLAISSE, O. & LONVAUD-FUNEL, A. 2000. Assimilation of glycerol by a strain of Lactobacillus collinoides isolated from cider. *Food Microbiology,* 17**,** 513 - 519.

CLAISSE, O. & LONVAUD-FUNEL, A. 2001a. Detection of lactic acid bacteria producing 3-hydroxypropionaldehyde (acrolein precursor) from glycerol by molecular tests. *Lait,* 81**,** 173 - 181.

CLAISSE, O. & LONVAUD-FUNEL, A. 2001b. Primers and a specific DNA probe for detecting lactic acid bacteria producing 3-hydroxypropionaldehyde from glycerol in spoiled ciders. *Journal of Food Protection,* 64**,** 833 - 837.

CLAUSSEN, N. H. 1903. Études sur les bactéries dites sarcines et sur les maladies quelles provoquent dans la bière. *C.R. Trav. Lab. Carlsberg,* 6**,** 64 - 83.

COENYE, T., GEVERS, D., VAN DE PEER, Y., VANDAMME, P. & SWINGS, J. 2005. Towards a prokaryotic genomic taxonomy. *Fems Microbiology Reviews,* 29**,** 147 - 167.

COHAN, F. M. 2001. Bacterial species and speciation. *Systematic Biology,* 50**,** 513 - 524.

COLLINS, M. D., PHILLIPS, B. A. & ZANONI, P. 1989. Deoxyribonucleic acid homology studies of Lactobacillus casei, Lactobacillus paracasei sp. nov., subsp. paracasei and subsp. tolerans, and Lactobacillus rhamnosus sp. nov., comb. nov. *International Journal of Systematic Bacteriology,* 39**,** 105 - 108.

COLLINS, M. D., RODRIGUES, U., ASH, C., AGUIRRE, M., FARROW, J. A. E., MARTINEZMURCIA, A., PHILLIPS, B. A., WILLIAMS, A. M. & WALLBANKS, S. 1991. Phylogenetic analysis of the genus Lactobacillus and related lactic acid bacteria as determined by reverse-transcriptase sequencing of 16S ribosomal RNA. *Fems Microbiology Letters,* 77**,** 5 - 12.

COOPER, J. E. & FEIL, E. J. 2004. Multilocus sequence typing - what is resolved? *Trends in Microbiology,* 12**,** 373 - 377.

CORSETTI, A., SETTANNI, L., VAN SINDEREN, D., FELIS, G. E., DELLAGLIO, F. & GOBBETTI, M. 2005. Lactobacillus rossii sp nov., isolated from wheat sourdough. *International Journal of Systematic and Evolutionary Microbiology,* 55**,** 35 - 40.

COSTER, E. & WHITE, H. R. 1964. Further studies of genus Pediococcus. *Journal of General Microbiology,* 37**,** 15 - 31.

CUNIN, R., GLANSDORFF, N., PIERARD, A. & STALON, V. 1986. Biosynthesis and metabolism of arginine in bacteria. *Microbiological Reviews,* 50**,** 314 - 352.

CURK, M. C., HUBERT, J. C. & BRINGEL, F. 1996. Lactobacillus paraplantarum sp. nov., a new species related to Lactobacillus plantarum. *International Journal of Systematic Bacteriology,* 46**,** 595 - 598.

CURK, M. C., PELADAN, F. & HUBERT, J. C. 1994. Fourier-transform infrared (FTIR) spectroscopy for identifying Lactobacillus species. *Fems Microbiology Letters,* 123**,** 241 - 248.

DA COSTA, M. S., ALBUQUERQUE, L., NOBRE, M. F. & WAIT, R. 2011. The identification of polar lipids in prokaryotes. *In:* RAINEY, F. & OREN, A. (eds.) *Methods in Microbiology, Vol 38: Taxonomy of Prokaryotes.*

DAIFAS, D. P., SMITH, J. P., BLANCHFIELD, B., CADIEUX, B., SANDERS, G. & AUSTIN, J. W. 2003. Effect of ethanol on the growth of Clostridium botulinum. *Journal of Food Protection,* 66**,** 610 - 617.

DAVIS, G. H. G. 1964. Notes on the phylogenetic background to Lactobacillus taxonomy. *Microbiology,* 34**,** 177 - 184.

DE ANGELIS, M. & GOBBETTI, M. 2011. Chapter 11: Stress response of Lactobacilli. *In:* TSAKALIDOU, E. & PAPADIMITRIOU, K. (eds.) *Stress responses of lactic acid bacteria.* Boston: Springer.

DE MAN, J. C., ROGOSA, M. & SHARPE, M. E. 1960. A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology,* 23**,** 130 - 135.

DE VUYST, L. & VANCANNEYT, M. 2007. Biodiversity and identification of sourdough lactic acid bacteria. *Food Microbiology,* 24**,** 120 - 127.

DENG, Y., LIU, J., LI, H., LI, L., TU, J., FANG, H., CHEN, J. & QIAN, F. 2014. An improved plate culture procedure for the rapid detection of beer-spoilage lactic acid bacteria. *Journal of the Institute of Brewing,* 120**,** 127 - 132.

DENG, Y., LIU, J. Y., LI, L., FANG, H. J., TU, J. X., LI, B., LIU, J., LI, H. P. & XU, Z. B. 2015. Reduction and restoration of culturability of beer-stressed and low-temperature-stressed Lactobacillus acetotolerans strain 2011-8. *International Journal of Food Microbiology,* 206**,** 96 - 101.

DERTLI, E., MERCAN, E., ARICI, M., YILMAZ, M. T. & SAGDIC, O. 2016. Characterisation of lactic acid bacteria from Turkish sourdough and determination of their exopolysaccharide (EPS) production characteristics. *Lwt-Food Science and Technology,* 71**,** 116 - 124.

DI CAGNO, R., DE ANGELIS, M., GALLO, G., SETTANNI, L., BERLOCO, M. G., SIRAGUSA, S., PARENTE, E., CORSETTI, A. & GOBBETTI, M. 2007. Genotypic and phenotypic diversity of Lactobacillus rossiae strains isolated from sourdough. *Journal of Applied Microbiology,* 103**,** 821 - 835.

DICKS, L. M. T., DUPLESSIS, E. M., DELLAGLIO, F. & LAUER, E. 1996. Reclassification of Lactobacillus casei subsp casei ATCC 393 and Lactobacillus rhamnosus ATCC 15820 as Lactobacillus zeae nom rev, designation of ATCC 334 as the neotype of L-casei subsp casei, and rejection of the Lactobacillus paracasei. *International Journal of Systematic Bacteriology,* 46**,** 337 - 340.

DICKS, L. M. T. & ENDO, A. 2009. Taxonomic status of lactic acid bacteria in wine and key characteristics to differentiate species. *South African Journal of Enology and Viticulture,* 30**,** 72 - 90.

DOBROGOSZ, W. J. & STONE, R. W. 1962a. Oxidative metabolism in Pediococcus pentosaceus. 1. Role of oxygen and catalase. *Journal of Bacteriology,* 84**,** 716 - 723.

DOBROGOSZ, W. J. & STONE, R. W. 1962b. Oxidative metabolism in Pediococcus pentosaceus. 2. Factors controlling formation of oxidative activities. *Journal of Bacteriology,* 84**,** 724 - 729.

DOBSON, C. M., DENEER H., LEE, S., HEMMINGSEN, S., GLAZE, S., ZIOLA, B. 2002. Phylogenetic analysis of the genus Pediococcus, including Pediococcus claussenii sp. nov., a novel lactic acid

bacterium isolated from beer. *International Journal of Systematic and Evolutionary Microbiology,* 52**,** 2003 - 2010.
DOLEZIL, L. & KIRSOP, B. H. 1980. Vatiations amongst beers and lactic acid bacteria relating to beer spoilage. *Journal of the Institute of Brewing,* 86**,** 122 - 124.
DOLS-LAFARGUE, M., LEE, H. Y., LE MARREC, C., HEYRAUD, A., CHAMBAT, G. & LONVAUD-FUNEL, A. 2008. Characterization of gtf, a glucosyltransferase gene in the genomes of Pediococcus parvulus and Oenococcus oeni, two bacterial species commonly found in wine. *Applied and Environmental Microbiology,* 74**,** 4079 - 4090.
DONHAUSER, S. & WAGNER, D. 1986. Ways to influence the zinc content of wort. *Monatsschrift für Brauwissenschaft,* 39**,** 223 - 230.
DÜRR, P. 1984. Detection of beer-spoiling bacteria in air. *Brauwelt international,* 2**,** 109 - 115.
DYKES, G. A. & VONHOLY, A. 1994. Taxonomic status of atypical Lactobacillus sake and Lactobacillus curvatus strains associated with vacuum-packaged meat spoilage. *Current Microbiology,* 28**,** 197 - 200.
DZIEWIT, L. & BARTOSIK, D. 2014. Plasmids of psychrophilic and psychrotolerant bacteria and their role in adaptation to cold environments. *Frontiers in Microbiology,* 5.
EATON, L. C., TEDDER, T. F. & INGRAM, L. O. 1982. Effects of fatty acid composition on the sensitivity of membrance functions to ethanol in Escherichia coli. *Subst. Alcohol Actions Misuse,* 3**,** 77 - 87.
EHRMANN, M. A., PREISSLER, P., DANNE, M. & VOGEL, R. F. 2010. Lactobacillus paucivorans sp. nov., isolated from a brewery environment. *International Journal of Systematic and Evolutionary Microbiology,* 60**,** 2353 - 2357.
EHRMANN, M. A. & VOGEL, R. F. 2005b. Taxonomic note "Lactobacillus pastorianus" (Van Laer, 1892) a former synonym for Lactobacillus paracollinoides. *Systematic and Applied Microbiology,* 28**,** 54 - 56.
EMEIS, C. C. 1969. Methoden der brauereibiologischen Betriebskontrolle. 3. VLB-S7 Agar zum Nachweis bierschädlicher Pediokokken. *Monatsschrift für Brauwissenschaft,* 22**,** 8 - 11.
ENGELMANN, U. & WEISS, N. 1985. Megasphaera cerevisiae sp. nov.: A new gram-negative obligately anaerobic coccus isolated from spoiled beer. *Systematic and Applied Microbiology,* 6**,** 287 - 290.
ENTANI, E., MASAI, H. & SUZUKI, K. I. 1986. Lactobacillus acetotolerans, a new species from fermented vinegar broth. *International Journal of Systematic Bacteriology,* 36**,** 544 - 549.
ESCHENBECHER, F. 1966. Lactobacilus pastorianus? Über die Artzugehörigkeit der "Biermilchsäurestäbchen". *Monatsschrift für Brauwissenschaft,* 19**,** 233 - 246.
ESCHENBECHER, F. 1968a. Zur Kenntnis der biersäuernden Laktobazillen Teil I. *Monatsschrift für Brauwissenschaft,* 11**,** 424 - 437.
ESCHENBECHER, F. 1968b. Zur Kenntnis der biersäuernden Laktobazillen Teil II. *Monatsschrift für Brauwissenschaft,* 12**,** 464 - 471.
ESCHENBECHER, F. 1969. Zur Kenntnis der biersäuernden Laktobazillen Teil III. *Monatsschrift für Brauwissenschaft,* 22**,** 14 - 28.
FARROW, J. A. E., FACKLAM, R. R. & COLLINS, M. D. 1989. Nucleic acid homologies of some vancomycin-resistant Leuconostocs and description of Leuconostoc citreum sp. nov. and Leuconostoc pseudomesenteroides sp. nov. *International Journal of Systematic Bacteriology,* 39**,** 279 - 283.
FARROW, J. A. E., PHILLIPS, B. A. & COLLINS, M. D. 1988. Nucleic acid studies on some heterogermentative Lactobacilli - description of Lactobacillus malefermentans sp. nov. and Lactobacillus parabuchneri sp. nov. *Fems Microbiology Letters,* 55**,** 163 - 167.
FELIS, G. E. & DELLAGLIO, F. 2007. Taxonomy of Lactobacilli and Bifidobacteria. *Current issues in intestinal microbiology,* 8**,** 44 - 61.
FERNANDEZ, J. L. & SIMPSON, W. J. 1993. Aspects of the resistance of lactic acid bacteria to hop bitter acids. *Journal of Applied Microbiology,* 75**,** 315 - 319.
FERNANDEZ, J. L. & SIMPSON, W. J. 1995. Measurement and prediction of the susceptibility of lager beer to spoilage by lactic acid bacteria. *Journal of Applied Microbiology,* 78**,** 419 - 425.

FIOCCO, D., CAPOZZI, V., GOFFIN, P., HOLS, P. & SPANO, G. 2007. Improved adaptation to heat, cold, and solvent tolerance in Lactobacillus plantarum. *Applied Microbiology and Biotechnology,* 77**,** 909 - 915.

FLANNIGAN, B. 2003. Chapter 4: The microbiota of barley and malt. *In:* PRIEST, F. G., CAMPBELL, I. (ed.) *Brewing Microbiology.* New York: Kluwer Academic/Plenum Publishers.

FLEMMING, H.-C. & WINGENDER, J. 2010. The biofilm matrix. *Nature reviews microbiology,* 8**,** 623 - 633.

FRAUNHOFER, M. E. 2018. *Characterization of EPS-producing brewery-associated lactobacilli.* PhD, TU München.

