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Categorization of *Lactobacillus brevis* along their beer-spoiling potential

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LIST OF ABBREVIATIONS

2D	two-dimensional
A	ampere
ADI	arginine deiminase
ATCC	American type culture collection, Manassas, Virginia, USA
ATP	adenosintriphosphate
BLAST	basic local alignment search tool
Da	Dalton
DDBJ	DNA Data Base of Japan
DW	Dry weight
DNA	desoxyribonucleic acid
DTT	dithiothreitol
E.	*Escherichia*
EDTA	ethylenediaminetetraacetic acid
g	g-force
h	hour
HI	hop stress inducible protein
HO	hop stress overexpressed protein
IEF	isoelectric focussing
IPG	immobilized pH gradient
l	liter
L.	*Lactobacillus*
LC-ESI MS/MS	liquid chromatography coupled to tandem mass spectrometry
m	milli (10^{-3}), meter
M	mega, molar

MALDI-TOF MS	Matrix-assisted laser desorption/ionization Time of Flight Mass Spectrometry
MIC	minimal inhibitory concentration
min	minute
NBB	Nachweismedium bierschädlicher Bakterien
Mw	molecular weight
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NCBI	National center for Biotechnology Information
NRAMP	natural resistance-associated macrophage protein
OD	optical density
O.	*Oenococcus*
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pI	isoelectric point
pmf	proton motive force
ppm	parts per million
RAPD	random amplified polymorphic DNA analysis
SDS	sodium n-dodecylsulfate
T	temperature, cycle time
TE	Tris, EDTA
TMW	Technische Mikrobiologie Weihenstephan
Tris	tris (hydroxymethyl) aminomethan
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
V	volt
v/v	volume / volume
w/v	mass / volume
w/w	mass / mass
µ	Micro

1 INTRODUCTION

1.1 Hop compounds and beer composite

The history of beer starts before approximately 5000–7000 years in Mesopotamia and Egypt, where production of beer is documented in reliefs and paintings (BEHRE, 1999). In contrast to wine, which is made from tasteful fruits, starch-containing materials like cereals are the starting point of beer production. However, cereals include less aroma components than fruits and flavouring agents have to be added to improve taste and beer quality (HELCK, 1971; BEHRE, 1999). Such agents were rosemary, anise, caraway, vermouth, but also toxic additives, such as the deadly nightshade and henbane. For this reason, Wilhelm IV introduced the German purity law for beer in 1516, which allows only water, barley and hop as ingredients for beer. The use of hops (*Humulus lupulus)* as an additive in beer is known since the 6th century BC. *Humulus lupulus* is a plant species within the genus of *Humulus* and belongs to the plant family of *Cannabaceae*. Abbess Hildegard noted the preservative qualities of hops in beverages in 1153 A.D. and described the antibiotic effects of hop, which prevents putrefaction by his bitterness (DELYSER and KASPER, 1994). The main component of hops, which is responsible for the bitterness, flavour and preservation effect in beer, is referred as to lupulin. The constituents of lupulin can be divided in hexane soluble soft resin (10-25 % of hop cones) and insoluble hard resin (3-5 %) (HOUGH et al., 1971). The most important component of the soft resin for brewers is the bitter substance humulone, which is converted to more water-soluble and bitter iso-α-acid during wort boiling (PALAMAND and ALDENHOFF, 1971). Furthermore, the complete process of brewage provokes the formation of a beverage. In addition to the described iso-α-acids, the presence of ethanol, low pH ranging from pH 3.8 – 4.7, the strongly reduced availability of nutrients and almost anaerobic conditions (oxygen content <0.1 ppm; high carbondioxide concentration ~ 0.5 % w/w) makes beer to an unfavorable medium for most bacteria (SAKAMOTO and KONINGS, 2003).

1.2 Hop inhibitory effects on bacteria

The antibiotic and bacteriostatic properties of hop components were investigated by several groups, which could identify effects on microorganisms at various cellular levels. SHIMWELL already recognized in 1937 that the ingredients of hops change the permeability of the cell membrane. TEUBER and SCHMALRECK (1973) studied the effects of hops on *Bacillus subtilis* and found that these cause a leak in the cell membrane. In addition, respiration and synthesis of DNA, RNA and protein is inhibited. Furthermore, SIMPSON (1993) discovered that leucine uptake was inhibited and previously enriched intracellular leucine was released. An important activity of hop compounds is their effect to act as proton ionophores, whereby intracellular pH is decreased and pmf-dependent nutrient uptake is hampered (SIMPSON 1993; SAKAMOTO and KONINGS 2003; YANSANJAV et al., 2004). BEHR and VOGEL (2009/2010) extended the mode of antibacterial action of hop compounds from the described proton ionophore activity, lowering the intracellular pH to pronounced redox reactivity in the presence of Mn^{2+}. In this connection the levels of intracellular Mn^{2+} apparently alter the redox properties of permeated hop compounds with regard to those that were extracellularly present, which results in trans-membrane redox reactions and concomitant increase of intracellular oxidative stress.

1.3 Beer-spoiling bacteria

The term „beer-spoiling bacteria" is not a taxonomic definition and comprises only of a few genera, which include Gram-positive and Gram-negative species (THELEN, 2009; HAIKARA et al., 1981; ENGELMANN and WEISS, 1985; BACK, 1981). The praxis shows, that more than 90 % of beer spoilage is caused by Gram-positive bacteria (BACK, 1994, THELEN et al., 2004 and 2006). Within the Gram-positive bacteria, *L. brevis*, *L. lindneri, L. backii*, *L. paracollinoides* and *P. damnosus* are recognized as the most potent beer-spoiling lactic acid species known in brewing microbiology (BOHAK et al., 2006; BACK et al., 1996; SUZUKI et al, 2004; SUZUKI et al., 2005; BACK, 1995; SUZUKI et al., 2008). Common for all beer-spoiling bacteria is their ability to grow in beer and changing this concerning appearance, organoleptic properties, taste and flavor (BACK, 2005). Thereby, Gram-positive bacteria cause spoilage due to formation of lactic- and

acetic acid and carbon dioxide, which decrease the pH value of beer. Furthermore, turbidity and formation of diacetyl could be observed (BACK, 1994). Interestingly, many beer-spoiling lactic acid bacteria prefer lower temperatures below 28ºC (BACK, 2005).

1.4 Sugar utilization of beer-spoiling lactobacilli

FERNANDEZ and SIMPSON (1993) reported that hop-sensitive and hop-resistant organisms could not be discriminated on the basis of their carbohydrate utilization patterns. They found out, that all tested strains utilized D-glucose and most utilized L-arabinose, ribose, D-xylose, galactose, D-fructose, N-acetylglucosamine, maltose, gluconate and 5-keto gluconate. Only a few utilized D-mannose, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, amygdalin, aesculin, salicin, cellobiose, melibiose, sucrose, trehalose, melezitose, beta-gentiobiose, D-turanose and D-tagatose (Fernandez, 1993). In contrast to the finding, that all tested strains in the study of FERNANDEZ and SIMPSON (1993) could utilize glucose, MOORE and RAINBOW (1955) reported, that only two of beer-spoiling strains utilized glucose for growth, whilst all strains grew well on L-arabinose, fructose or maltose and release glucose at the presence of maltose. This is due to the existence of maltosephosphorylase and might reflect an adaptation of these lactobacilli to their natural environment beer, which contains maltose as predominant carbon source (MOORE and RAINBOW, 1955; WOOD and RAINBOW, 1961). Depending on the beer type, maltose concentrations are in a range from 0.7 – 7.1 g/l (mean value 2.19 g/l), whereas glucose concentration varying between 0.1 – 12.1 g/l (mean value 1.38 g/l) (FERNANDEZ and SIMPSON, 1995). In most bacteria, glucose is consumed first followed by secondary sugar utilization (GÖRKE and STÜLKE, 2008). Only few bacteria have been reported to metabolize different sugars simultaneously. KIM et al. (2009) examined simultaneous carbohydrate utilization of glucose and a second carbohydrate in *L. brevis*. They confirmed that fructose, galactose, xylose, arabinose, ribose and maltose could be fermented simultaneously with glucose and concluded, that *L. brevis* possess a relaxed control of carbohydrate utilization.