FUNAHASHI, W., SUZUKI, K., OHTAKE, Y. & YAMASHITA, H. 1998. Two novel beer-spoilage Lactobacillus species isolated from breweries. *Journal of the American Society of Brewing Chemists,* 56**,** 64 - 69.

GANCHEVA, A., POT, B., VANHONACKER, K., HOSTE, B. & KERSTERS, K. 1999. A polyphasic approach towards the identification of strains belonging to Lactobacillus acidophilus and related species. *Systematic and Applied Microbiology,* 22**,** 573 - 585.

GARES, S. L., WHITING, M. S., INGLEDEW, W. M. & ZIOLA, B. 1993. Detection and identification of Pectinatus cerevisiiphilus using surface-reactive monoclonal antibodies in a membrane filter-based fluoroimmunoassay. *Journal of the American Society of Brewing Chemists,* 51**,** 158 - 163.

GARVIE, E. I. 1974. Nomenclature problems of Pediococci - request for an opinion. *International Journal of Systematic Bacteriology,* 24**,** 301 - 306.

GARVIE, E. I. 1983. Leuconostoc mesenteroides subsp. cremoris (Knudsen and Sorensen) comb. nov. and Leuconostoc mesenteroides subsp. dextranicum (Beijerinck) comb. nov. . *International Journal of Systematic Bacteriology,* 33**,** 118 - 119.

GARVIE, E. I. 1984a. Genus Leuconostoc. *In:* SNEATH, P. H. A., MAIR, N. S., SHARPE, M. E. & HOLT, J. G. (eds.) *Bergey's Manual of Systematic Microbiology.* London: Williams & Wilkins.

GARVIE, E. I. 1984b. Genus Pediococcus. *In:* SNEATH, P. H. A., MAIR, N. S., SHARPE, M. E. & HOLT, J. G. (eds.) *Bergey's Manual of Systematic Microbiology.* London: Williams & Wilkins.

GEIßLER, A. 2016. *Lifestyle of beer spoiling lactic acid bacteria.* PhD, TU München.

GEIßLER, A. J., BEHR, J., SCHMID, J., ZEHE, A. & VOGEL, R. F. 2017. Beer spoilage ability of lactic acid bacteria is a plasmid-encoded trait. *Brewing Science,* 70**,** 57 - 73.

GEVERS, D., COHAN, F. M., LAWRENCE, J. G., SPRATT, B. G., COENYE, T., FEIL, E. J., STACKEBRANDT, E., VAN DE PEER, Y., VANDAMME, P., THOMPSON, F. L. & SWINGS, J. 2005. Re-evaluating prokaryotic species. *Nature reviews microbiology,* 3**,** 733 - 739.

GEVERS, D., HUYS, G. & SWINGS, J. 2001. Applicability of rep-PCR fingerprinting for identification of Lactobacillus species. *Fems Microbiology Letters,* 205**,** 31 - 36.

GHYSELINCK, J., VAN HOORDE, K., HOSTE, B., HEYLEN, K. & DE VOS, P. 2011. Evaluation of MALDI-TOF MS as a tool for high-throughput dereplication. *Journal of Microbiological Methods,* 86**,** 327 - 336.

GILLILAND, R. B. 1971. Yeast classification. *Journal of the Institute of Brewing,* 77**,** 276 - 284.

GONZALEZ, A., HIERRO, N., POBLET, M., MAS, A. & GUILLAMON, J. M. 2005. Application of molecular methods to demonstrate species and strain evolution of acetic acid bacteria population during wine production. *International Journal of Food Microbiology,* 102**,** 295 - 304.

GONZALEZ, J. M., JURADO, V., LAIZ, L., ZIMMERMANN, J., HERMOSIN, B. & SAIZ-JIMENEZ, C. 2004. Pectinatus portalensis nov sp., a relatively fast-growing, coccoidal, novel Pectinatus species isolated from a wastewater treatment plant. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology,* 86**,** 241 - 248.

GORIS, J., KONSTANTINIDIS, K. T., KLAPPENBACH, J. A., COENYE, T., VANDAMME, P. & TIEDJE, J. M. 2007. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *International Journal of Systematic and Evolutionary Microbiology,* 57**,** 81 - 91.

GUNTHER, H. L., COSTER, E. & WHITE, H. R. 1962. Designation of the type strain of Pediococcus parvulus Gunther and White. *International Bulletin of Bacteriological Nomenclature and Taxonomy,* 12**,** 189 - 190.

HAAKENSEN, M. 2009. *Genetic markers for beer-spoilage by lactobacilli and pediococci.* PhD, University of Saskatchewan.

HAAKENSEN, M., DOBSON, C. M., HILL, J. E. & ZIOLA, B. 2009a. Reclassification of Pediococcus dextrinicus (Coster and White 1964) Back 1978 (Approved Lists 1980) as Lactobacillus dextrinicus comb. nov., and emended description of the genus Lactobacillus. *International Journal of Systematic and Evolutionary Microbiology,* 59**,** 615 - 621.

HAAKENSEN, M., PITTET, V., MORROW, K., SCHUBERT, A., FERGUSON, J. & ZIOLA, B. 2009b. Ability of novel ATP-binding cassette multidrug resistance genes to predict growth of Pediococcus isolates in beer. *Journal of the American Society of Brewing Chemists,* 67**,** 170 - 176.

HAAKENSEN, M., PITTET, V. & ZIOLA, B. 2011. Reclassification of Paralactobacillus selangorensis Leisner et al. 2000 as Lactobacillus selangorensis comb. nov. *International Journal of Systematic and Evolutionary Microbiology,* 61**,** 2979 - 2983.

HAAKENSEN, M. C., BUTT, L., CHABAN, B., DENEER, H., ZIOLA, B. & DOWGIERT, T. 2007. HorA-specific real-time PCR for detection of beer-spoilage lactic acid bacteria. *Journal of the American Society of Brewing Chemists,* 65**,** 157 - 165.

HAFT, R. J. F., KEATING, D. H., SCHWAEGLER, T., SCHWALBACH, M. S., VINOKUR, J., TREMAINE, M., PETERS, J. M., KOTLAJICH, M. V., POHLMANN, E. L., ONG, I. M., GRASS, J. A., KILEY, P. J. & LANDICK, R. 2014. Correcting direct effects of ethanol on translation and transcription machinery confers ethanol tolerance in bacteria. *Proceedings of the National Academy of Sciences of the United States of America,* 111**,** E2576 - E2585.

HAIKARA, A. 1980. A new gram-negative anaerobic beer spoilage organism. *Monatsschrift für Brauwissenschaft,* 33**,** 278 - 278.

HAIKARA, A. 1985a. Detection of anaerobic, gram-negative bacteria in beer. *Monatsschrift Fur Brauwissenschaft,* 38**,** 239 - 243.

HAIKARA, A. 1985b. Detection of Pectinatus contaminants in beer. *Journal of the American Society of Brewing Chemists,* 43**,** 43 - 46.

HAIKARA, A. & HELANDER, I. 2006. Pectinatus, Megasphaera and Zymophilus. *In:* DWORKIN, M. (ed.) *The Prokaryotes.* 3rd ed. Singapur: Springer Science + Business Media, LLC.

HAIKARA, A. & LOUNATMAA, K. 1987. Characterization of Megasphaera sp., a new anaerobic beer spoilage coccus. *Journal of the Institute of Brewing,* 93**,** 161 - 161.

HAIKARA, A., MATTILA-SANDHOLM, T. & MANNINEN, M. 1990. Rapid detection of contaminants in pitching yeast using automated turbidometry. *Journal of the American Society of Brewing Chemists,* 48**,** 92 - 95.

HAIKARA, A., PENTTILA, L., ENARI, T. M. & LOUNATMAA, K. 1981. Microbiological, biochemical, and electron-microscopic characterization of a Pectinatus strain. *Applied and Environmental Microbiology,* 41**,** 511 - 517.

HAMMES, W. P. & HERTEL, C. 2009. Genus I. Lactobacillus Beijerink, 1901. *In:* DE VOS, P., GARRITY, G. M., JONES, D., KRIEG, N. R., LUDWIG, W., RAINEY, F. A., SCHLEIFER, K. H. & WHITMAN, W. B. (eds.) *Bergey's manual of systematic bacteriology.* Berlin: Springer

HAMMES, W. P., WEISS, N. & HOLZAPFEL, W. H. 1992. The genera Lactobacillus and Carnobacterium. *In:* BALOWS, A., TRUPER, H. G., DWORKIN, M., HARDER, W. & SCHLEIFER, K. H. (eds.) *Ecophysiology, Isolation, Identification, Applications.* New York: Springer Verlag.

HAYASHI, N., ITO, M., HORIIKE, S. & TAGUCHI, H. 2001. Molecular cloning of a putative divalent-cation transporter gene as a new genetic marker for the identification of Lactobacillus brevis strains capable of growing in beer. *Applied Microbiology and Biotechnology,* 55**,** 596 - 603.

HELANDER, I. M., HAIKARA, A., SADOVSKAYA, I., VINOGRADOV, E. & SALKINOJA-SALONEN, M. S. 2004. Lipopolysaccharides of anaerobic beer spoilage bacteria of the genus Pectinatus - lipopolysaccharides of a Gram-positive genus. *Fems Microbiology Reviews,* 28**,** 543 - 552.

HENNEBERG, W. 1903. Zur Kenntnis der Milchsäurebakterien der Brennereimaische, der Milch, und des Bieres. *Wochenschrift Brau,* 18**,** 381 - 384.

HENNEBERG, W. 1926. *Handbuch der Gärungsbakteriologie,* Berlin, Paul Parey.

HENRIKSSON, E. & HAIKARA, A. 1991. Airborne microorganisms in the brewery filling area and their effect on microbiological stability of beer. *Monatsschrift Fur Brauwissenschaft,* 44**,** 4 - 8.

HILL, A. E. 2009. Chapter 5 - Microbiological stability of beer. *In:* CHARLES, W. B., INGE, R., GRAHAM STEWARTA2 - CHARLES W. BAMFORTH, I. R. & GRAHAM, S. (eds.) *Beer.* San Diego: Academic Press.

HOLLEROVA, I. & KUBIZNIAKOVA, P. 2001. Monitoring Gram positive bacterial contamination in Czech breweries. *Journal of the Institute of Brewing,* 107**,** 355 - 358.

HOLZAPFEL, W. H. 1992. Culture media for non-sporulating gram-positive food spoilage bacteria. *International Journal of Food Microbiology,* 17**,** 113 - 133.

HOLZAPFEL, W. H. & WOOD, B. J. B. 2014. *Lactic acid bacteria: Biodiversity and Taxonomy,* Chichester, UK, John Wiley & Sons Ltd.

HOMANN, F., BREMER, Z. & MÖLLER-HERGT, G. 2002. Etablierung der LightCycler-PCR als Schnellnachweismethode bierschädlicher Bakterien in der mikrobiologischen Routineanalytik. *Brauwelt,* 142**,** 798 - 804.

HOMANN, F. & KÜHLE, A. 2017. MALDI-TOF: Routineanwendung im mikrobiologischen Brauereilabor? *Brauwelt,* 10**,** 274 - 277.

HOUGH, J. S., BRIGGS, D. E., STEVENS, R. & YOUNG, T. W. 1982. *Malting and brewing science,* London, Chapman and Hall ltd.

HUSS, V. A. R., FESTL, H. & SCHLEIFER, K. H. 1983. Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Systematic and Applied Microbiology,* 4**,** 184 - 192.

HUTZLER, M., KOOB, J., GRAMMER, M., RIEDL, R. & JACOB, F. 2012a. Statistische Auswertung der PCR-Analyse bierschädlicher Bakterien in den Jahren 2010 und 2011. *Brauwelt,* 152**,** 546 - 547.

HUTZLER, M., KOOB, J., RIEDL, R. & JACOB, F. 2012b. Classification, Identification and Detection of Beer Spoiling Microorganisms - A Review. *World Brewing Congress 2012.* Portland / Oregon.

HUTZLER, M., MÜLLER-AUFFERMANN, K., KOOB, J., RIEDL, R. & JACOB, F. 2013. Bierschädliche Bakterien - Eine aktuelle Übersicht. *Brauwelt,* 3**,** 58 - 60.

HYYTIA-TREES, E. K., COOPER, K., RIBOT, E. M. & GERNER-SMIDT, P. 2007. Recent developments and future prospects in subtyping of foodborne bacterial pathogens. *Future Microbiology,* 2**,** 175 - 185.

IDLER, F. & ANNEMÜLLER, G. 2001. Bacteriocinbildende Milchsäurebakterien bei der Bierherstellung. *Brauwelt,* 30/31**,** 1161 - 1168.

IIJIMA, K., SUZUKI, K., ASANO, S., KURIYAMA, H. & KITAGAWA, Y. 2007. Isolation and identification of potential beer-spoilage Pediococcus inopinatus and beer-spoilage Lactobacillus backi strains carrying the horA and horC gene clusters. *Journal of the Institute of Brewing,* 113**,** 96 - 101.

IIJIMA, K., SUZUKI, K., ASANO, S., OGATA, T. & KITAGAWA, Y. 2009. HorC, a hop-resistance related protein, presumably functions in homodimer form. *Bioscience Biotechnology and Biochemistry,* 73**,** 1880 - 1882.

IIJIMA, K., SUZUKI, K., OZAKI, K. & YAMASHITA, H. 2006. HorC confers beer-spoilage ability on hop-sensitive Lactobacillus brevis ABBC45(cc). *Journal of Applied Microbiology,* 100**,** 1282 - 1288.

INGLEDEW, W. M. & CASEY, G. P. 1982. The use and understanding of media used in brewing microbiology - 1. Media for wild yeast. *Brewers Digest,* 57**,** 18 - 22.

INGRAM, L. O. 1990. Ethanol tolerance in bacteria. *Critical Reviews in Biotechnology,* 9**,** 305 - 319.

ISHIWA, H. & IWATA, S. 1980. Drug-resistance plasmids in Lactobacillus fermentum. *Journal of General and Applied Microbiology,* 26**,** 71 - 74.

JACK, R. W., TAGG, J. R. & RAY, B. 1995. Bacteriocins of gram-positive bacteria. *Microbiological Reviews,* 59**,** 171 - 200.

JESPERSEN, L. & JAKOBSEN, M. 1996. Specific spoilage organisms in breweries and laboratory media for their detection. *International Journal of Food Microbiology,* 33**,** 139 - 155.

JUVONEN, R. 2009. *DNA-based detection and characterisation of strictly anaerobic beer-spoilage bacteria.* PhD, University of Helsinki.

JUVONEN, R. 2015. Strictly anaerobic beer-spoilage bacteria. *In:* HILL, A. E. (ed.) *Brewing Microbiology - Managing Microbes, Ensuring Quality and Valorising Waste.* Heidelberg: Woodhead Publishing.

JUVONEN, R. & SUIHKO, M. L. 2006. Megasphaera paucivorans sp nov., Megasphaera sueciensis sp nov and Pectinatus haikarae sp nov., isolated from brewery samples, and emended description of the genus Pectinatus. *International Journal of Systematic and Evolutionary Microbiology,* 56**,** 695 - 702.

KALAC, P., SAVEL, J., KRIZEK, M., PELIKANOVA, T. & PROKOPOVA, M. 2002. Biogenic amine formation in bottled beer. *Food Chemistry,* 79**,** 431 - 434.

KANDLER, O. & WEISS, N. 1984. Genus Lactobacillus *In:* SNEATH, P. H. A., MAIR, N. S., SHARPE, M. E. & HOLT, J. G. (eds.) *Bergey's Manual of Systematic Microbiology.* London: Williams & Wilkins.

KANDLER, O. & WEISS, N. 1986. Regular, nonsporing gram-positive rods. *In:* SNEATH, P. H. A., MAIR, N. S., SHARPE, M. E. & HOLT, J. G. (eds.) *Bergey's Manual of Systematic Bacteriology.* Lippincott: Williams & Wilkins.

KATLA, A. K., KRUSE, H., JOHNSEN, G. & HERIKSTAD, H. 2001. Antimicrobial susceptibility of starter culture bacteria used in Norwegian dairy products. *International Journal of Food Microbiology,* 67**,** 147 - 152.

KEHRES, D. G., LAWYER, C. H. & MAGUIRE, M. E. 1998. The CorA magnesium transporter gene family. *Microbial & Comparative Proteomics,* 3**,** 151 - 169.

KERN, C. C., USBECK, J. C., VOGEL, R. F. & BEHR, J. 2013. Optimization of Matrix-Assisted-Laser-Desorption-Ionization-Time-Of-Flight Mass Spectrometry for the identification of bacterial contaminants in beverages. *Journal of Microbiological Methods,* 93**,** 185 - 191.

KERN, C. C., VOGEL, R. F. & BEHR, J. 2014a. Differentiation of Lactobacillus brevis strains using Matrix-Assisted-Laser-Desorption-Ionization-Time-of-Flight Mass Spectrometry with respect to their beer spoilage potential. *Food Microbiology,* 40**,** 18 - 24.

KERN, C. C., VOGEL, R. F. & BEHR, J. 2014b. Identification and differentiation of brewery isolates of Pectinatus sp by Matrix-Assisted-Laser Desorption-Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS). *European Food Research and Technology,* 238**,** 875 - 880.

KIM, M., OH, H.-S., PARK, S.-C. & CHUN, J. 2014a. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *International Journal of Systematic and Evolutionary Microbiology,* 64**,** 346 - 351.

KITAHARA, K. 1974. Genus III, Pediococcus Blacke 1884. *In:* BUCHANAN, R. E. & GIBBONS, N. E. (eds.) *Bergey's Manual of Determinative Bacteriology.* 8th ed. Baltimore.

KLAENHAMMER, T. R. & DE VOS, W. M. 2011. An incredible scientific journey. The evolutionary tale of lactic acid bacteria. *In:* LEDEBOER, A., HUGENHOLTZ, J., KOK, J., KONINGS, W. N. & WOUTERS, J. (eds.) *The 10th LAB symposium. Thirty years of research on lactic acid bacteria.* 24 Media Labs.

KLEIN, G., DICKS, L. M. T., PACK, A., HACK, B., ZIMMERMANN, K., DELLAGLIO, F. & REUTER, G. 1996. Emended descriptions of Lactobacillus sake (Katagiri, Kitahara, and Fukami) and Lactobacillus curvatus (Abo-Elnaga and Kandler): Numerical classification revealed by protein fingerprinting and identification based on biochemical patterns and DNA-DNA hybridizations. *International Journal of Systematic Bacteriology,* 46**,** 367 - 376.

KLOOS, W. E., TORNABENE, T. G. & SCHLEIFER, K. H. 1974. Isolation and characterization of Micrococci from human skin, including 2 new species - Micrococcus lylae and Micrococcus kristinae. *International Journal of Systematic Bacteriology,* 24**,** 79 - 101.

KOIVULA, T. T., JUVONEN, R., HAIKARA, A. & SUIHKO, M. L. 2006. Characterization of the brewery spoilage bacterium Obesumbacterium proteus by automated ribotyping and development of PCR methods for its biotype 1. *Journal of Applied Microbiology,* 100**,** 398 - 406.

KONINGS, W. N., LOLKEMA, J. S., BOLHUIS, H., VANVEEN, H. W., POOLMAN, B. & DRIESSEN, A. J. M. 1997. The role of transport processes in survival of lactic acid bacteria - Energy transduction and multidrug resistance. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology,* 71**,** 117 - 128.

KONSTANTINIDIS, K. T., RAMETTE, A. & TIEDJE, J. M. 2006. The bacterial species definition in the genomic era. *Philosophical Transactions of the Royal Society B-Biological Sciences,* 361**,** 1929 - 1940.

KONSTANTINIDIS, K. T. & TIEDJE, J. M. 2005. Genomic insights that advance the species definition for prokaryotes. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 2567 - 2572.

KOOB, J., BREITBACH, A., HUTZLER, M. & JACOB, F. 2015. Molekularbiologischer Nachweis bierschädigender Organismen im Format eines Schwangerschaftstests. *Der Weihenstephaner,* 04/2015**,** 156 - 159.

KOOB, J., JACOB, F., GRAMMER, M., RIEDL, R. & HUTZLER, M. 2014. PCR-Analysen bierschädlicher Bakterien 2012 und 2013. *Brauwelt,* 10**,** 288 - 290.

KOOB, J., JACOB, F., METHNER, F.-J. & HUTZLER, M. 2016. Lactobacillus sp. brewery isolate: A new threat to the brewing industry? *Brewing Science,* 69**,** 42 - 49.

KOOB, J., JACOB, F., WENNING, M. & HUTZLER, M. 2017. Lactobacillus cerevisiae sp. nov., isolated from a spoiled brewery sample. *International Journal of Systematic and Evolutionary Microbiology,* 67**,** 3452 - 3457.

KOORT, J., VANDAMME, P., SCHILLINGER, U., HOLZAPFEL, W. & BJORKROTH, J. 2004. Lactobacillus curvatus subsp melibiosus is a later synonym of Lactobacillus sakei subsp carnosus. *International Journal of Systematic and Evolutionary Microbiology,* 54**,** 1621 - 1626.

KOZULIS, J. A. & PAGE, H. E. 1968. A new universal beer agar medium for the enumeration of wort and beer microorganisms. *Proceedings of the American Society of Brewing Chemists***,** 52 - 58.

KRUSKA, L. & SCHNEEGANS, S. 2010. Identifizierung von Mikroorganismen jenseits der klassischen Mikrobiologie. *Brauwelt,* 31 - 32**,** 946 - 947.

KUBOTA, H., SENDA, S., NOMURA, N., TOKUDA, H. & UCHIYAMA, H. 2008. Biofilm formation by lactic acid bacteria and resistance to environmental stress. *Journal of Bioscience and Bioengineering,* 106**,** 381 - 386.

KUHLE, A. V. & JESPERSEN, L. 1998. Detection and identification of wild yeasts in lager breweries. *International Journal of Food Microbiology,* 43**,** 205 - 213.

LANTHOEN, N. C. & INGLEDEW, W. M. 1996. Heat resistance of bacteria in alcohol-free beer. *Journal of the American Society of Brewing Chemists,* 54**,** 32 - 36.

LAWRENCE, D. R. 1988. Spoilage organisms in beer. *In:* ROBINSON, R. K. (ed.) *Developments in Food Microbiology.* London: Elsevier

LAY, J. O. 2001. MALDI-TOF mass spectrometry of bacteria. *Mass Spectrometry Reviews,* 20**,** 172 - 194.

LEE, S. Y. 1994. SMMP - a medium for selective isolation of Megasphaera and Pectinatus from the brewery. *Journal of the American Society of Brewing Chemists,* 52**,** 115 - 119.

LEE, S. Y., MABEE, M. S. & JANGAARD, N. O. 1978. Pectinatus, a new genus of family Bacteriodaceae. *International Journal of Systematic Bacteriology,* 28**,** 582 - 594.

LEE, S. Y., MABEE, M. S., JANGAARD, N. O. & HORIUCHI, E. K. 1980. Pectinatus, a new genus of bacteria capable of growth in hopped beer. *Journal of the Institute of Brewing,* 86**,** 28 - 30.

LEE, S. Y., MOORE, S. E. & MABEE, M. S. 1981. Selective differential medium for isolation and differentation of Pectinatus from other brewery microorganisms. *Applied and Environmental Microbiology,* 41**,** 386 - 387.

LEISNER, J. J., VANCANNEYT, M., GORIS, J., CHRISTENSEN, H. & RUSUL, G. 2000. Description of Paralactobacillus selangorensis gen. nov., sp nov., a new lactic acid bacterium isolated from chili bo, a Malaysian food ingredient. *International Journal of Systematic and Evolutionary Microbiology,* 50**,** 19 - 24.

LEISTNER, L. 2000. Basic aspects of food preservation by hurdle technology. *International Journal of Food Microbiology,* 55**,** 181 - 186.

LINDNER, P. 1909. *Mikroskopische Betriebskontrolle in den Gärungsgewerben,* Berlin, Paul Parey.

LIU, J. Y., DENG, Y., LI, L., LI, B., LI, Y. Y., ZHOU, S. S., SHIRTLIFF, M. E., XU, Z. B. & PETERS, B. M. 2018. Discovery and control of culturable and viable but non-culturable cells of a distinctive Lactobacillus harbinensis strain from spoiled beer. *Scientific Reports,* 8.

LIU, J. Y., LI, L., LI, B., PETERS, B. M., DENG, Y., XU, Z. B. & SHIRTLIFF, M. E. 2017a. First study on the formation and resuscitation of viable but nonculturable state and beer spoilage capability of Lactobacillus lindneri. *Microbial Pathogenesis,* 107**,** 219 - 224.

LIU, J. Y., LI, L., LI, B., PETERS, B. M., DENG, Y., XU, Z. B. & SHIRTLIFF, M. E. 2017b. Study on spoilage capability and VBNC state formation and recovery of Lactobacillus plantarum. *Microbial Pathogenesis,* 110**,** 257 - 261.

LIU, J. Y., LI, L., PETERS, B. M., LI, B., CHEN, L. Q., DENG, Y., XU, Z. B. & SHIRTLIFF, M. E. 2017c. The viable but nonculturable state induction and genomic analyses of Lactobacillus casei BM-LC14617, a beer-spoilage bacterium. *Microbiologyopen,* 6.

LIU, W. T., MARSH, T. L., CHENG, H. & FORNEY, L. J. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology,* 63**,** 4516 - 4522.

LOPEZ, I., RUIZ-LARREA, F., COCOLIN, L., ORR, E., PHISTER, T., MARSHALL, M., VANDERGHEYNST, J. & MILLS, D. A. 2003. Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology,* 69**,** 6801 - 6807.

LOUWS, F. J., BELL, J., MEDINA-MORA, C. M., SMART, C. D., OPGENORTH, D., ISHIMARU, C. A., HAUSBECK, M. K., DE BRUIJN, F. J. & FULBRIGHT, D. W. 1998. rep-PCR-mediated genomic fingerprinting: A rapid and effective method to identify Clavibacter michiganensis. *Phytopathology,* 88**,** 862 - 868.

LOWE, D. P. & ARENDT, E. K. 2004. The use and effects of lactic acid bacteria in malting and brewing with their relationships to antifungal activity, mycotoxins and gushing: A review. *Journal of the Institute of Brewing,* 110**,** 163 - 180.

LOWE, D. P., ULMER, H. M., VAN SINDEREN, D. & ARENDT, E. K. 2004. Application of biological acidification to improve the quality and processability of wort produced from 50% raw barley. *Journal of the Institute of Brewing,* 110**,** 133 - 140.