1.5 Beer-spoiling *Lactobacillus brevis*

The most prominent beer-spoiling species, which is found in breweries and causes more than 50 % of spoilage cases, is *L. brevis* (BACK, 2004). In addition to beer isolates, *L. brevis* has been found in faeces, sourdough and fermented herbs. *L. brevis* is a rod-shaped, non-motile and Gram-positive bacterium. Thereby, two types concerning cell morphology can be differentiated. Type I forms long and slim rods (0.7 – 4 µm), whereas the cell shape of type II is shorter and plump (0.8 – 0.9 µm) (Figure 1). Common for both types is the occurrence of single cells or couples. The morphology of colonies can be described as partially round, non-transparent, glossy, smooth, convex, entire (type R) or matt and marmorate finish, flat, but in the center upraised, non-transparent until opaque, irregular lobed, precision toothed or rhizoid edge (type U) (BACK, 2004, Figure 1).

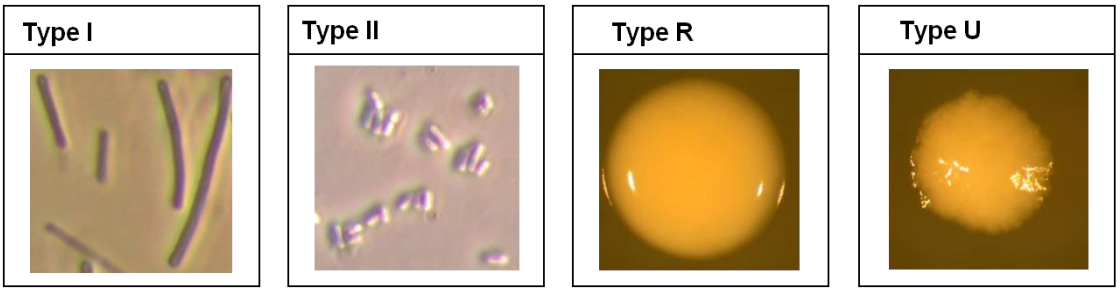


Figure 1. Different types of cell and colony morphology of *L. brevis*

L. brevis is obligatorily heterofermentative, ferments pentoses and most strains can cleave arginine. Hexoses are metabolized by the 6-phosphogluconate pathway, in which lactic acid, ethanol, acetic acid and carbon dioxide is produced (KANDLER, 1983). Furthermore, this species forms no spores and grows optimal at 30 °C in the range from pH 4 to 6. The beer-spoiling potential is depending on strain and origin (FERNANDEZ and SIMPSON, 1993; PREISSLER et al., 2010)

1.6 Definition of tolerance, adaptation and resistance

Several authors described hop compounds as effective inhibitors of Gram-positive bacteria (HAAS and BARSUMIAN, 1994; SCHMALRECK et al., 1975;

SHIMWELL. 1937) and hop extracts are known to have bacteriostatic and antibiotic effects (BHATTACHARYA et al, 2003). To survive these effects, mechanisms in tolerance or resistance are needed. Therefore, tolerance or intrinsic resistance means the absence of a bactericidal, but not a bacteriostatic effect. In presence of antibiotic agents, cells stop growing but can survive. These species-specific characteristics are inherited and should be present in the majority of the representatives of a species. Within one species, some strains possess increased or acquired resistance, which is strain-specific and beyond natural variability. Such acquired resistance can be also achieved by adaptation. Adaptation is a temporary effect and can be lost again. In contrast to that, resistance means the insensitivity against an antibiotic due to missing target structures and bacterial growth is unaffected (DIEHL et al, 2000, LEVIN, 2004, BALABAN, et al. 2004, MILLER et al. 2004).

1.7 General mechanisms of antibiotic tolerance and resistance

1.7.1 Limited intake

The effect of antibiotics can be limited by reduced intake. On the one hand, this can be caused by modification of cell wall (teichoic acids) or cell membrane (change in lipid composition) (BEHR et al., 2006). On the other hand, the formation of biofilms prevents the permeation of antibiotics into bacterial cells (STEWART and COSTERTON, 2001).

1.7.2 Inactivating mechanisms

The antimicrobial agent can be inactivated by chemical modification in its structure by intra- or extra-cellular detoxifying enzymes. One example is the inactivation of chloramphenicol by chloramphenicol acetyltransferase (SHAW et al., 1985).

1.7.3 Bypass mechanisms

In a bypass mechanism of tolerance, the effect of the antibiotic is invalid, because either an alternative pathway is taken, or even the inhibited enzymes

are replaced by new and more effective enzymes with the same function but lack of affinity for the antibiotic. An example is the resistance to trimethoprim by synthesis of an alternative, trimethoprim-insensitive dihydrofolate reductase enzyme (YOUNG et al., 1987).

1.7.4 Target modifications

Target modification results in a structural change in the target, which provokes a significantly reduced affinity for the antibiotic. Such target modification is described for the resistance to streptogramin type B (UCHIYAMA and WEISBLUM, 1985). In case of antibiotics, which access more than one target, resistance by target modification is rather infrequent (SPRATT, 1994).

1.7.5 Efflux mechanisms

The antimicrobial agent can be transported out of the cells by active transport efflux pumps. Two major classes of multidrug resistance transporters are known. The family of ABC transporter use ATP to extrude antibiotics whereas the secondary class intrudes H^+ to remove the drug from intracellular room (WEBBER and PIDDOCK, 2003; BAMBEKE et al, 2000).

1.7.6 Sequestration

In sequestration the antibiotic is specifically and stoichiometrically bound to a cellular protein, so that its antimicrobial action is blocked. For example, glycopeptides antibiotics can be inactivated by reduced activity peptidoglycan transpeptidase, thicker cell wall with more non-specific binding, increased rate of turnover of peptidoglycan in wall with more non-specific binding in growth media or overproduction and excretion of cell wall intermediates in medium, which immobilize glycopeptides (REYNOLD, 2002).

1.8 Mechanisms of hop tolerance in *Lactobacillus brevis*

L. brevis comprises hop-tolerant and hop sensitive strains (SUZUKI et al., 2006). Such intraspecies differences in hop tolerance could not be predicted based on differences in cell or colony morphology, pH range for growth, carbohydrate utilization profile, products of metabolism, manganese requirement, sensitivity

to superoxide radicals or expression of cellular proteins (FERNANDEZ and SIMPSON, 1993). BACK (1994) described most strains of *L. brevis* as obligate beer-spoiling bacteria, which is indicated by cell growth in beer without longer lag-phase. Rarely found are potential beer-spoiling strains, which can propagate only in some beers under special conditions (higher content of oxygen and nutrients, reduced alcohol concentration, higher storage temperature, BACK, 1994). Several groups investigated mechanisms, which confer hop tolerance to beer-spoiling bacteria. Thus, the cell wall of beer-spoiling lactic acid bacteria shows galactosylated glycerol teichoic acids, which inhibit the penetration of hop acids into the cell (YASUI, 1997). In hop- and acid stress adapted beer-spoiling *L. brevis* TMW 1.465, the LTA content in the cell wall was also increased (BEHR et al., 2006). Furthermore, in hop-tolerant strains an increased ATP pool and ATPase activity could be measured (OKAZAKI et al., 1997). A key role plays the gene *horA*, which is coding for a multidrug resistance transporter and is responsible for extrusion of hop acids under consumption of ATP. In strain ABBC45 the gene is located on the plasmid pRH45I (SAMI et al, 1998). Another transporter, HorC, presumably belonging to the resistance-nodulation-cell division superfamily is regulated by *horB* and seems to extrude also hop compounds (SUZUKI et al., 2005). In contrast to HorA, HorC is independent of ATP and uses proton motive force. The gene *horC* and *horB* is located on a second plasmid pRH45II also found in *L. brevis* (IIJIMA et al, 2006). Another transporter, only found in *L. brevis* is HitA. The role of HitA in hop resistance was suggested as divalent-cation transporters, which transport H^+ and Mn^{2+} (HAYASHI et al., 2001). In the case of trans-isohumulone, the target site is the cell membrane and under acid and hop stress condition, the membrane fluidity was reduced (BEHR et al., 2006; TEUBER and SCHMALRECK, 1973). Probably, a change in membrane lipid composition and fluidity lowers the permeability to hop compounds. Furthermore, hop stress resistance mechanisms imply mechanisms to cope with intracellular acidification and mechanisms for energy generation, economy, genetic information fidelity, and enzyme functionality (BEHR et al., 2007).

1.9 Effect of manganese in beer-spoiling lactobacilli

Manganese is an important trace metal, which is accumulated in high intracellular concentrations in lactobacilli and is required for growth and survival. In the cell, oxidation of Mn^{2+} ions can serve as scavenger of harmful oxygen species (ARCHIBALD and FRIDOVICH, 1981). Furthermore, many enzymes require manganese as co-factor for reactivity. For beer-spoiling bacteria the role of manganese for survival in beer is still unclear. In comparison with culture media like MRS, the levels of manganese in beer are limited and in the range of 31 - 182 µg/L (FERNANDEZ and SIMPSONS, 1995). It was shown that *L. brevis* requires manganese to grow in media supplemented with hop bitter compounds (HAYASHI et al., 2001). Furthermore, there is a weak relationship between susceptibility of a beer to spoil and manganese content of the beer. It seems that beer with low manganese is more resistant against spoilage, because the availability of this essential trace element limits growth of lactic acid bacteria (FERNANDEZ and SIMPSONS, 1995). Previous studies suggested that some hop bitter compounds (e.g. trans-isohumulone) trap protons from extracellular and exchange them for intracellular Mn^{2+} (SIMPSON, 1993). HitA, a protein, which confers tolerance to hop by the assumed transport of Mn^{2+} and H^{+} ions was only induced in the presence of iso-α-acids but not in manganese-free MRS broth (HAYASHI et al., 2001).

1.10 Detection and differentiation of beer-spoiling bacteria

In standard methods for detecting beer-spoiling bacteria, the growth of organisms or utilization of metabolic products was observed on selective media (JESPERSEN and JAKOBSEN, 1997). These methods are proven in practice and ensure detection of contamination in beer. Breweries use modified culture media like NBB (Nachweismedium für bierschädliche Bakterien), UBA (Universal beer agar) or MRS (de Man-Rogosa-Sharpe-medium) for isolating spoilage microorganisms (NISHIKAWA and KOHGO, 1985; Kozulis and Page, 1968; DEMAN et al., 1960). The major disadvantage of these classical methods is the high expenditure of time and results will only be available after 5-7 days. Furthermore, some media e.g. MRS are only selective for lactic acid bacteria but cannot differentiate between beer-spoiling and non-spoiling strains (DEMAN

et al., 1960; REUTER, 1985, SUZUKI et al, 2008). To evaluate beer-spoiling potential, further analyses have to be performed. The so-called „forcing test" is an essential and reliable method with a high degree of accuracy to separate beer-spoiling and nonspoiling strains. This method consists of an inoculation and incubation of bacteria in beer until visible growth can be detected. Depending on beers hop concentration and pH, which are the main factors influencing microbial stability, time until visible growth occurred can be up to 3 month (SAKOMOTO et al., 2003). Due to this reason, this method is not very practical. Nevertheless, methods based on assaying for spoilage-associated genetic markers such as by PCR allow faster detection. For *L. brevis*, the *hitA*, *horA* and *horC* genes have been proposed as the beer-spoilage marker genes (FUJII et al, 2005; HAYASHI et al, 2001; IIJIMA et al., 2006, SAMI et al., 1997; SUZUKI et al, 2005). However, the interpretation of the results of the assessment of beer-spoiling potential using these markers has to be done carefully as mutations in resistance genes or the presence of other unknown resistance mechanisms may lead to false-positive or false-negative results (FUJII et al, 2005, HAAKENSEN et al., 2008, TEICHERT, 2008). Other researchers designed beer-based media (SUZUKI et al., 2007) and could differentiate beer-spoiling lactobacilli from non-spoiling within two to five days depending on species and physiological status. In contrast to that MRS-based agar plates containing 9 BU hop compounds and 5% v/v alcohol (HGA+E) by HAAKENSEN et al. (2009) were positive for 9.1 % of non-beer lactobacilli and 100 % of beer-spoiling lactobacilli. YANSANJAV et al. (2004) applied a method for noninvasive measurement of intracellular pH and predicted the beer-spoiling potential of lactic acid bacteria towards the resistance to tetrahydroiso-α-acids. MARCH et al. (2005) used a monoclonal antibody-based immuno-chemiluminescence assay for rapid detection and enumeration of viable and culturable bacteria of six different breweries and could recognize 18 out of 19 unknown different beerspoilers. ASANO et al. (2009) trapped cells on a polycarbonate membrane filters and cultured them on ABD medium. After short-time incubation, they stained viable cells, which formed microcolonies with carboxyfluorescein diacetate (CFDA). Cells were counted with µFinder Inspection System. This so-called microcolony-CFDA method could discriminate beer-spoiling strains from non-spoiling strains upon detection of microcolonies. Furthermore, this method was useful to detect

beer-spoiling ability of LAB species, such as *L. brevis*, *L. lindneri* and *L. paracollinoides*. Another approach to identify spoilage potential is the measurement of the manganese efflux during incubation in beer (VOGEL et al., 2010). Thereby, so-called „wheat beer *L. brevis*“, which possess only low hop tolerance exhibit decreased mananese efflux, whereas highly tolerant strains („Pilsner-*L. brevis*“) release high amounts within 3 h.

1.11 Tools to identify constitutive mechanisms of hop tolerance

Even before antibiotics were introduced for the treatment of common microbial infections in the 1950s, ABRAHAM and CHAIN (1940) identified a bacterial enzyme that eliminated the antibacterial activity of penicillin by hydrolysis of the beta-lactam ring. Since this time, research is focused on detecting bacterial resistance mechanisms to several antibiotics. The knowledge of resistance to antibiotics allows not only the development of medicals but also brewers to detect infections or contaminations with antibiotic resistant strains. In case of beer infection, the species *L. brevis* is particularly suitable for investigation of hop tolerance mechanisms, because hop-sensitive and hop-resistant strains are available for comparison (SUZUKI et al., 2004). Furthermore, a careful analysis of physiological characteristics of each strain is an important prerequisite for identifying mechanisms of hop tolerance (VOGEL et al., 2010; PREISSLER et al., 2010).

1.11.1 Genetic tools

RAPD-PCR

Among PCR-based techniques, RAPD-PCR is described as a simple method for detecting genetic variation among individuals based on the amplification of unspecific DNA fragments with primers of arbitrary nucleotide sequence (WILLIAMS et al. 1990). When 16S DNA-PCR is used for the identification of species, RAPD-PCR is a powerful tool for detection of differences between several strains within one species. HAYASHI et al. (2001) and FUJII et al. (2005) applied this technique to identify genetic markers, which correlate with tolerance to hop and beer-spoiling ability.

Plasmid profile

Beer-spoiling strains could be highly adapted to high tolerance against hop (BEHR et al., 2006). Like other antibiotics hop compounds possess bacteriostatic or bacteriocidal properties. Defense mechanisms for protecting cells are chromosomally encoded or are reside on plasmids (HAYASHI et al., 2001, SAMI et al., 1998). Plasmids are conserved in presence of selective pressure. Dehabituation of hop compounds can induce the loss of plasmid, which is associated to hop tolerance. SAMI et al. (1997) isolated a hop-resistant mutant by adapting a strain of *L. brevis* to the hop compounds. They found, that the copy number of plasmid pRH45 in the hop-adapted strain was higher than in the wild type (SAMI, 1997). Furthermore, SAMI et al. (1998) repeatedly subcultured the wild-type strain ABBC45 every 2–3 d over 15 subcultures and induced the loss of plasmid pRH45, which was harboring *horA*. Subsequently, this strain was repeatedly subcultured at 37 °C by SUZUKI et al. (2004) and a non-spoiling variant was obtained, which lost a second plasmid designated pRH45II, harboring the gene for the multidrug transporter HorC.

1.11.2 Proteomic tools

Proteins are responsible for many important biological processes. They control growth and differentiation, act as catalysts (enzymes) or transport molecules. Numerous studies have shown that the amount of each expressed protein cannot be predicted by the quantitative determination of its mRNA. The mRNA levels of a protein may remain unchanged, while the number of the expressed protein molecules is increased or decreased (GYGI et al., 1999). Furthermore, the specific functions of proteins are often determined by post-translational modifications (MATA et al. 2005). Posttranslational modifications are phosphorylation, glycosylation, sulfation or acetylation. For this reason, the function of a specific protein cannot be predicted only by the knowledge of the mRNA quantity. Therefore, for quantification and functional characterization, the proteins must be characterized even closer. This is the task of proteomics, which has itself the goal to identify and characterize proteins or the proteome of an organism with regard to its function. In contrast to the genome, which is comparatively static, the proteome is highly dynamic and qualitative and

quantitative changes in its protein composition can be induced by variety of conditions (environmental factors, stress response, starvation, temperature, etc).