LUDWIG, W. & SCHLEIFER, K. H. 1994. Bacterial phylogeny based on 16S and 23S ribosomal RNA sequence analysis. *Fems Microbiology Reviews,* 15**,** 155 - 173.

LUPSKI, J. R. & WEINSTOCK, G. M. 1992. Short, interspersed repetitive DNA sequences in prokaryotic genomes. *Journal of Bacteriology,* 174**,** 4525 - 4529.

MANZANO, M., GIUSTO, C., BARTOLOMEOLI, I., BUIATTI, S. & COMI, G. 2005. Microbiological analyses of dry and slurry yeasts for brewing. *Journal of the Institute of Brewing,* 111**,** 203 - 208.

MARCH, C., MANCLUS, J. J., ABAD, A., NAVARRO, A. & MONTOYA, A. 2005. Rapid detection and counting of viable beer-spoilage lactic acid bacteria using a monoclonal chemiluminescence enzyme immunoassay and a CCD camera. *Journal of Immunological Methods,* 303**,** 92 - 104.

MARCHANDIN, H., TEYSSIER, C., CAMPOS, J., JEAN-PIERRE, H., ROGER, F., GAY, B., CARLIER, J. P. & JUMAS-BILAK, E. 2010. Negativicoccus succinicivorans gen. nov., sp nov., isolated from human clinical samples, emended description of the family Veillonellaceae and description of Negativicutes classis nov., Selenomonadales ord. nov and Acidaminococcaceae fam. nov in the bacterial phylum Firmicutes. *International Journal of Systematic and Evolutionary Microbiology,* 60**,** 1271 - 1279.

MATOULKOVA, D., KOSAR, K. & SIGLER, K. 2012a. Rapid, simple, and specific cultivation-based method for detection of Pectinatus spp. in brewery samples. *Journal of the American Society of Brewing Chemists,* 70**,** 29 - 34.

MATOULKOVA, D., KOSAR, K., SLABY, M. & SIGLER, K. 2012b. Occurrence and species distribution of strictly anaerobic bacterium Pectinatus in brewery bottling halls. *Journal of the American Society of Brewing Chemists,* 70**,** 262 - 267.

MATOULKOVA, D. & KUBIZNIAKOVA, P. 2018. Brewing microbiology - Kocuria (Micrococcus) and cultivation methods for their detection - part 1. *Kvasny Prumysl,* 64**,** 10 - 13.

MATSUZUWA, K., KIRCHNER, G. & WACKERBAUER, K. 1979. Vergleichende Untersuchungen mit bekannten Nährböden zum Nachweis bierschädlicher Milchsäurebakterien. *Monatsschrift für Brauwissenschaft,* 321**,** 321 - 318.

MATTARELLI, P., HOLZAPFEL, W., FRANZ, C. M. A. P., ENDO, A., FELIS, G. E., HAMMES, W., POT, B., DICKS, L. & DELLAGLIO, F. 2014. Recommended minimal standards for description of new taxa of the genera Bifidobacterium, Lactobacillus and related genera. *International Journal of Systematic and Evolutionary Microbiology,* 64**,** 1434 - 1451.

MCCAIG, R., WEAVER, R.L. 1983. Physiological Studies on Pediococcus. *Master Brewers Association of the Americas Technical Quarterly,* 20**,** 31 - 46.

MCKAY, L. L. & BALDWIN, K. A. 1990. Applications for biotechnology - present and future improvements in lactic acid bacteria. *Fems Microbiology Letters,* 87**,** 3 - 14.

MEMBRÉ, J. M., THOLOZAN, J. L., DELATTRE, G., EULALIE, B. & ALBAGNAC, G. 1994. Volatile fatty acid production during beer spoilage by Pectinatus sp. *Food Quality and Preference,* 5**,** 25 - 29.

MENZ, G., ALDRED, P. & VRIESEKOOP, F. 2011. Growth and survival of foodborne pathogens in beer. *Journal of Food Protection,* 74**,** 1670 - 1675.

MENZ, G., ANDRIGHETTO, C., LOMBARDI, A., CORICH, V., ALDRED, P. & VRIESEKOOP, F. 2010. Isolation, identification, and characterisation of beer-spoilage lactic acid bacteria from microbrewed beer from Victoria, Australia. *Journal of the Institute of Brewing,* 116**,** 14 - 22.

MENZ, G. & VRIESEKOOP, F. 2009. Pathogens in beer. *In:* PREEDY, V. R. & WATSON, R. R. (eds.) *Beer in health and disease prevention.* Amsterdam: Elsevier Academic Press.

MIYAMOTO, M., SETO, Y., HAO, D. H., TESHIMA, T., SUN, Y. B., KABUKI, T., YAO, L. B. & NAKAJIMA, H. 2005. Lactobacillus harbinensis sp nov., consisted of strains isolated from traditional fermented vegetables 'Suan cai' in Harbin, Northeastern China and Lactobacillus perolens DSM 12745. *Systematic and Applied Microbiology,* 28**,** 688 - 694.

MOHANIA, D., NAGPAL, R., KUMAR, M., BHARDWAJ, A., YADAV, M., JAIN, S., MAROTTA, F., SINGH, V., PARKASH, O. & YADAV, H. 2008. Molecular approaches for identification and characterization of lactic acid bacteria. *Journal of Digestive Diseases,* 9**,** 190 - 198.

MOORE, W. B. & RAINBOW, C. 1955. Nutritional requirements and biochemical activities of brewery Lactobacilli. *Journal of General Microbiology,* 13**,** 190 - 197.

MOTOYAMA, Y., OGATA, T. & SAKAI, K. 1998. Characterization of Pectinatus cerevisiiphilus and P. frisingensis by ribotyping. *Journal of the American Society of Brewing Chemists,* 56**,** 19 - 23.

MULLER-AUFFERMANN, K., GRIJALVA, F., JACOB, F. & HUTZLER, M. 2015a. Nisin-producing microorganisms and their implementation in brewers' wort. *Journal of the Institute of Brewing,* 121**,** 320 - 331.

MULLER-AUFFERMANN, K., GRIJALVA, F., JACOB, F. & HUTZLER, M. 2015b. Nisin and its usage in breweries: a review and discussion. *Journal of the Institute of Brewing,* 121**,** 309 - 319.

MUNFORD, A. R. G., ALVARENGA, V. O., DO PRADO-SILVA, L., CRUCELLO, A., CAMPAGNOLLO, F. B., CHAVES, R. D., OTEIZA, J. M. & SANT'ANA, A. S. 2017. Sporeforming bacteria in beer: Occurrence, diversity, presence of hop resistance genes and fate in alcohol-free and lager beers. *Food Control,* 81**,** 126 - 136.

NAKAGAWA, A. 1978. Beer spoilage lactic acid bacteria. Prinicipal characteristics and simple methods of their selective detection. *Bulletin of Brewing Science,* 24**,** 1 - 10.

NAKAKITA, Y., MAEBA, H. & TAKASHIO, M. 2003. Grouping of Lactobacillus brevis strains using the gyrB gene. *Journal of the American Society of Brewing Chemists,* 61**,** 157 - 160.

NASER, S. M., DAWYNDT, P., HOSTE, B., GEVERS, D., VANDEMEULEBROECKE, K., CLEENWERCK, I., VANCANNEYT, M. & SWINGS, J. 2007. Identification of lactobacilli by pheS and rpoA gene sequence analyses. *International Journal of Systematic and Evolutionary Microbiology,* 57**,** 2777 - 2789.

NISHIKAWA, N., KOHGO, M. 1985. Microbial control in the brewery. *Master Brewers Association of the Americas Technical Quarterly,* 22**,** 61 - 66.

NOVY, R., TIPPMANN, J. & BECKER, T. 2013. Marktanalyse: Mikrobiologische Qualitätssicherung. *Brauwelt,* 20**,** 582 - 585.

O'SULLIVAN, T. F., WALSH, Y., O'MAHONY, A., FITZGERALD, G. F. & VAN SINDEREN, D. 1999. A comparative study of malthouse and brewhouse microflora. *Journal of the Institute of Brewing,* 105**,** 55 - 61.

OGDEN, K. 1986. Nisin - a bacteriocin with a potential use in brewing. *Journal of the Institute of Brewing,* 92**,** 379 - 383.

OGDEN, K., WAITES, M. J. & HAMMOND, J. R. M. 1988. Nisin and brewing. *Journal of the Institute of Brewing,* 94**,** 233 - 238.

OLIVER, J. D. 2005. The viable but nonculturable state in bacteria. *Journal of Microbiology,* 43**,** 93 - 100.

ORLA-JENSEN, S. 1916. *Milchwirtschaftliches Zentralblatt,* 9**,** 129.

ORLA-JENSEN, S. 1919. *The lactic acid bacteria,* Copenhagen, Host and son.

PARADH, A. D. 2015. Gram-negative spoilage bacteria in brewing. *In:* HILL, A. E. (ed.) *Brewing Microbiology - Managing Microbes, Ensuring Quality and Valorising Waste.* Heidelberg: Woodhead Publishing.

PARADH, A. D., MITCHELL, W. J. & HILL, A. E. 2011. Occurrence of Pectinatus and Megasphaera in the major UK breweries. *Journal of the Institute of Brewing,* 117**,** 498 - 506.

PASTEUR, L. 1876. Recherche des causes des maladies de la bière et de celles du moût qui sert à la produire. *In:* GAUTHIER-VILLARS (ed.) *Etudes sur la bière, ses maladies, causes qui les provoquent, Procèdè pour la rendre inàlterable, avec une thèorie nouvelle de la fermentation.* Paris.

PETTERS, H. I., FLANNIGAN, B. & AUSTIN, B. 1988. Quantitative and qualitative studies of the microflora of barley malt production. *Journal of Applied Bacteriology,* 65**,** 279 - 297.

PITTET, V., MORROW, K. & ZIOLA, B. 2011. Ethanol tolerance of lactic acid bacteria, including relevance of the exopolysaccharide gene gtf. *Journal of the American Society of Brewing Chemists,* 69**,** 57 - 61.

PITTET, V., PHISTER, T. G. & ZIOLA, B. 2013. Transcriptome sequence and plasmid copy number analysis of the brewery Iisolate Pediococcus claussenii ATCC BAA-344(T) during growth in beer. *Plos One,* 8.

PLENGVIDHYA, V., BREIDT, F., JR., LU, Z. & FLEMING, H. P. 2007. DNA fingerpnntmg of lactic acid bacteria in sauerkraut fermentations. *Applied and Environmental Microbiology,* 73**,** 7697 - 7702.

PREISSLER, P. 2011. *Categorization of Lactobacillus brevis along their beer-spoiling potential.* PhD, Technische Universität München.

PRIEST, F. G. 2003. Chapter 5: Gram-positive brewery bacteria. *In:* PRIEST, F. G., CAMPBELL, I. (ed.) *Brewing Microbiology.* New York: Kluwer Academic/Plenum Publishers.

PRIEST, F. G., COWBOURN.MA & HOUGH, J. S. 1974. Wort enterobacteria - a review. *Journal of the Institute of Brewing,* 80**,** 342 - 356.

PRIEST, F. G. & HOUGH, J. S. 1974. Influence of Hafnia protea (Obesumbacterium proteus) on beer flavor. *Journal of the Institute of Brewing,* 80**,** 370 - 376.

QIAN, F. 2009. Einfluss der Bierzusammensetzung auf die mikrobiologische Stabilität von Reisbier. *42. Technologisches Seminar.* Weihenstephan.

QUAIN, D. E. 2015. Assuring the microbiological quality of draught beer. *In:* HILL, A. E. (ed.) *Brewing Microbiology - Managing Microbes, Ensuring Quality and Valorising Waste.* Heidelberg: Woodhead Publishing.

RAINBOW, C. 1973. Beer spoilage lactic acid bacteria. *In:* CARR, J. G., CUTTING, C.V., WHITING, G.C. (ed.) *Lactic acid bacteria in beverages and food.* New York: Academic Press.

RAINBOW, C. 1981. Beer spoilage microorganisms. *In:* POLLOCK, J. R. A. (ed.) *Brewing Science.* New York: Academic Press.

RICHTER, M. & ROSSELLO-MORA, R. 2009. Shifting the genomic gold standard for the prokaryotic species definition. *Proceedings of the National Academy of Sciences of the United States of America,* 106**,** 19126 - 19131.

RIEDL, R., DÜNZER, N., MICHEL, M., JACOB, F. & HUTZLER, M. 2019. Beer enemy number one: genetic diversity, physiology and biofilm formation of Lactobacillus brevis. *Journal of the Institute of Brewing*.

RIEDL, R., GODERBAUER, P., BRANDL, A., JACOB, F. & HUTZLER, M. 2017. Bavarian wheat beer, an example of a special microbe habitat - cultivation, detection, biofilm formation,

characterization of selected lactic acid cacteria hygiene indicators and spoilers. *Brewing Science,* 70**,** 39 - 50.

RÖCKEN, W. & SCHULTE, S. 1986. Nachweis von Fremdhefen. *Brauwelt,* 41**,** 1921 - 1927.

ROGOSA, M. 1974. *In:* BUCHANAN, R. E. & GIBBONS, N. E. (eds.) *Bergey's Manual of Determinative Bacteriology.* 8th ed. Baltimore: Williams & Wilkins.

ROGOSA, M. & SHARPE, M. E. 1959. An approach to the classification of the lactobacilli. *Journal of Applied Bacteriology,* 12**,** 329 ff.

ROSS, R. P., MORGAN, S. & HILL, C. 2002. Preservation and fermentation: past, present and future. *International Journal of Food Microbiology,* 79**,** 3 - 16.

ROSSELLO-MORA, R. 2006. DNA-DNA reassociation methods applied in microbial taxonomy and their critical evaluation. *In:* STACKEBRANDT, E. (ed.) *Molecular identification, systematics and population structure of prokaryotes.* Berlin: Springer.

ROUSE, S., SUN, F., VAUGHAN, A. & VAN SINDEREN, D. 2007. High-throughput isolation of bacteriocin-producing lactic acid bacteria, with potential application in the brewing industry. *Journal of the Institute of Brewing,* 113**,** 256 - 262.

RUSSELL, C. & WALKER, T. K. 1953a. Lactobacillus malefermentans n.sp. isolated from beer. *Journal of General Microbiology,* 8**,** 160 - 162.

RUSSELL, C. & WALKER, T. K. 1953b. Lactobacillus parvus n.sp. isolated from beer. *Journal of General Microbiology,* 8**,** 310 - 313.

SAKAGUCHI, K. 1960. Vitamin and amino acid requirements of Pediococcus soyae and Pediococcus acidilactici Kitahara strain. 9. Studies on the activities of bacteria in soy sauce brewing. *Bulletin of the Agricultural Chemical Society of Japan,* 24**,** 638 - 643.

SAKAMOTO, K. & KONINGS, W. N. 2003. Beer spoilage bacteria and hop resistance. *International Journal of Food Microbiology,* 89**,** 105 - 124.

SAKAMOTO, K., MARGOLLES, A., VAN VEEN, H. W. & KONINGS, W. N. 2001. Hop resistance in the beer spoilage bacterium Lactobacillus brevis is mediated by the ATP-binding cassette multidrug transporter HorA. *Journal of Bacteriology,* 183**,** 5371 - 5375.

SALVETTI, E., HARRIS, H. M. B., FELIS, G. E. & O'TOOLE, P. W. 2018. Comparative genomics of the genus Lactobacillus reveals robust phylogroups that provide the basis for reclassification. *Applied and Environmental Microbiology,* 84.

SALVETTI, E., TORRIANI, S. & FELIS, G. E. 2012. The genus Lactobacillus: A taxonomic update. *Probiotics and Antimicrobial Proteins,* 4**,** 217 - 226.

SANDERS, J. W., LEENHOUTS, K. J., HAANDRIKMAN, A. J., VENEMA, G. & KOK, J. 1995. Stress response in Lactococcus lactis - cloning, expression analysis, and mutation of the lactococcal superoxide dismutase gene. *Journal of Bacteriology,* 177**,** 5254 - 5260.

SANDERS, J. W., OOMES, S., MEMBRE, J. M., WEGKAMP, A. & WELS, M. 2015. Biodiversity of spoilage lactobacilli: Phenotypic characterisation. *Food Microbiology,* 45**,** 34 - 44.

SANDRIN, T. R., GOLDSTEIN, J. E. & SCHUMAKER, S. 2013. MALDI TOF MS profiling of bacteria at the strain level: A review. *Mass Spectrometry Reviews,* 32**,** 188 - 217.

SANTOS, S. R. & OCHMAN, H. 2004. Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. *Environmental Microbiology,* 6**,** 754 - 759.

SCHEIRLINCK, I., VAN DER MEULEN, R., VAN SCHOOR, A., VANCANNEYT, M., DE VUYST, L., VANDAMME, P. & HUYS, G. 2007. Influence of geographical origin and flour type on diversity of lactic acid bacteria in traditional Belgian sourdoughs. *Applied and Environmental Microbiology,* 73**,** 6262 - 6269.

SCHEIRLINCK, I., VAN DER MEULEN, R., VAN SCHOOR, A., VANCANNEYT, M., DE VUYST, L., VANDAMME, P. & HUYS, G. 2008. Taxonomic structure and stability of the bacterial community in Belgian sourdough ecosystems as assessed by culture and population fingerprinting. *Applied and Environmental Microbiology,* 74**,** 2414 - 2423.

SCHEIRLINCK, I., VAN DER MEULEN, R., VRANCKEN, G., DE VUYST, L., SETTANNI, L., VANDAMME, P. & HUYS, G. 2009. Polyphasic taxonomic characterization of Lactobacillus rossiae isolates from

Belgian and Italian sourdoughs reveals intraspecific heterogeneity. *Systematic and Applied Microbiology,* 32**,** 151 - 156.

SCHLEIFER, K. H. 2009a. Classification of bacteria and archaea: Past, present and future. *Systematic and Applied Microbiology,* 32**,** 533 - 542.

SCHLEIFER, K. H. 2009b. Family V *Leuconostocaceae* fam. nov. *In:* DE VOS, P., GARRITY, G. M., JONES, D., KRIEG, N. R., LUDWIG, W., RAINEY, F. A., SCHLEIFER, K. H. & WHITMAN, W. B. (eds.) *Bergey's manual of systematic bacteriology.* Berlin: Springer

SCHLEIFER, K. H. & KANDLER, O. 1972. Peptidoglycan types of bacterial cell-walls and their taxonomic implications. *Bacteriological Reviews,* 36**,** 407 - 477.

SCHLEIFER, K. H., KILPPERBALZ, R., KRAUS, J., LUDWIG, W., DVORAK, C. & STACKEBRANDT, E. 1986. Classification of streptococci. *Zentralblatt Fur Bakteriologie Mikrobiologie Und Hygiene Series a-Medical Microbiology Infectious Diseases Virology Parasitology,* 262**,** 275 - 275.

SCHLEIFER, K. H., LEUTERITZ, M., WEISS, N., LUDWIG, W., KIRCHHOF, G. & SEIDELRUFER, H. 1990. Taxonomic study of anaerobic, gram-negative, rod-shaped bacteria from breweries - emended description of Pectinatus cerevisiiphilus and description of Pectinatus frisingensis sp. nov., Selenomonas lacticifex sp. nov.. Zymophilus raffinosivorans gen. nov., and Zymophilus paucivorans sp. nov. *International Journal of Systematic Bacteriology,* 40**,** 19 - 27.

SCHLEIFER, K. H. & LUDWIG, W. 1996. Phylogeny of the genus Lactobacillus and related genera. *Systematic and Applied Microbiology,* 18**,** 461 - 467.

SCHNEIDERBANGER, J., JACOB, F. & HUTZLER, M. 2019. Genotypic and phenotypic diversity of Lactobacillus rossiae isolated from beer. *Journal of Applied Microbiology,* 126**,** 1187 - 1197.

SCHURR, B. C., HAHNE, H., KUSTER, B., BEHR, J. & VOGEL, R. F. 2015. Molecular mechanisms behind the antimicrobial activity of hop iso-alpha-acids in Lactobacillus brevis. *Food Microbiology,* 46**,** 553 - 563.

SCOLARI, G. & VESCOVO, M. 2004. Use of X-prolyl-dipeptidyl aminopeptidase electrophoretic mobility as a tool to differentiate Lactobacillus species of dairy relevance. *Annals of Microbiology,* 54**,** 529 - 542.

SEIDEL-RÜFER, H. 1990. Pectinatus and other morphologically simiar Gram-negative anaerobic bacilli from brewing. *Monatsschrift für Brauwissenschaft,* 43**,** 101 - 105.

SEIDEL, H., BACK, W., WEIß, N. 1979. Isolierung und systematische Zuordnung bierschädlicher gramnegativer Bakterien - III.: Welche Gefahr stellen die in den beiden vorausgegangenen Mitteilungen gramnegativen Kokken und Stäbchen für das Bier dar? *Monatsschrift für Brauwissenschaft,* 32**,** 262 - 270.

SHARPE, M. E. 1959. A note on the source and identification of the strains of heterofermentative Lactobaccilli submitted to chromatographic analysis. *Journal of Applied Bacteriology,* 22**,** 294 - 304.

SHIMWELL, J. L. 1935. The resistance of beer towards Saccharobacillus pastorianus. *Journal of the Institute of Brewing,* 41**,** 245 - 258.

SHIMWELL, J. L. 1945. *Wallerstein Laboratories Communication,* 8**,** 23.

SHIMWELL, J. L. 1948. A rational nomenclature for the brewery lactic acid bacteria. *Journal of the Institute of Brewing,* 54**,** 100 - 104.

SHIMWELL, J. L. & KIRKPATRICK, W. F. 1939. A new light on the Sarcina question. *Journal of the Institute of Brewing,* 45**,** 137 - 145.

SIEGRIST, J., KOHLSTOCK, M., MERX, K. & VETTER, K. 2015. Rapid detection and identification of spoilage bacteria in beer. *In:* HILL, A. E. (ed.) *Brewing Microbiology - Managing Microbes, Ensuring Quality and Valorising Waste.* Heidelberg: Woodhead Publishing.

SIEZEN, R. J., TZENEVA, V. A., CASTIONI, A., WELS, M., PHAN, H. T. K., RADEMAKER, J. L. W., STARRENBURG, M. J. C., KLEEREBEZEM, M., MOLENAAR, D. & VLIEG, J. 2010. Phenotypic and genomic diversity of Lactobacillus plantarum strains isolated from various environmental niches. *Environmental Microbiology,* 12**,** 758 - 773.

SIEZEN, R. J. & VLIEG, J. 2011. Genomic diversity and versatility of Lactobacillus plantarum, a natural metabolic engineer. *Microbial Cell Factories,* 10.

SILVEIRA, M. G., BAUMGARTNER, M., ROMBOUTS, F. M. & ABEE, T. 2004. Effect of adaptation to ethanol on cytoplasmic, and membrane protein profiles of Oenococcus oeni. *Applied and Environmental Microbiology,* 70**,** 2748 - 2755.

SIMPSON, W. J. 1993a. Ionophoric action of trans-isohumulone on Lactobacillus brevis. *Journal of General Microbiology,* 139**,** 1041 - 1045.

SIMPSON, W. J. 1993b. Studies on the sensitivity of lactic acid bacteria to hop bitter acids. *Journal of the Institute of Brewing,* 99**,** 405 - 411.

SIMPSON, W. J. & FERNANDEZ, J. L. 1992. Selection of beer-spoilage lactic acid bacteria and induction of their ability to grow in beer. *Letters in Applied Microbiology,* 14**,** 13 - 16.

SIMPSON, W. J. & FERNANDEZ, J. L. 1994. Mechanism of resistance of lactic acid bacteria to trans-isohumulone. *Journal of the American Society of Brewing Chemists,* 52**,** 9 - 11.

SIMPSON, W. J. & HAMMOND, J. R. M. 1991. *Antibacterial action of hop resin materials,* Oxford, IRL Press.

SKYTTA, E., HAIKARA, A. & MATTILASANDHOLM, T. 1993. Production and characterization of antibacterial compounds produced by Pediococcus damnosus and Pediococcus pentosaceus. *Journal of Applied Bacteriology,* 74**,** 134 - 142.

SMITH, M. R., VANDERSCHAAF, A., DEREE, E. M., DEBONT, J. A. M. & HUGENHOLTZ, J. 1993. The physiology of Lactococcus lactis subsp. lactis biovar diacetylactis immobilized in hollow-fiber bioreactors - glucose and citrate metabolism at high cell densities. *Applied Microbiology and Biotechnology,* 39**,** 94 - 98.

SOBERKA, R., SCIAZKO, D. & WARZECHA, A. 1988. Pectinatus: nouvelle bactérie pouvant affecter la stabilité biologique du moût et de la bière. *Bios,* 19**,** 31 - 37.

STACKEBRANDT, E. & EBERS, J. 2006. Taxonomic parameters revisited: tarnished gold standards. *Microbiology today,* 33**,** 152 - 155.

STACKEBRANDT, E., FREDERIKSEN, W., GARRITY, G. M., GRIMONT, P. A. D., KAMPFER, P., MAIDEN, M. C. J., NESME, X., ROSSELLO-MORA, R., SWINGS, J., TRUPER, H. G., VAUTERIN, L., WARD, A. C. & WHITMAN, W. B. 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology,* 52**,** 1043 - 1047.

STACKEBRANDT, E. & GOEBEL, B. M. 1994. A place for DNA-DNA reassociation and 16S ribosomal RNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology,* 44**,** 846 - 849.

STACKEBRANDT, E., KOCH, C., GVOZDIAK, O. & SCHUMANN, P. 1995. Taxonomic dissection of the genus Micrococcus - Kocuria gen. nov., Nesterenkonia gen. nov., Kytococcus gen. nov., Dermacoccus gen. nov., and Micrococcus Cohn 1872 gen. emend. *International Journal of Systematic Bacteriology,* 45**,** 682 - 692.

STACKEBRANDT, E., LUDWIG, W., SEEWALDT, E. & SCHLEIFER, K. H. 1983. Phylogeny of sporeforming members of the order Actinomycetales. *International Journal of Systematic Bacteriology,* 33**,** 173 - 180.