2-D gel-electrophoresis

The most important method of proteomics until today is the 2D gel-electrophoresis (2DGE), which has been used for more than 30 years by researchers to separate complex samples of more than 2000 proteins. The separation of proteins by mass in polyacrylamide gel is described 50 years ago by RAYMOND and WEINTRAUB (1959). Independent of each other, KLOSE (1975) and O´FARRELL (1975) demonstrated that proteins can be also separated by charge. The isoelectrical focusing is based on the principle that proteins are uncharged at the pH corresponding to their isolectrical point (pI). In an IPG strip with pH gradient, proteins in cup-loaded samples migrate under high voltage through the strip and stop moving, when pH is equal to pI and net charge is zero. The combination of isoelectrical focusing and SDS-PAGE (LAEMMLI, 1970) results in high-resolution 2D gels. The resolving power of 2-DE in combination with subcellular prefractionation and narrow-range immobilized pH gradients has been estimated to reveal up to 75 % of the protein genome (CORDWELL et al., 2000).

Differential proteomics

Strains possess diverse phenotypes including resistance or tolerance to antibiotics or other chemicals. The comparison of such antibiotic resistant strains with sensitive strains on proteomic level leads to an identification of possible mechanisms of resistance or tolerance. Another approach to identify mechanisms of tolerance is the adaptation of a given strain to the antibiotic. BEHR et al. (2007) adapted *L. brevis* TMW 1.465 to increasing concentrations of hop and compared the proteome expressed under reference conditions with the proteome appeared under acid- and hop stress. Furthermore, species- or strain specific strategies as response to several stress conditions such as acid stress, nutrient limitation, and manganese deficiency can be investigated by differential proteomics.

Matrix-assisted Laser Desorption/Ionization Time of Flight Mass spectroscopy

Matrix-assisted laser desorption/ionisation (MALDI) as ionisation methods for mass spectrometry was first developed by the group of HILLENKAMP (1985). Most MALDI mass spectrometers used 20 Hz pulsed ultraviolet nitrogen laser with wavelength of 337 nm, which generated ions by photon bombardment of a sample served on a stainless steel target. For this laser type, proteins are often successfully ionized with sinapinic acid (BEAVIS and CHAIT, 1989) or α-cyano-4-hydroxycinnamic acid (BEAVIS et al. 1992). By measuring the flight time of ions in a drift path, the mass of produced protein or –peptide ions can be estimated. This can be used to identify proteins after previous tryptic digestion or for bacterial identification based on peptidic spectra from whole bacterial cells, which can be used as protein fingerprint signature. Thereby, such fingerprint of unknown bacteria can be compared with reference fingerprints in a database by the use of various algorithms and bacteria can be identified rapidly and cost-effective (FENSELAU and DEMIREV, 2001). Futhermore, MALDI-TOF mass spectrometry can be used for the identification of antibiotic resistance strains, e.g. methicillin-resistant *S. aureus*, which can be separated from methicillin-susceptible of the same species (EDWARDS-JONES et al., 2000).

In conclusion, hop tolerance is a multifactorial process, which is based on constitutive or adaptive properties of bacterial cells. The initial physiological state (adapted or non-adapted) of beer isolates is mostly unknown. Therefore, constitutively expressed properties cannot easily be distinguished from properties acquired by adaptation. This can lead to a misinterpretation of the spoilage potential of isolates, and the comparison of partial or highly beer-adapted strains gives only limited information about their constitutive beer-spoiling potential. A categorization of beer-spoiling strains along the differentiation of constitutive versus acquired properties allows the objective assessment of beer-spoiling potential and identification of new hop tolerance mechanisms and markers for their detection.

1.12 Objectives of the work

It was the aim of this thesis to categorize non-adapted *L. brevis* strains along their constitutive property to spoil beer and identify mechanisms of basal hop tolerance. Based on the understanding of these tolerance mechanisms, the development of new and more reliable markers for quality management in breweries can be advanced. Therefore, different isolates of *L. brevis* (beer, sour dough, plant fermentation) should be propagated several times on mMRS4 laboratory medium to wean hop-adapted isolates and bring all strains in the same physiological state. Subsequent „forcing tests" in beer should categorize all tested strains into constitutive beer-spoilers and non-spoilers in dependence on their non-adapted beer-spoiling potential. This categorization should be the basis to identify typical traits and mechanism of stress response within the category of constitutive beer-spoiling strains in sugar utilization, occurrence of hop- and acid stress associated genes, protein expression pattern in different growth phases, composition of cell membrane and differences in MALDI mass spectra. Furthermore, the influence of divalent manganese ions, which contribute to a change in hop tolerance, should be investigated on cellular level in non-adapted *L. brevis* strains to advance the understanding of hop inhibitory effects along cellular stress response.

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Equipment

Table 1. Overview about used equipment

Device	Model	Manufacturer
Agarose gel chamber 25 x 20 cm	Easy Cast electrophoresis system	Owl Separation Systems, Portsmouth, NH, USA
Agarose gel chamber 13.8 x 12 cm	Easy Cast electrophoresis system	Owl Separation Systems, Portsmouth, NH, USA
Autoclaves	2540 ELV	Systec GmbH, Wettenberg, Germany
	Varioklav	H + P Labortechnik, Oberschleißheim, Germany
Breeding/incubation	Certomat BS-1	B. Braun Biotech International, Melsungen, Germany
	Hereaus B5042E	Hereaus Instruments, Hanau, Germany
	Memmert INB series	Memmert GmbH & Co. KG, Schwabach, Germany
	WiseCube®WIS-ML02	Witeg Labortechnik GmbH, Wertheim, Germany
Centrifuges	Sigma 1 K 15	Sigma Labortechnik, Osterrode am Harz, Germany
	Sigma 6-16K	Sigma Labortechnik, Osterrode am Harz, Germany
	J-6	Beckman, Palo alto, CA, USA
	J-2	Beckman, Palo alto, CA, USA
	Hermle Z383 K	Hermle Labortechnik, Wehningen, Germany
	Hermle Z382 K	Hermle Labortechnik, Wehningen, Germany
Electroporation system	Bio-Rad Gene pulser device	Bio-Rad Laboratories, Hercules, CA, USA
Incubation hood	Certomat H	B. Braun Biotech International, Melsungen, Germany
Laminar flow sterile work bench	HERA safe	Heraeus Instruments, Hanau, Germany
MALDI-TOF MS	microflex LT	Bruker Daltonics GmbH, Bremen
Microscope	Axiolab	Carl Zeiss MicroImaging GmbH, Germany
Nanodrop	Nanodrop1000	Peqlab Biotechnologie GmbH,

		Erlangen, Germany
PCR-Cycler	Primus 96 plus	MWG Biotech, AG, Ebersberg, Germany
	Mastercycler gradient	Eppendorf AG, Hamburg, Germany
pH determination (electrode)	InLab 412, pH 0-14	Mettler-Toledo, Gießen, Germany
pH determination (measuring device)	Knick pH 761 Calimatic	Knick elektronische Geräte, Berlin, Germany
Photometer	NovaspeIIq	Pharmacia Biotech, Cambridge, England
Pipettes	Pipetman	Gilson-Abomed, Langenfeld, Germany
Plate readers	TECAN SPECTRAFluor	TECAN Deutschlan GmbH, Crailsheim, Germany
	TECAN SUNRISE	TECAN Deutschlan GmbH, Crailsheim, Germany
Power supplies	MPP 2 x 3000 Power Supply	MWG Biotech AG, Ebersberg, Germany
	Electroophoresis Power Supply EPS 3000	Pharmacia Biotech, Cambridge, England
	2197 Supply PPS 200-1D	MWG Biotech AG, Ebersberg, Germany
Pure water	Euro 25 and RS 90-4/UF pure water system	SG Wasseraufbereitung GmbH, Barsbüttel, Germany
Shaking	Certomat R	B. Braun Biotech International, Melsungen, Germany
	Vortex 2 Genie	Scientific Industries Inc., Bohemia, NY, USA
Stirring	RCT-Basic	Mettler-Toledo, Gießen, Germany
Thermo block	Techne DRI-Block DB3	Thermo-Dux Gesellschaft für Laborgerätebau mbH, Wertheim, Germany
Ultra sonic water bath	Sonorex Super RK 103H	Bandelin electronic, Berlin, Germany
Ultra sonification	UP 200S	Dr. Hielscher GmbH, Teltow, Germany
	SONOPLUS/SH70G	Bandelin electronic, Berlin, Germany
UV table	Herolab UVT 28M	Herlab GmbH Laborgeräte, Wiesloch, Germany
Water bath	Lauda BD	LAUDA Dr. D. Wobser GmbH & Co., Lauda-Königshofen, Germany