STACKEBRANDT, E., POHLA, H., KROPPENSTEDT, R., HIPPE, H. & WOESE, C. R. 1985. 16S ribosomal RNA analysis of Sporomusa, Selenomonas, and Megasphaera - on the phylogenetic origin of gram-positive eubacteria. *Archives of Microbiology,* 143**,** 270 - 276.

STORGARDS, E. 2000. *Process hygiene control in beer produkction and dispensing.* PhD, VTT Tecnical Research Center of Finland.

STORGARDS, E., HAIKARA, A. & JUVONEN, R. 2006. *Brewing control systems: microbiological analysis,* Abington, UK, Woodhead Publishing Limited.

STORGARDS, E., PIHLAJAMAKI, O. & HAIKARA, A. 1997. Biofilms in the brewing process: A new approach to hygiene management. *Journal of the Institute of Brewing,* 103**,** 157 ff.

STORGARDS, E., SUIHKO, M. L., POT, B., VANHONACKER, K., JANSSENS, D., BROOMFIELD, P. L. E. & BANKS, J. G. 1998. Detection and identification of Lactobacillus lindneri from brewery environments. *Journal of the Institute of Brewing,* 104**,** 47 - 54.

STORGARDS, E., TAPANI, K., HARTWALL, P., SALEVA, R. & SUIHKO, M. L. 2006b. Microbial attachment and biofilm formation in brewery bottling plants. *Journal of the American Society of Brewing Chemists,* 64**,** 8 - 15.

STRACHOTTA, T. 2003. Mikrobiologische Qualitätssicherung im Brauereibetrieb. Wichtige Gesichtspunkte. *Brauwelt,* 143**,** 1020 - 1022.

SUIHKO, M., STORGARDS, E. & HAIKARA, A. A fingerprint database for characterisation and identification of microbial contaminants. Proc. Eur. Brew. Conv. Congress, 2003 Dublin. Fachverlag Hans Carl.

SUIHKO, M. L. & HAIKARA, A. 1990. Maintenance of the anaerobic beer spoilage bacteria Pectinatus and Megasphaera. *Food Microbiology (London),* 7**,** 33 - 42.

SUIHKO, M. L. & HAIKARA, A. 2001. Characterization of Pectinatus and Megasphaera strains by automated ribotyping. *Journal of the Institute of Brewing,* 107**,** 175 - 184.

SUZUKI, K. 2009. Beer spoilage lactic acid bacteria. *In:* PREEDY, V. R. & WATSON, R. R. (eds.) *Beer in health and desease prevention.* Amsterdam: Elsevier Academic Press.

SUZUKI, K. 2011. 125th Anniversary Review: Microbiological Instability of Beer Caused by Spoilage Bacteria. *Journal of the Institute of Brewing,* 117**,** 131 - 155.

SUZUKI, K. 2011b. Recent developments of the studies for spoilage lactic acid bacteria in alcoholic beverages. *Biol. Chem.,* 49**,** 209 - 223.

SUZUKI, K. 2015. Gram-positive spoilage bacteria in brewing. *In:* HILL, A. E. (ed.) *Brewing Microbiology - Managing Microbes, Ensuring Quality and Valorising Waste.* Heidelberg: Woodhead Publishing.

SUZUKI, K., ASANO, S., IIJIMA, K. & KITAMOTO, K. 2008a. Sake and beer spoilage lactic acid bacteria - a review. *Journal of the Institute of Brewing,* 114**,** 209 - 223.

SUZUKI, K., ASANO, S., IIJIMA, K., KURIYAMA, H. & KITAGAWA, Y. 2008b. Development of detection medium for hard-to-culture beer-spoilage lactic acid bacteria. *Journal of Applied Microbiology,* 104**,** 1458 - 1470.

SUZUKI, K., FUNAHASHI, W., KOYANAGI, M. & YAMASHITA, H. 2004a. Lactobacillus paracollinoides sp nov., isolated from brewery environments. *International Journal of Systematic and Evolutionary Microbiology,* 54**,** 115 - 117.

SUZUKI, K., IIJIMA, K., ASANO, S., KURIYAMA, H. & KITAGAWA, Y. 2006a. Induction of viable but nonculturable state in beer spoilage lactic acid bacteria. *Journal of the Institute of Brewing,* 112**,** 295 - 301.

SUZUKI, K., IIJIMA, K., OZAKI, K. & YAMASHITA, H. 2005a. Isolation of a hop-sensitive variant of Lactobacillus lindneri and identification of genetic markers for beer spoilage ability of lactic acid bacteria. *Applied and Environmental Microbiology,* 71**,** 5089 - 5097.

SUZUKI, K., IIJIMA, K., OZAKI, K. & YAMASHITA, H. 2005b. Study on ATP production of lactic acid bacteria in beer and development of a rapid pre-screening method for beer-spoilage bacteria. *Journal of the Institute of Brewing,* 111**,** 328 - 335.

SUZUKI, K., IIJIMA, K., SAKAMOTO, K., SAMI, M. & YAMASHITA, H. 2006b. A review of hop resistance in beer spoilage lactic acid bacteria. *Journal of the Institute of Brewing,* 112**,** 173 - 191.

SUZUKI, K., KOYANAGI, M. & YAMASHITA, H. 2004b. Isolation of hop-sensitive variants from beer-spoilage Lactobacillus brevis strains. *Journal of the American Society of Brewing Chemists,* 62**,** 71 - 74.

SUZUKI, K., OZAKI, K. & YAMASHITA, H. 2004c. Genetic marker for differentiating beer-spoilage ability of Lactobacillus paracollinoides strains. *Journal of Applied Microbiology,* 97**,** 712 - 718.

TAGUCHI, H., PHKOCHI, M., UEHARA, H., KOJIMA, K. & MAWATARI, M. 1990. KOT medium, a new medium for the detection of beer spoilage bacteria *Journal of the American Society of Brewing Chemists,* 48**,** 72 - 75.

TAKAHASHI, T., NAKAKITA, Y., SUGIYAMA, H., SHIGYO, T. & SHINOTSUKA, K. 1999. Classification and identification of strains of Lactobacillus brevis based on electrophoretic characterization of D-Lactate dehydrogenase: Relationship between D-Lactate dehydrogenase and beer-spoilage ability. *Journal of Bioscience and Bioengineering,* 88**,** 500 - 506.

TAMAOKA, J. 1994. Determination of DNA base composition. *In:* GOODFELLOW, M. & O'DONNELL, A. G. (eds.) *Chemical Methods in prokaryotic Systematics.* Chichester: John Qiley and Sons.

TANIGAWA, K. & WATANABE, K. 2011. Multilocus sequence typing reveals a novel subspeciation of Lactobacillus delbrueckii. *Microbiology-Sgm,* 157**,** 727 - 738.

TASKILA, S., KRONLOF, J. & OJAMO, H. 2011. Enrichment cultivation of beer-spoiling lactic scid bacteria. *Journal of the Institute of Brewing,* 117**,** 285 - 294.

TASKILA, S., TUOMOLA, M., KRONLOF, J. & NEUBAUER, P. 2010. Comparison of enrichment media for routine detection of beer spoiling lactic acid cacteria and development of trouble-shooting medium for Lactobacillus backi. *Journal of the Institute of Brewing,* 116**,** 151 - 156.

TEICHERT, H. 2009. *Behaviour of membrane transport proteins under high hydrostatic pressure.* PhD, Technische Universität München.

TEMMERMAN, R., HUYS, G. & SWINGS, J. 2004. Identification of lactic acid bacteria: culture-dependent and culture-independent methods. *Trends in Food Science & Technology,* 15**,** 348 - 359.

TEUBER, M., MEILE, L. & SCHWARZ, F. 1999. Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology,* 76**,** 115 - 137.

TIMKE, M., WANG-LIEU, N. Q., ALTENDORF, K. & LIPSKI, A. 2005. Community structure and diversity of biofilms from a beer bottling plant as revealed using 16S rRNA gene clone libraries. *Applied and Environmental Microbiology,* 71**,** 6446 - 6452.

TINDALL, B. J., ROSSELLO-MORA, R., BUSSE, H. J., LUDWIG, W. & KAEMPFER, P. 2010. Notes on the characterization of prokaryote strains for taxonomic purposes. *International Journal of Systematic and Evolutionary Microbiology,* 60**,** 249 - 266.

TOHNO, M., KITAHARA, M., IRISAWA, T., MASUDA, T., UEGAKI, R., OHKUMA, M. & TAJIMA, K. 2013. Description of Lactobacillus iwatensis sp nov., isolated from orchardgrass (Dactylis glomerata L) silage, and Lactobacillus backii sp nov. *International Journal of Systematic and Evolutionary Microbiology,* 63**,** 3854 - 3860.

TORRIANI, S., VANREENEN, C. A., KLEIN, G., REUTER, G., DELLAGLIO, F. & DICKS, L. M. T. 1996. Lactobacillus curvatus subsp. curvatus subsp. nov. and Lactobacillus curvatus subsp. melibiosus subsp. nov. and Lactobacillus sake subsp. sake subsp. nov. and Lactobacillus sake subsp. carnosus subsp. nov., new subspecies of Lactobacillus curvatus Abo-Elnaga and Kandler 1965 and Lactobacillus sake Katagiri, Kitahara, and Fukami 1934 (Klein et al 1996, emended descriptions), respectively. *International Journal of Systematic Bacteriology,* 46**,** 1158 - 1163.

TSUCHIYA, Y., KANO, Y. & KOSHINO, S. 1994. Identification of lactic acid bacteria using temperature-gradient gel-electrophoresis for DNA fragments amplified by polymerase chain reaction. *Journal of the American Society of Brewing Chemists,* 52**,** 95 - 99.

UCHIDA, K. 1974. Lipids of alcoholophilic Lactobacilli. 2. Occurrence of polar lipids with unusually long chains in Lactobacillus heterohiochii. *Biochimica Et Biophysica Acta,* 369**,** 146 - 155.

UEKI, A., WATANABE, M., OHTAKI, Y., KAKU, N. & UEKI, K. 2014. Description of Propionispira arcuata sp. nov., isolated from a methanogenic reactor of cattle waste, reclassification of Zymophilus raffinosivorans and Zymophilus paucivorans as Propionispira raffinosivorans comb. nov. and Propionispira paucivorans comb. nov. and emended description of the genus Propionispira. *International Journal of Systematic and Evolutionary Microbiology,* 64**,** 3571 - 3577.

VAN BELKUM, A. 2007. Tracing isolates of bacterial species by multilocus variable number of tandem repeat analysis (MLVA). *Fems Immunology and Medical Microbiology,* 49**,** 22 - 27.

VAN DE GUCHTE, M., PENAUD, S., GRIMALDI, C., BARBE, V., BRYSON, K., NICOLAS, P., ROBERT, C., OZTAS, S., MANGENOT, S., COULOUX, A., LOUX, V., DERVYN, R., BOSSY, R., BOLOTIN, A., BATTO, J. M., WALUNAS, T., GIBRAT, J. F., BESSIERES, P., WEISSENBACH, J., EHRLICH, S. D. & MAGUIN, E. 2006. The complete genome sequence of Lactobacillus bulgaricus reveals extensive and ongoing reductive evolution. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 9274 - 9279.

VAN DE GUCHTE, M., SERROR, P., CHERVAUX, C., SMOKVINA, T., EHRLICH, S. D. & MAGUIN, E. 2002. Stress responses in lactic acid bacteria. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology,* 82**,** 187 - 216.

VAN LEAR, H. 1892. Contributions à l'histoire des ferments des hydrates de carbone. *Acad. Roy. Belg. Cl. Sci. Collect. Octavo. Mem.***,** 1 - 37.

VAN VUUREN, H. J. J. & PRIEST, F. G. 2003. Chapter 6: Gram-negative brewery bacteria. *Brewing Microbiology.* New York: Kluwer Academic/Plenum Publishers.

VANDAMME, P., POT, B., GILLIS, M., DEVOS, P., KERSTERS, K. & SWINGS, J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiological Reviews,* 60**,** 407 - 438.

VAUGHAN, A., O'SULLIVAN, T. & VAN SINDEREN, D. 2005. Enhancing the microbiological stability of malt and beer - A review. *Journal of the Institute of Brewing,* 111**,** 355 - 371.

VENTURA, M., CASAS, I. A., MORELLI, L. & CALLEGARI, M. L. 2000. Rapid amplified ribosomal DNA restriction analysis (ARDRA) identification of Lactobacillus spp. isolated from fecal and vaginal samples. *Systematic and Applied Microbiology,* 23**,** 504 - 509.

VEREECKE, C. & ARAHAL, D. R. 2008. The status of the species Pectinatus portalensis Gonzalez et al. 2005. Request for an opinion. *International Journal of Systematic and Evolutionary Microbiology,* 58**,** 1507 - 1507.

VERSALOVIC, J., DEBRUIJN, F. J. & LUPSKI, J. R. 1998. Repetitive sequence-based PCR (rep-PCR) DNA fingerprinting of bacterial genomes. *In:* DEBRUIJN, F. J., LUPSKI, J. R. & WEINSTOCK, G. M. (eds.) *Bacterial Genomes.*

VERSALOVIC, J., KOEUTH, T. & LUPSKI, J. R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingeprinting of bacterial genomes. *Nucleic Acids Research,* 19**,** 6823 - 6831.

VERSALOVIC, J., SCHNEIDER, M., DE BRUIJN, F. J. & LUPSKI, J. R. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology,* 5**,** 25 - 40.