2.1.2 Chemicals

Table 2. Overview about used chemicals

Chemicals	**Purity**	**Manufacturer**
6 x DNA loading dye	-	Fermentas GmbH, St. Leon-Rot, Germany
Acetic acid	99 - 100 % (glacial)	Merck, Darmstadt, Germany
Acrylamid-Bis solution	(19:1); 30 % (w/v)	SERVA, Heidelberg, Germany

Agar	european agar	Difco, BD Sciences, Heidelberg
Agarose	for electrophoresis	Biozym Scientific GmbH, Oldendorf, Germany
Amino acids	research grade	SERVA, Heidelberg, Germany
Ampicillin sodium salt	93.3 %	Gerbu Biotechnik GmbH, Gaiberg, Germany
Ammonium chloride	≥99.5 % p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ammonium persulfat (APS)	electrophoresis grade	SERVA, Heidelberg, Germany
Anaerocult C mini	-	Merck, Darmstadt, Germany
Boric acid	≥99.5 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Bromphenol blue	for electrophoresis	SIGMA-Aldrich, Steinheim, Germany
$CaCl_2 * 2H_2O$	p.a.	Merck, Darmstadt, Germany
Cysteinhydrochloride * H_2O	p.a.	Merck, Darmstadt, Germany
Dimidium bromide	≥98 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
DTT (1,4 Dithio-D,L-Threitol)	high purity	GERBU Biotechnik, GmbH, Gaiberg, Germany
EDTA	for molecular biology	SIGMA-Aldrich, Steinheim, Germany
Erythromycin		SIGMA-Aldrich, Steinheim, Germany
Ethanol, denatured	99 % with 1 % methylethylketone	Chemikalien und Laborbedarf Nierle, Freising, Germany
Ethanol, absolute	≥99,8 %	VWR, Prolabo, Foutenay-sous-Bois, France
Ethidium bromide	1 % in H_2O for electrophoresis	Merck, Darmstadt, Germany
FD restriction buffer		Fermentas GmbH, St. Leon-Rot, Germany
FD restriction enzymes	-	Fermentas GmbH, St. Leon-Rot, Germany
Glucose	for biochemical use	Merck, Darmstadt, Germany
Glycerol	99.5 %, high purity	GERBU Biotechnik, GmbH, Gaiberg, Germany
Glycine	p.a.	Merck, Darmstadt, Germany
HCl 37 %	p.a.	Merck, Darmstadt, Germany
KH_2PO_4	p. a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$K_2HPO_4 * 3 H_2O$	p.a.	Merck, Darmstadt, Germany
L-amino acids	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Lysozyme	-	SERVA, Heidelberg, Germany
Meat extract	for microbiology	Merck, Darmstadt, Germany
Methanol	HPLC-grade	Mallinkrodt Baker B. V., Deventer, NL

$MgCl_2$ * 6 H_2O	p.a.	Merck, Darmstadt, Germany
$MgSO_4$ * 7 H_2O	p.a.	Merck, Darmstadt, Germany
$MnCl_2$	p.a.	Merck, Darmstadt, Germany
$MnSO_4$ * 4 H_2O	p.a.	Merck, Darmstadt, Germany
NaCl	p.a.	Merck, Darmstadt, Germany
NaH_2PO_4	p.a.	Merck, Darmstadt, Germany
NaOH	p.a.	Merck, Darmstadt, Germany
NH_4Cl	p.a.	Merck, Darmstadt, Germany
Paraffin oil	-	SIGMA-Aldrich, Steinheim, Germany
Pepton from casein	for microbiology	Merck, Darmstadt, Germany
Primer	-	MWG-BiotechAG, Ebersberg, Germany
SDS	research grade	SERVA, Heidelberg, Germany
Sucrose	HPLC-grade	Gerbu Biotechnik GmbH, Gaiberg, Germany
T4 DNA ligase	-	Fermentas GmbH, St. Leon-Rot, Germany
Taq Core Kit	-	MP Biomedicals Solon, Ohio, USA
TEMED	p.a.	Merck, Darmstadt, Germany
Tris	ultra pure	MP Biomedicals Solon, Ohio, USA
Tris-HCl	p.a.	Merck, Darmstadt, Germany
Tween 80	-	Mallinkrodt Baker B. v., Deventer, NL
Yeast extract	for microbiology	Merck, Darmstadt, Germany

2.1.3 Consumables

Table 3. Overview about used consumables

Material	**Type**	**Manufacturer**
Electroporation cuvettes		Biozym scientific GmbH, Oldendorf, Germany
Microtiter plates	multi well plate 96-well flat bottom with lid	Sarstedt, Nümbrecht, Germany
Reaction tubes	2 ml, 1.5 ml, 200 µl	Eppendorf, Hamburg, Germany
Sterile ml tubes	5 ml, 15 ml, 50 ml	Sarstedt, Nümbrecht, Germany
Sterile filter	Filtropur S 0.2 (0.2 µm)	Sarstedt, Nümbrecht, Germany
Anaerocult		Merck, Darmstadt, Germany

2.1.4 Molecular-biological kits

Table 4. Overview about used molecular-biological kits

Kit	Type	Manufacturer
E.Z.N.A. Bacterial DNA Kit	DNA isolation	Omega Bio-Tek Inc., Norcross, GA, USA
peqGOLD Gelextraction Kit	Gel extraction	PEQLAB Biotechnologie GmbH, Erlangen, Germany
KOD hot start DNA polymerase	DNA polymerase	Novagen, EMD chemicals Inc., San Diego, CA, USA
peqGOLD plasmid miniprep kit	Plasmid miniprep kit	PEQLAB Biotechnologie GmbH, Erlangen, Germany
QIAquick PCR purification Kit	PCR purification Kit	Qiagen GmbH, Hilden, Germany
Taq Core Kit	DNA polymerase	MP Biomedicals Solon, Ohio, USA

2.1.5 Types of beer used in this study and characteristic

All beers used in this study are commercial available. The characteristics of every beer are shown in Table 5.

Table 5. Overview about properties of used beers

beer type	fermentation type	hop content (ppm)	alcohol (vol%)	pH (adjusted)
wheat beer	top fermented	8	5	4.3
wheat beer	top fermented	8	0.5	4.3
lager beer	bottom fermented	16	5	4.3
lager beer	bottom fermented	16	0.5	4.3
pilsner I	bottom fermented	36	4.9	4.3
pilsner I	bottom fermented	36	0.5	4.3
pilsner II	bottom fermented	26	4.9	4.3
pilsner III	bottom fermented	25	4.9	4.3

2.1.6 Bacterial strains

The isolates used in this study were *L. brevis* strains from diverse origins (Table 6), which included isolates from beverage industry, human faeces, plant

fermentations, silage and sourdough. All strains from different environments were sub-cultured ten times on mMRS4 plates (STOLZ et al., 1993), in order to achieve a comparable initial physiologic state with no adaptation to beer/hop for all isolates.

Table 6. *L. brevis* strains used in this study (TMW = Technische Mikrobiologie Weihenstephan)

Species	Strain	Source
L. brevis	TMW 1.313	beer
L. brevis	TMW 1.465	softrink
L. brevis	TMW 1.485	beer
L. brevis	TMW 1.230	beer
L. brevis	TMW 1.315	beer
L. brevis	TMW 1.317	beer
L. brevis	TMW 1.240	beer
L. brevis	TMW 1.476	beer
L. brevis	TMW 1.474	beer
L. brevis	TMW 1.484	beer
L. brevis	TMW 1.310	beer
L. brevis	TMW 1.1282	beer
L. brevis	TMW 1.1283	beer
L. brevis	TMW 1.316	beer
L. brevis	TMW 1.336	beer
L. brevis	TMW 1.305	beer
L. brevis	TMW 1.302	beer
L. brevis	TMW 1.1284	beer
L. brevis	TMW 1.337	beer
L. brevis	TMW 1.1205	sourdough
L. brevis	TMW 1.1216	sourdough
L. brevis	TMW 1.939	Spiros Paranithiotis
L. brevis	TMW 1.473	Tank cleaning water
L. brevis	TMW 1.841	ULICE (Fr)
L. brevis	TMW 1.507	wheat beer
L. brevis	TMW 1.6	faeces
L. brevis	TMW 1.1371	fermentation

L. brevis		
L. brevis	TMW 1.362	beer
L. brevis	TMW 1.483	beer
L. brevis	TMW 1.1369	fermentation
L. brevis	TMW 1.1370	fermentation
L. brevis	TMW 1.1421	fermentation
L. brevis	TMW 1.1326	silage
L. brevis	TMW 1.100	sourdough

2.2 Methods

2.2.1 Microbiological methods

2.2.1.1 Media and growth conditions

2.2.1.1.1 Cultivation and growth conditions of LAB

All *L. brevis* strains were cultivated in modified mMRS4 medium (Stolz 1995, Table 7). In case of agar plates, 1.5 % agar was additionally added. All components expect for sugars were dissolved in 800 ml deionised water and pH of 6.2 was adjustted. Subsequently, media was autoclaved for 20 min at 121 °C. Sugar solutions (200 ml) were autoclaved seperatly to avoid formation of Maillard products and were mixed with the basic medium after cooling.

Table 7. Composition of MRS4 medium used for lactobacilli

Compound	Concentration (g/l)
Peptone	10
Meat extract	5
Yeast extract	5
Tween 80	1
K_2HPO_4 * 3 H_2O	4
KH_2PO_4	2.6
NH_4Cl	3
Tween 80	1
cysteine HCl	0.5
maltose	15
fructose	5
$MgSO_4$ x 7 H_2O	0.2
$MnSO_4$ x H_2O	0.03

2.2.1.1.2 Cultivation and growth conditions of of *E. coli*

E. coli was grown in LB (Luria-Bertani) medium. All components were dissolved in deionised water and medium pH was adjusted to 7.2 with 2 M NaOH according the recipe in Table 8. LB medium was sterilized by autoclaving at

121 °C for 20 min. For agar plates, 1.5 % agarose was added. Furthermore, depending on transformed plasmid, antibotics were added (750 ppm ampicilin). Inoculated medium and agar plates of *E. coli* were incubated at 37 °C. Liquid cultures were shaked in 100 ml flask with 50 ml growth medium at 200 ppm.

Table 8. Composition of LB growth medium

Compound	Concentration
NaCl	5 g/L
Yeast extract	5 g/L
Casein-pepton	10 g/L

2.2.1.2 Determination of growth in presence of iso-α-acids

Minimal inhibitory concentration (MIC) was determined in mMRS4 (STOLZ et al., 1993) broth with pH 4.3 and manganese (0.16 mg/l), and magnesium (98 mg/l) which were similar in composition to beer. Furthermore, there was no addition cysteine hydrochloride to the medium. The bacterial cells used for this experiment were grown in mMRS4 broth (with manganese content: 30 mg/l) for four days at 30 °C. OD_{590} of 2 was adjusted as mentioned above and 5 µl was used as inoculum. The MIC test was performed in a sterile 96-well microtiter plate and each well contained 200 µl test media. The concentration of iso-α-acids was gradually increased from 5 ppm to 35 ppm. After inoculation, wells were overlaid with sterile paraffin oil and trays were incubated for two days at 30 °C. After incubation, 10 µl of resazurin indicator buffer was added to all wells and a second incubation step was done for one hour at 30 °C. Finally, wells were assessed visually for colour changes.

2.2.1.3 Growth challenges in different beers

The growth of all strains after cultivation on mMRS4 (STOLZ et al., 1993) (disregarding hop stress) was investigated upon direct transfer into beers varying in cereal base, fermentation type, ethanol and hop content. The optical density (OD) of the preculture was adjusted at 590 nm to OD_{590} of 2 in beer using the Novaspec II photometer and 10 x 4 x 45 mm cuvettes (Amersham Pharmacia Biotech, New Jersey; Cuvette, Sarstedt GmbH Germany). In a 96 well microtiter plates, the pH indicator bromophenol blue was added at a

concentration of 0.02 % (w/v) and the beers were filter sterilized (non-pyrogenic, 0.2 µm). Beer was adjusted to OD_{590} = 0.2 by adding 20 µl cell suspension to 180 µl beer. Every well was then overlaid with 100 µl paraffin oil and incubated for 30 days at 30°C. Sample with visible growth or colour changes from from blue to green were considered to be positive for the test. NBB bouillon was aliquoted in 1.5 ml sterile Eppendorf tubes and inoculated with a single colony and incubated at 30 °C for two days.

2.2.1.4 Detection of metabolic activity in beer

Metabolic activity in beer was determined by reduction of the redox dyes tetrazolim or resazurin by $NADH_2$, which results in color change.

2.2.1.4.1 Analysis of metabolic activity in beer by tetrazolium salt

For the tetrazolium assay in all beer samples, a tetrazolium salt solution at a concentration of 0.25 mM and a phenazinmethosulfate solution at 0.01 mM were prepared. All strains were grown in hop-free mMRS4 broth at 30 °C for four days and a cell suspension with OD_{590} = 2 was prepared. For determining metabolic activity in beer, 4 µl of the cell suspension was mixed with 100 µl of beer and overlaid with 80 µl paraffin oil. Metabolic activity was monitored semi-quantitatively along the reduction of tetrazolium salt by cellular dehydrogenases during a two week period and changes in OD_{485} were determined using a Tecan spectrafluor plate reader.

2.2.1.4.2 Determination of metabolic activity in beer by resazurin

For the resazurin assay, cells were harvested in late stationary phase, washed twice with 50 mM phosphate buffer and suspended in beer to OD_{590} = 2. Subsequently, 5 µl of the cell suspension was mixed with 200 µl of beer in a 96 well microtiter plate and overlaid with 100 µl paraffin oil. Finally, cells were incubated for 48 h at 30 °C. For determination of cell metabolism in beer, 5 µl of resazurin indication buffer was added. After one hour incubation, absorbance was measured at 590 nm using a Tecan spectraflour plate reader and wells were assessed visually for colour change.

2.2.1.5 Detection of arginine utilization

Overnight cultures were incubated in 2 ml arginine growth medium (Table 9) for 24 h or 48 h at 30 °C. Subsequently, cultures were centrifuged and 100 µl supernatant was used for analysis. Ammonia was determined by adding 100 µl Neßler reagent (E. Merck, Darmstadt, Deutschland).

Table 9. Composition of used culture media for detection of arginine utilization

	Arginine medium
Yeast extract (g/l)	4
Meat peptone (g/l)	10
Casein peptone (g/l)	2
L-arginine	3
Tween 80 (g/l)	1
Maltose (g/l)	7
Fructose (g/l)	7
Glucose (g/l)	7
Cystein-HCl	0.5
MgSO4 x $7H_2O$ (mg/l)	20
MnSO4 x H_2O (mg/l)	30

2.2.1.6 Growth challenges in the presence/absence of manganese cations

The experiment was performed in a 96 well microtiter plate whereas an optical cell inoculation density of 0.05 at 590 nm (OD_{590}) was adjusted in 200 µl of medium I or II (Table 10). Finally, every well was overlaid with 100 µl paraffin oil and incubated for 4 days at 30°C. Bacterial growth was monitored by measuring OD_{590} using a Tecan spectrafluor plate reader.