VIZOSO PINTO, M. G., PASTERIS, S. E. & STRASSER DE SAAD, A. M. 2004. Glycerol catabolism by Pediococcus pentosaceus isolated from beer. *Food Microbiology,* 21**,** 111 - 118.

VOETZ, M., PAHL, R. & FOLZ, R. 2010. Der etwas andere Pectinatus. *Brauwelt* 150**,** 261 - 263.

VOGEL, R. F., PREISSLER, P., BEHR, J. 2010. Towards an understanding of hop tolerance in beer spoiling Lactobacillus brevis. *Brewing Science,* 63**,** 23 - 30.

VOS, P., HOGERS, R., BLEEKER, M., REIJANS, M., VANDELEE, T., HORNES, M., FRIJTERS, A., POT, J., PELEMAN, J., KUIPER, M. & ZABEAU, M. 1995. AFLP - a new technique for DNA-fingerprinting. *Nucleic Acids Research,* 23**,** 4407 - 4414.

VRIESEKOOP, F., KRAHL, M., HUCKER, B. & MENZ, G. 2012. 125th anniversary review: Bacteria in brewing: The good, the bad and the ugly. *Journal of the Institute of Brewing,* 118**,** 335 - 345.

WACKERBAUER, K. & EMEIS, C. C. 1969. Über die bierschädlichen Bakterien der Gattung Lactobacillus (Biermilchsäurestäbchen). *Monatsschrift für Brauwissenschaft,* 22**,** 3 - 8.

WACKERBAUER, K. & METHNER, F. J. 1988. The microorganisms of "Berliner Weissbier" and their influence on the beer flavour. *EBC Microbiology Group Meeting.* Wuppertal, Germany.

WARD, L. J. H. & TIMMINS, M. J. 1999. Differentiation of Lactobacillus casei, Lactobacillus paracasei and Lactobacillus rhamnosus by polymerase chain reaction. *Letters in Applied Microbiology,* 29**,** 90 - 92.

WATIER, D., LEGUERINEL, I., HORNEZ, J. P., CHOWDHURY, I. & DUBOURGUIER, H. C. 1995. Heat-resistance of Pectinatus sp., a beer spoilage anaerobic bacterium. *Journal of Applied Bacteriology,* 78**,** 164 - 168.

WAYNE, L. G., BRENNER, D. J., COLWELL, R. R., GRIMONT, P. A. D., KANDLER, O., KRICHEVSKY, M. I., MOORE, L. H., MOORE, W. E. C., MURRAY, R. G. E., STACKEBRANDT, E., STARR, M. P. & TRUPER, H. G. 1987. Report of the ad-hoc-committee on reconciliation of approaches to bacterial systematics. *International Journal of Systematic Bacteriology,* 37**,** 463 - 464.

WEIß, N., SEIDEL, H., BACK, W. 1979. Isolierung und systematische Zuordnung bierschädlicher gramnegativer Bakterien - I.: Gramnegative strikt anaerobe Kokken. *Brauwissenschaft,* 32**,** 179 - 194.

WELKER, M. & MOORE, E. R. B. 2011. Applications of whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry in systematic microbiology. *Systematic and Applied Microbiology,* 34**,** 2 - 11.

WELSH, J. & MCCLELLAND, M. 1990. Fingerprinting genomes using PCR with arbitraty primers. *Nucleic Acids Research,* 18**,** 7213 - 7218.

WENNING, M., BREITENWIESER, F., KONRAD, R., HUBER, I., BUSCH, U. & SCHERER, S. 2014. Identification and differentiation of food-related bacteria: A comparison of FTIR spectroscopy and MALDI-TOF mass spectrometry. *Journal of Microbiological Methods,* 103**,** 44 - 52.

WENNING, M. & SCHERER, S. 2013. Identification of microorganisms by FTIR spectroscopy: perspectives and limitations of the method. *Applied Microbiology and Biotechnology,* 97**,** 7111 - 7120.

WERNING, M. L., IBARBURU, I., DUENAS, M. T., IRASTORZA, A., NAVAS, J. & LOPEZ, P. 2006. Pediococcus parvulus gtf gene encoding the GTF glycosyltransferase and its application for specific PCR detection of β-D-Glucan-producing bacteria in foods and beverages. *Journal of Food Protection,* 69**,** 161 - 169.

WHITE, D., DRUMMOND, J. & FUQUA, C. 2012. Chapter 17: Solute transport. *In:* WHITE, D., DRUMMOND, J. & FUQUA, C. (eds.) *The physiology and biochemistry of prokaryotes.* New York: Oxford University Press.

WIEME, A. D., SPITAELS, F., AERTS, M., DE BRUYNE, K., VAN LANDSCHOOT, A. & VANDAMME, P. 2014. Identification of beer-spoilage bacteria using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *International Journal of Food Microbiology,* 185**,** 41 - 50.

WILLIAMS, J. G. K., KUBELIK, A. R., LIVAK, K. J., RAFALSKI, J. A. & TINGEY, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research,* 18**,** 6531 - 6535.

WOESE, C. R. 1987. Bacterial evolution. *Microbiological Reviews,* 51**,** 221 - 271.

WOOD, B. J. B. & HOLZAPFEL, W. (eds.) 1995. *The lactic acid bacteria,* London: Blackie Academic & Professional.

WRAY, E. 2015. Reducing microbial spoilage of beer using pasteurisation. *In:* HILL, A. E. (ed.) *Brewing Microbiology - Managing Microbes, Ensuring Quality and Valorising Waste.* Heidelberg: Woodhead Publishing.

XU, H. S., ROBERTS, N., SINGLETON, F. L., ATTWELL, R. W., GRIMES, D. J. & COLWELL, R. R. 1982. Survival and viability of nonculturable Escherichia coli and Vibrio cholerae in the estuarine and marine environment. *Microbial Ecology,* 8**,** 313 - 323.

YAMADA, Y., HOSHINO, K. & ISHIKAWA, T. 1997. Taxonomic studies of acetic acid bacteria and allied organisms .11. The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: The elevation of the subgenus Gluconoacetobacter to the generic level. *Bioscience Biotechnology and Biochemistry,* 61**,** 1244 - 1251.

YASUI, T., OKAMOTO, T. & TAGUCHI, H. 1997. A specific oligonucleotide primer for the rapid detection of Lactobacillus lindneri by polymerase chain reaction. *Canadian Journal of Microbiology,* 43**,** 157 - 163.

YASUI, T. & YODA, K. 1997. Purification and partial characterization of an antigen specific to Lactobacillus brevis strains with beer spoilage activity. *Fems Microbiology Letters,* 151**,** 169 - 176.

ZHAO, Y., KNOCHEL, S. & SIEGUMFELDT, H. 2017. Heterogeneity between and within strains of Lactobacillus brevis exposed to beer compounds. *Frontiers in Microbiology,* 8.

ZHENG, J. S., RUAN, L. F., SUN, M. & GANZLE, M. 2015. A genomic ciew of Lactobacilli and Pediococci demonstrates that phylogeny matches ecology and physiology. *Applied and Environmental Microbiology,* 81**,** 7233 - 7243.

# 6. APPENDIX

## 6.1 Peer-reviewed publications

SCHNEIDERBANGER, J., JACOB, F., HUTZLER, M. 2019. Genotypic and phenotypic diversity of *Lactobacillus rossiae* isolated from beer. *Journal of Applied Microbiology*, 126, 1187-1197.

SCHNEIDERBANGER, J., GRAMMER, M., JACOB, F. & HUTZLER, M. 2018. Statistical evaluation of beer spoilage bacteria by real-time PCR analyses from 2010 to 2016. *Journal of the Institute of Brewing*, 124 (2), 173-181.

SCHNEIDERBANGER, J., SCHNEIDERBANGER, H., JACOB, F. & HUTZLER, M. 2017. Enhanced cultivation of beer spoilage bacteria in propagation yeast by enforced yeast suppression. *Brewing Science*, 70 (7/8), 142-147.

KOOB, J., JACOB, F., WENNING, M. & HUTZLER M. 2017. *Lactobacillus cerevisiae* sp. nov., isolated from a spoiled brewery sample. *International Journal of Systematic and Evolutionary Microbiology*, 76, 3452-3457.

KOOB, J., JACOB, F., METHNER, F.-J. & HUTZLER M. 2016. *Lactobacillus* sp. brewery isolate: A new threat to the brewing industry? *Brewing Science*, 69 (7/8), 42-49.

SCHNEIDERBANGER, H., KOOB, J., POLTINGER, S., JACOB, F. & HUTZLER M. 2016. Gene expression in wheat beer yeast strains and the synthesis of acetate esters. *Journal of the Institute of Brewing*, 122 (3), 403-411.

HUTZLER, M., RIEDL, R., KOOB, J. & JACOB, F. 2012. Fermentation and spoilage yeasts and their relevance for the beverage industry – A Review. *Brewing Science*, 65/66 (3/4), 33-52.

## 6.2 Non-reviewed publications

SCHAPPALS, L., KOOB, J. & JACOB, F. 2016. Physiologische Charakterisierung ausgewählter bierschädlicher Bakterien anhand ihrer Alkoholtoleranz. *Brauwelt*, 9-10, 262-265.

BREITBACH, A., JACOB, F., HUTZLER, M. & KOOB, J. 2015. Einfacher Schnelltest zum Nachweis bierschädigender Organismen. *Brauwelt*, 33, 961-966.

KOOB, J., BREITBACH, A., HUTZLER, M. & JACOB, F. 2015. Molekularbiologischer Nachweis bierschädigender Organismen im Format eines Schwangerschaftstests. *Der Weihenstephaner*, 4, 155-159.

KOOB, J., MARQUARDT, P., TAUPP, M., JACOB, F. & HUTZLER, M. 2015. A different kind of product challenge test. *Brauwelt international*, III, 164-167.

KOOB, J., MARQUARDT, P., TAUPP, M., JACOB, F. & HUTZLER, M. 2015. Produktschädlichkeitstest mal anders. *Brauwelt*, 04/05, 100-103.

KOOB, J., JACOB, F., GRAMMER, M., KLEUCKER, A., RIEDL, R. & HUTZLER, M. 2014. PCR-Analysen bierschädlicher Bakterien 2012 und 2013. *Brauwelt*, 10, 288-290.

HUTZLER, M., MUELLER-AUFFERMANN, K., KOOB, J., RIEDL, R. & JACOB, F. 2013. Beer spoiling microorganisms – a current overview. *Brauwelt en Espagnol*, I, 2-4.

HUTZLER, M., MUELLER-AUFFERMANN, K., KOOB, J., RIEDL, R. & JACOB, F. 2013. Beer spoiling microorganisms – a current overview. *Brauwelt International*, I, 23-25.

HUTZLER, M., MUELLER-AUFFERMANN, K., KOOB, J., RIEDL, R. & JACOB, F. 2013. Bierschädliche Mikroorganismen – eine aktuelle Übersicht. *Brauwelt*, 3, 58-60.

RIEDL, R., KOOB, J., HUTZLER, M., HACKL, C. & JACOB, F. 2012. Mikrobiologisch sensible Getränke - Ein Risikobewertungssystem. *Brauwelt*, 27-28, 787-792.

HUTZLER, M., KOOB, J., RIEDL, R., PETERS, W. & JACOB, F. 2012. Sauberkeit kontrollieren – Hygienemonitoring in Fruchtsaftbetrieben. *Getränkeindustrie*, 5, 12-15.

HUTZLER, M., KOOB, J., GRAMMER, M., RIEDL, R. & JACOB, F. 2012. Statistische Auswertung der PCR-Analysen bierschädlicher Bakterien in den Jahren 2010 und 2011. *Brauwelt*, 18-19, 546-547.

## 6.3 Posters and oral presentations

Oral presentations

KOOB, J. 2016. Charakterisierung eines neues Bierschädlings. *4. Seminar Hefe und Mikrobiologie*, Weihenstephan, 15-16 März 2016.

RIEDL, R., KOOB, J., JACOB, F. & HUTZLER M. 2016. Genetic strain differentiation of *Lactobacillus brevis* – know the number one enemy. *Young Scientist Symposium*, Chico (USA), 21-23 April 2016.

KOOB, J., JACOB, F., DOSTATNI, R., BREITBACH, A. & HUTZLER, M. 2015. A new approach to bacterial identification in a point-of-care format. *EBC Congress*, Porto, 24-28 Mai 2015.

DOSTATNI, R., BREITBACH, A. & KOOB, J. 2015. „Schwangerschaftstest“ zum Nachweis von Bierschädlingen – Evaluierungsphase. *3. Seminar Hefe und Mikrobiologie*, Weihenstephan 25-26 März 2015.

KOOB, J. 2015. PCR-Schnellsystem zur Detektion und Identifikation bierschädlicher Mikroorganismen im Streifen-Format. 10. Weihenstephaner Praxisseminar,

HUTZLER, M., RIEDL, R. & KOOB, J. 2014. Brauereimikrobiologie 2013 – aktuelle Forschungsprojekte und Praxiserfahrungen. *47. Technologisches Seminar*, Freising-Weihenstephan, 18-20 Februar 2014.

KOOB, J. 2014. Bierschädliche Mikroorganismen – Übersicht und Aktuelles (+ Mikroskopische Bilder). *2. Seminar Hefe und Mikrobiologie*, Weihenstephan, 3-4 April 2014.