Table 10. Composition of used culture media with different manganese content

	Medium I (Mn⊕)	Medium II (Mn-)
Yeast extract (g/l)	5	5
Meat extract (g/l)	5	5
Casein peptone (g/l)	10	10
NH_4Cl (g/l)	3	3
Tween 80 (g/)	1	1
Maltose (g/l)	15	15
Fructose (g/l)	5	5
MgSO4 x $7H_2O$ (mg/l)	20	20
MnSO4 x H_2O (mg/l)	30	0.16

2.2.1.7 Measurement of intracellular manganese content

Cell pellets of 72 h cultures were washed with 50 mM MES buffer (twice) and the cell density was adjusted to OD_{590} = 1. Subsequently, samples were permeabilized by cooking for 10 min, centrifuged and 500 µl of the supernatant was mixed with an equal volume of 0.5 M Tris buffer (pH 8.8). In every sample, a final concentration of calcein (0.1 µM), DTT (0.1 mM) and magnesium (0.16 M) was adjusted and measured at Ex. 488 nm, Em. 507 nm (HASINOFF, 2003; TOMITA et al. 2008). Released manganese concentrations were determined using calibration curve (personal communication Jürgen Behr, Figure 2).

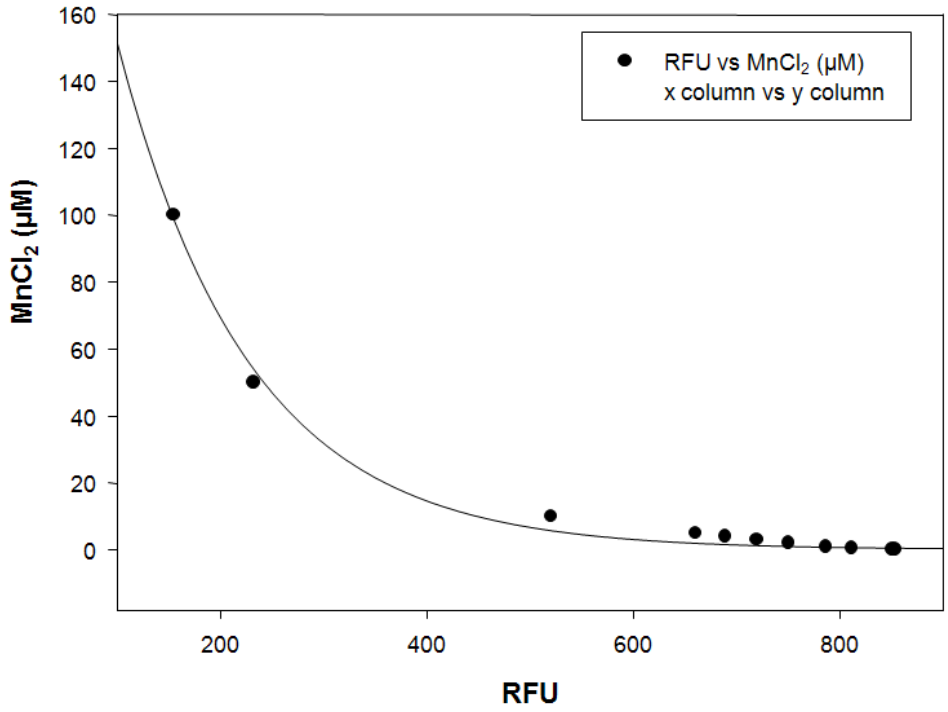


Figure 2. Calibration curve for divalent manganese concentrations in dependency of Relative Fluorescence Units (RFU)

2.2.2 Molecular-biological methods

2.2.2.1 Identification of genes up-regulated at hop- and acid stress conditions

A set of 17 strains of *Lactobacillus brevis* with different origin of isolate (beer, sourdough, silage, faeces, plant fermentation) were used for PCR screening to

detect the occurrence of genes (Table 11), which are induced or up-regulated under hop- and acid stress conditions concerning to BEHR et al. (2008).

Table 11. Overview about gene function and deduced forward and reverse primer sequences of screened genes induced or overexpressed under hop- and acid stress conditions

function	forward (5‘-3‘)	reverese (5‘-3‘)
phosphopentomutase	TTCCCTAACGGCTTCCCC	CCAAAGTCAGCAAATGGCG
purine-nucleosid phosphorylase	AGCCTCAACCGACTCCTCAAT	GACTTTTCTCGTTCTTCTGGCG
RecR	AGAACCCATTGCCCAGTTGA	TACATCTCTGTCCGCCCTTCA
formamidopyrimidine-DNA glycolase	TCATTCAGGGCATCTTGCG	GCACCACAACGGGAACATTT
ornithine carbamoyltransferase	AGGACGGAGTGTTCTTGCTGA	GTTCTTCCCAGTTGCTTTCACC
Cysteine sulfinate desulfinase/cysteine desulfurase related enzyme	CCGCTCACGACGAAGAGATTA	CCACCCGAAACGGCAA
peptidylpropyl isomerase	GGACCAAAAGCAACAATCAAAA	GCCGCAATCTTATCAACGA
glutamine-fructose-6-phosphate transaminase	GTGGTTAGCGTGGATAATGCG	GGTCAGGAAAATAAAGAATGGCTTC
Malate/lactate dehydrogenase	TAGACTGTGTGGTGATGCCTGATAG	CCTCCGTTTGCTGATTTTCAAT
2-hydroxyacid dehydrogenase	AAATCGGTGAAGTGAAGCGG	GTGTAAGCAGAGGTATGTGGGGTTA
3-hexulose-6-phosphate synthase related protein	GAACAGATTCACGCCCAGC	CGCTTCCCACAATAACCGAT
phosphoenolpyruvate carboxykinase	TAAAATCTCCGCCCAAAA	CAGTATGAAGTAAACGGCTCTCA
ATP-dependent protease	GGCACAACGGTCACAATGC	GGATGGCACTTTCGGCAA
Glutamate decarboxylase	CTAATGCCTGCTCTATTGGCGTA	CTGGTTTGATGTATTCTTTATCTTCCGT
Acetoin dehydrogenase	GGCATCAAATGGAAAAGTAGCAAT	TAAGAAAGCCACCCCGTTAGC
Glycerol dehydrogenase	AGGCAAGACGCTGGATTCTG	CCGCTTCACTCCGACCTTC
Mn2+ and Fe2+ transporter of the NRAMP family (1)	AACCGATACGAAAACCAAGCAC	GCCCCAACAATCCCGAG
Mn2+ and Fe2+ transporter of the NRAMP family (2)	ATTGAAGCCATTGTGGTCTGC	GCTCGCCCATTTCCAACTT
Mn2+ and Fe2+ transporter of the NRAMP family (3)	GCCCTCGTTGCCGTTG	CGCCGCACCTAACAATAGAAGTA
ABC-type Mn2+/Zn2+ transport system	GCGGCGGGAACCATTATT	TATTCTGCTGTTTGAAGTCGTTGC
hitA gene for putative manganese transporter	GGCTTCTGGCGAACATTATTTG	CCCGACCGTGCTATTGGTT

2.2.2.2 Identification of *horA, horC, hitA* - Primer design and PCR conditions

All strains were checked for the presence or absence of the *horA, horC* and *hitA* genes using PCR with their respective primer pairs:

forward primer: 5‘-tatatctagaatgcaagctcagtccaag-3‘
reverse primer: 5‘-tataaagctttcacccgttgctgctgcc-3‘ for *horA*;

forward primer: 5‘-tatatctagaatgttcgatgtaattcgt-3‘
reverse primer: 5‘-tataaagcttttatttaattttgcggtg-3‘ for *horC;* and
forward primer: 5‘-tatatctgaaatgaaagagggtattgat-3‘
reverse primer: 5‘-tataaagcttttaaccaatcacgccaac-3‘ for *hit*).

The PCR program consisted of an initial denaturation step of 2 min at 94°C, followed by 32 cycles of step 1: 94 °C for 45 sec; step 2: 61 °C (*horA*), 55 °C (*horC*), 56 °C (*hitA*) for 45 sec; step 3: 72°C for 120 sec (*horA*) and 90 sec (*horC*, *hitA*), and a final extension step of 72°C for 5 min. Amplicons were detected by electrophoresis in 1.2% agarose gels containing ethidium bromide.

2.2.2.3 Sequence analysis of *hitA* and *horC*

For sequencing of the genes *horC* and *hitA*, proofreading KOD-polymerase was used to generate error-free ampflicons with blunt ends. Primers were onstructed with restriction endonuclease sites for HindIII and NocI:

*horC*for_hindIII 5'-TATAAAGCTTATGTTCGATGTAATTCGTAGTA-3'
T_M = 60.5 °C,
*horC*rev_ncoI 5'-TATACCATGGTTATTTAATTTTGCGGTGTGGGC-3'
T_M = 65.8 °C;
*hitA*for_hindIII 5‘-TATAAAGCTTATTCATCTGGTTTAAGTA-3‘
T_M = 56.3 °C,
hitArev_ncoI 5‘-TATACCATGGAAAAATGTAATTTTCATT-3‘
T_M = 54.9 °C).