KOOB, J. 2014. *L. brevis* Fingerprint – ein probates Werkzeug. *2. Seminar Hefe und Mikrobiologie*, Weihenstephan, 3-4 April 2014.

KOOB, J. 2013. Differenzierungsmöglichkeit von *Lactobacillus brevis*. *8. Weihenstephaner Praxisseminar*, Teisendorf, 24-25 Oktober 2013.

HUTZLER, M., RIEDL, R. & KOOB, J. 2013. Brauereimikrobiom – Brauereimikrobiologie der nächsten Generation. *8. Weihenstephaner Praxisseminar*, Teisendorf, 24-25 Oktober 2013.

HUTZLER, M., KOOB, J. & RIEDL, R. 2013. Biofilm-Hygiene-Schnellmonitoring. *46. Technologisches Seminar*, Freising-Weihenstephan, 19-21 Februar 2013.

KOOB, J., RIEDL, R. & HUTZLER, M. 2013. Brauereikeimflora 2012 – Umfassende Statistik und Fallbeispiele. *46. Technologisches Seminar*, Freising-Weihenstephan, 19-21 Februar 2013.

RIEDL, R., KOOB, J. & HUTZLER, M. 2013. Optimierte mikrobiologische Weizenbier-Qualitätssicherung. *46. Technologisches Seminar*, Freising-Weihenstephan, 19-21 Februar 2013.

HUTZLER, M., RIEDL, R., KOOB, J. & JACOB, F. 2013. Evaluation of biofilm composition and maturation using Real-time-PCR. *EBC Congress*, Luxemburg, 26-30 Mai 2013.

RIEDL, R., HUTZLER, M., KOOB, J. & JACOB, F. 2012. Quantitative evaluation of biofilm composition using Real-time-PCR. *World Brewing Congress*, Portland, 28 July - 01 August 2012.

KOOB, J. 2012. Brauereikeimflora 2012 – Umfassende Statistik und Fallbeispiele. *1. Seminar Hefe und Mikrobiologie*, Weihenstephan, 28. Februar-1. März 2012

HUTZLER, M., RIEDL, R. & KOOB, J. 2012. Mikroorganismenbedingte Aroma- und Geschmacksbeeinflussung bei der Produktion und im fertigen Bier. *7. Weihenstephaner Praxisseminar*, Schaffhausen, 18-19 Oktober 2012.

KOOB, J. & RIEDL, R. 2012. Beschreibung von Geruchs- und Geschmacksfehlern - Praxisbeispiele mit Verkostung. *7. Weihenstephaner Praxisseminar*, Schaffhausen, 18-19 Oktober 2012.

HUTZLER, M., RIEDL, R. & KOOB, J. 2012. Sinnvoller Einsatz moderner Analysenmethoden zur Untersuchung spezifischer brauereimikrobiologischer Problemstellungen. *45. Technologisches Seminar*, Freising-Weihenstephan, 14-16 Februar 2012.

Posters

HUTZLER, M., KOOB, J.,RIEDL, R. & JACOB, F. 2015. DNA fingerprinting of beer spoiler No. 1 (L. brevis) - tracing & troubleshooting tool. *EBC Congress*, Porto, 24-28 Mai 2015.

MICHEL, M., KOOB, J., JACOB, F. & HUTZLER M. 2014. Investigation of the thermo tolerance of three different beer spoiling bacteria. *4th International Young Scientists Symposium on Malting, Brewing, and Distilling*, Ghent, 28-30 Oktober 2014.

KOOB, J., JACOB, F., DOSTATNI, R., BREITBACH, A. & HUTZLER M. 2014. A new approach to bacterial identification in a point-of-care format. *4th International Young Scientists Symposium on Malting, Brewing, and Distilling*, Ghent, 28-30 Oktober 2014.

HUTZLER, M., KOOB, J., RIEDL, R. & JACOB, F. 2014. Classification, identification, and detection of beer spoiling microorganisms - a review. *MBAA District Ontario Technical Conference*, 100th District anniversary (Master Brewers' Association of Canada), Toronto, 30-31 Januar 2014.

HUTZLER, M., KOOB, J., RIEDL, R. & JACOB, F. 2013. Classification, identification, and detection of beer spoiling microorganisms - A review. *40th Annual 2013 M. B. A. C.* (Master Brewers' Association of Canada) *Ontario Technical Conference*, Toronto, 25 Januar 2013.

HUTZLER, M., KOOB, J., RIEDL, R. & JACOB, F. 2012. Classification, identification, and detection of beer spoiling microorganisms - a review. *World Brewing Congress*, Portland, 28 Juli - 01 August 2012.

KOOB, J., HUTZLER, M., RIEDL, R., COELHAN, M. & JACOB, F. 2012. Indirect detection of microbial contamination in beer by chemical fingerprints. *World Brewing Congress*, Portland, 28 Juli - 01 August 2012.

## 6.4 Book chapter and awards

Book chapter

HUTZLER, M., KOOB, J., RIEDL, R., SCHNEIDERBANGER, H., MÜLLER-AUFFERMANN, K. & JACOB, F. 2015. Yeast identification and characterization (Chapter 6) in *Brewing Microbiology - Managing Microbes, Ensuring Quality and Valorising Waste*. Editor Hill, A. E., Woodhead Publishing, London, ISBN 9781782423317

Awards

Heinrich Funke Pschorr Award of the German brewing industry 2015: Jennifer Koob (PhD project)

Dr. Nienaber Student Award 2011 Jennifer Koob (Master project)

Dr. Nienaber Student Award 2014 Lisa Maria Iwo (Master project, supervising)

## 6.5 Publisher permissions to reproduce publications

Koob, J.; Jacob, F.; Methner, F.-J.; Hutzler, M. (2016): Lactobacillus sp. brewery isolate: A new threat to the brewing industry? Brewing Science, Vol. 69, pp. 42 – 49

DOI: 10.23763/BRSC16-09KOOB

---

Dear Ms Schneiderbanger, dear Mr Hutzler, we hereby grant the permission to include the following publications in your theses:

**J. Koob, F. Jacob, F.-J. Methner and M. Hutzler:**
**Lactobacillus sp. brewery isolate: A new threat to the brewing industry?**
**BrewingScience July/August 2016 (Vol. 69), 42 – 49**

**J. Schneiderbanger (former Koob), H. Schneiderbanger, F. Jacob and M. Hutzler:**
**Enhanced Cultivation of Beer Spoilage Bacteria in Propagation Yeast by Enforced Yeast Suppression**
**BrewingScience September/October 2017 (Vol. 70), 142 - 147**

Yours sincerely

i. V. Sabine Raab

Assistant to the manager

Fachverlag Hans Carl GmbH
Geschäftsführer: Michael Schmitt
Registergericht: HRB 17388
USt-Id.-Nr.: DE211630158

Andernacher Str. 33a
D-90411 Nürnberg
Tel. ++49 (0)911 95285-20
Fax ++ 49 (0)911 95285-71
mailto:raab@hanscarl.com

---

Koob, J.; Jacob, F.; Wenning, M.; Hutzler, M. (2017): Lactobacillus cerevisiae sp. nov., isolated from a spoiled brewery sample. International Journal of Systematic and Evolutionary Microbiology, Vol. 67, pp. 3452 – 3457

DOI: 10.1099/ijsem.0.002139

---

Dear Jennifer,

Thank you for your permission request. The Microbiology Society no longer responds to individual permission requests; these are now handled by the Copyright Clearance Center.

If you are an author (or an author's representative) of the content for which you are requesting permission:
The copyright transfer agreement/licence to publish that was signed by the authors gives express permission for authors to reuse their own content, provided that the journal is acknowledged as the original source. No further permission is required.

If another publisher requires confirmation that permission has been granted, please forward this email to them.
If you are NOT an author of the content for which you are requesting permission:

Please click on the '© Get Permissions' link that appears with the online article. Alternatively, you can visit www.copyright.com.

Please note that the Copyright Clearance Center will charge a processing fee for each request. Additional charges may apply for reuse for "for-profit" purposes.

For manuscripts published with open access or beyond 12 months of publication, all of the content in the articles are published under an open access license called "CC-BY". This license allows you to download, reuse, reprint, modify, distribute, and/or copy articles or images. You must give appropriate credit, provide a link to the license, and indicate if changes were made. You may do so in any reasonable manner, but not in any way that suggests the licensor endorses you or your use.

Kind regards,

Wendy Tilley
Editorial Office Ltd
International Journal of Systematic and Evolutionary Microbiology

---

Schneiderbanger, J.; Schneiderbanger, H.; Jacob, F.; Hutzler, M. (2017): Enhanced cultivation of beer spoilage bacteria in propagation by enforced yeast suppression. Brewing Science, Vol. 70, pp. 142 – 147

DOI: 10.23763/BRSC17-14SCHNEIDERBANGER

---

Dear Ms Schneiderbanger, dear Mr Hutzler, we hereby grant the permission to include the following publications in your theses:

**J. Koob, F. Jacob, F.-J. Methner and M. Hutzler:**
**Lactobacillus sp. brewery isolate: A new threat to the brewing industry?**
**BrewingScience July/August 2016 (Vol. 69), 42 – 49**

**J. Schneiderbanger (former Koob), H. Schneiderbanger, F. Jacob and M. Hutzler:**
**Enhanced Cultivation of Beer Spoilage Bacteria in Propagation Yeast by Enforced Yeast Suppression**
**BrewingScience September/October 2017 (Vol. 70), 142 - 147**

Yours sincerely

i. V. Sabine Raab

Assistant to the manager

Fachverlag Hans Carl GmbH
Geschäftsführer: Michael Schmitt
Registergericht: HRB 17388
USt-Id.-Nr.: DE211630158

Andernacher Str. 33a
D-90411 Nürnberg
Tel. ++49 (0)911 95285-20
Fax ++ 49 (0)911 95285-71
mailto:raab@hanscarl.com

---

Schneiderbanger, J.; Grammer, M.; Jacob, F.; Hutzler, M. (2018): Statistical evaluation of beer spoilage bacteria by real-time PCR analyses from 2010 – 2016. Journal of the Institute of Brewing, Vol. 124, pp. 173 – 181

DOI: 10.1002/jib.486

**JOHN WILEY AND SONS LICENSE**
**TERMS AND CONDITIONS**

Feb 19, 2019

This Agreement between Mrs. Jennifer Schneiderbanger ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

| | |
|---|---|
| License Number | 4482530842159 |
| License date | Dec 05, 2018 |
| Licensed Content Publisher | John Wiley and Sons |
| Licensed Content Publication | Journal of the Institute of Brewing |
| Licensed Content Title | Statistical evaluation of beer spoilage bacteria by real-time PCR analyses from 2010 to 2016 |
| Licensed Content Author | Jennifer Schneiderbanger, Margit Grammer, Fritz Jacob, et al |
| Licensed Content Date | Mar 30, 2018 |
| Licensed Content Volume | 124 |
| Licensed Content Issue | 2 |
| Licensed Content Pages | 9 |
| Type of use | Dissertation/Thesis |
| Requestor type | Author of this Wiley article |
| Format | Print and electronic |
| Portion | Full article |
| Will you be translating? | No |
| Order reference number | 20181205 |
| Title of your thesis / dissertation | Occurrence, Detection, Characterization and Description of Selected Beer Spoilage Lactic Acid Bacteria |
| Expected completion date | Feb 2019 |
| Expected size (number of pages) | 95 |
| Requestor Location | Mrs. Jennifer Schneiderbanger<br>Alte Akademie 3<br><br>Freising, Bayern 85354<br>Germany<br>Attn: Mrs. Jennifer Schneiderbanger |
| Publisher Tax ID | EU826007151 |
| Total | 0.00 EUR |

Schneiderbanger, J.; Jacob, F.; Hutzler, M. (2019): Genotypic and phenotypic diversity of *Lactobacillus rossiae* isolated from beer. Journal of Applied Microbiology, published online on January 13th 2019

DOI: 10.1111/jam.14202

**JOHN WILEY AND SONS LICENSE**
**TERMS AND CONDITIONS**

Feb 22, 2019

This Agreement between Mrs. Jennifer Schneiderbanger ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

| | |
|---|---|
| License Number | 4533670105876 |
| License date | Feb 21, 2019 |
| Licensed Content Publisher | John Wiley and Sons |
| Licensed Content Publication | Journal of Applied Microbiology |
| Licensed Content Title | Genotypic and phenotypic diversity of Lactobacillus rossiae isolated from beer |
| Licensed Content Author | J. Schneiderbanger, F. Jacob, M. Hutzler |
| Licensed Content Date | Feb 20, 2019 |
| Licensed Content Volume | 0 |
| Licensed Content Issue | 0 |
| Licensed Content Pages | 12 |
| Type of use | Dissertation/Thesis |
| Requestor type | Author of this Wiley article |
| Format | Print and electronic |
| Portion | Full article |
| Will you be translating? | No |
| Title of your thesis / dissertation | Occurrence, Detection, Characterization and Description of Selected Beer Spoilage Lactic Acid Bacteria |
| Expected completion date | Feb 2019 |
| Expected size (number of pages) | 95 |
| Requestor Location | Mrs. Jennifer Schneiderbanger<br>Alte Akademie 3<br><br>Freising, Bayern 85354<br>Germany<br>Attn: Mrs. Jennifer Schneiderbanger |
| Publisher Tax ID | EU826007151 |
| Total | 0.00 EUR |