The plasmid pBAD-Myc/His A possesses within the multiple cloning site restriction endonuclease sites for HindIII and NocI. Both, PCR-amplicon and plasmid were restricted with HindIII and NocI in separate reaction mixture. Following, restricted DNA was purified by gel extraction. Purified amplicons and plasmid DNA were ligated and transformed in *E. coli*. Positive clones were detected by PCR and sequenced by GATC using following primers:

pBAD-for 5‘-CTACTGTTTCTCCATACCCG-3‘ and
pBAD-rev 5‘-CTGATTTAATCTGTATCA-3‘.

2.2.2.4 Extraction of whole-cell protein for 2D gel electrophoresis

The cell pellets of investigated strains were digested with lysozyme (0.1 g/ml) in TE buffer (10 mM Tris-HCl, 0.01 M EDTA, pH 8.0) for 60 min at 37°C. Cells were centrifuged (10,000 x g for 5 min), and the supernatant was discarded. The pellet was resuspended in 600 µl SDS buffer (0.9% sodium dodecyl sulfate [SDS], 0.1% Pefabloc, 100 mM Tris base, pH 8.6) and disrupted by sonication (HD-70/Bandelin, three cycles of 30 s each; power, 90%; cycle, 70%; on ice). The suspension was diluted 3.5-fold with thiourea lysis buffer (6.10 M urea, 1.79 M thiourea, 65.06 mM Chaps, 1% [wt/vol] DTT, 0.5% [vol/vol] Pharmalyte 3-10). The proteins were solubilized by vortexing for 20 min. The remaining cell wall fragments were removed by centrifugation at 17,500 x g at 4°C for 30 min. The clear supernatants were stored at - 80°C (HARDER, 2001).

2.2.2.5 2D gelelectrophoresis

Isoelectric focusing (IEF) was carried out using IEF 100 Isoelectric Focusing System (Hoefer Inc., USA) with 24-cm immobilized-pH-gradient (IPG) 4 to 7, 4 – 5, 4.5 - 5.5, 5.5 – 6.7 strips (Figure 3) at 20°C. The IPG strips were rehydrated with an excess of rehydration solution (6.10 M urea, 1.79 M thiourea, 8.13 mM DDM, 0.2% [wt/vol] DTT, 0.2% [vol/vol] Pharmalyte 3-10). For analytical gels, 200 µl protein extract was applied by sequential anodic cup loading. Initial IEF was run for 10 h at 250 V. IEF to steady state at 12,000 V was carried out according to manufactures protocol. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on a vertical system with gels of a total acrylamide concentration of 11% at 15°C. The proteins were visualized by silver staining according to BLUM et al. (1987) and the two groups of manganese-starved and non-starved cells were analyzed using Progenesis SameSpots (Nonlinear Dynamics Limited, Newcastle, UK).

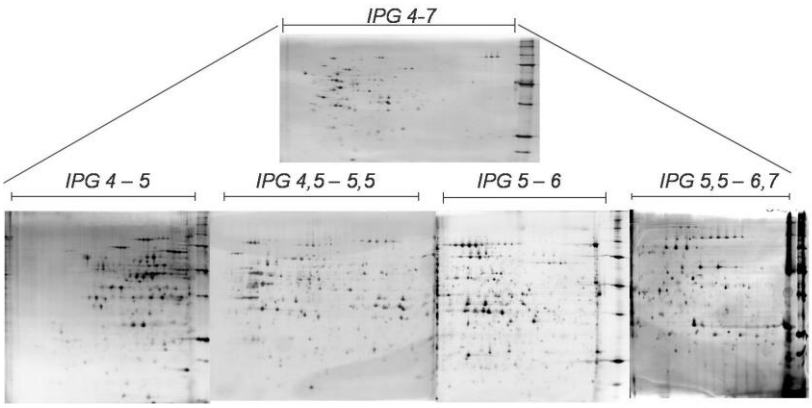


Figure 3. Overview about analyzed range of isoelectrical focusing

2.2.2.6 Protein identification

For identification, 2D gels were visualized by colloidal Coomassie staining (Roti-Blue; Carl Roth GMBH & Co., Karlsruhe, Germany) and spots were picked under laminar flow. Samples were sent to the Zentrallabor für Proteinanalytik (Ludwig-Maximilians-Universität München, Munich, Germany) for LC MS/MS.

2.2.2.7 RAPD-PCR, clustering and fragment analysis

For strain level differentiation, RAPD-PCR was performed with Taq Core Kit and the random M13V primer. Ingredients for PCR were mixed according to recipe in Table 12.

Table 12. Composition of the used master mixes for RAPD-PCR

Component	**volume (µl)**
PCR-H_2O	32.25
10x PCR Taq-Buffer (without. $MgSO_4$)	5
$MgCl_2$ (25 mM)	7
dNTPs (each 10 mM)	2
M13V	0.50
Taq polymerase (5 U/µl)	0.25
Template DNA	0.30
Total volume	50

The following amplification was carry out in a Primus 96 cycler using program parameters, which are illustrated in Table 13.

Table 13. PCR cycler program used for RAPD-PCR with M13V primers

Process	step	temperature [°C]	time [min]
Lid		103	
Loop 3 x	1	94	3
	2	40	5
	3	72	5
Loop 32 x	1	94	1
	2	60	2
	3	72	3

Subsequently, 10 µl of the amplification products were separated by agarose gel electrophoresis (1.2 % agarose in TBE buffer according to SAMBROOK et al., 1989) and gels were stained and documented as described above. Electrophoretic profiles were analyzed with the software Bionumerics (Applied Maths, Belgium). The analysis included the registration of the electrophretic patterns normalization of the densitometric traces and substraction of background noise, grouping of strains by Pearson correlation coefficient and cluster analysis by UPGMA or Ward.

2.2.3 Determination of fatty acid composition of cell membrane

Cells of *L. brevis* TMW 1.313, 1.465, 1.6 and 1.1369 were grown under reference conditions (mMRS4, 30 mg/l manganese) in 500 ml to the stationary growth phase, washed and lyophilized. Furthermore, cells of *L. brevis* TMW 1.313 were grown under reference conditions (mMRS4, 30 mg/l manganese) to OD_{590} = 0.5 in 500 ml medium and subsequently incubated in manganese-deficiency medium (mMRS4, 0.16 mg/l manganese) and beer (lager beer) for 5 days. As described above, cells were also washed and lyophilized. All samples were sent to Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) for analysis by gas chromatography.

2.2.4 Determination of intracellular trace elements

Cells of *L. brevis* TMW 1.313 were grown under reference conditions (mMRS4, 30 mg/l manganese) and manganese-starvation (mMRS4, 0.16 mg/l manganese) to OD_{590} = 0.5 in 500 ml medium and subsequently incubated in

pilsner beer for 5 days. After incubation cells were washed and lyophilized and sent to Forschungszentrum Weihenstephan f. Brau- und Lebensmittelqualität (Freising-Weihenstephan, Germany). Samples were analyzed according to EN ISO 11885 E22.

2.3 MALDI-TOF/MS analysis

2.3.1 Extraction of whole-cell protein

Cell protein extraction for MALDI-TOF analysis was performed using the standard protocol of Bruker daltonics. Cells were harvest and centrifuged. Cell pellet was resuspended in 300 µl water and 900 µl ethanol was added. Sample was centrifuged, supernatant removed and pellet dried. Subsequently, pellet was resuspended in 50 µl 70 % formic acid and 50 µl acetonitrile was added. Finally, sample was centrifuged and 1 µl of the supernatant was used for MALDI analysis.

2.3.2 Preparation of MALDI matrix

The MALDI-matrix was prepared by the use of: α-Cyano-4-hydroxy-cinnamic acid (Sigma-Aldrich Chemie GmbH, Taufenkirchen, Germany), acetonitrile (ChemLab GmbH, Bensheim, Germany), trifluoroacetic acid (Merck KGaA, Darmstadt, Germany) and de-ionized water.

2.3.3 Sample application onto MALDI target

The MALDI target was prepared at room temperature under sterile work flow. Initially, 0.5 µl of extracted samples were applicated on the MALDI target and dried at room temperature. Subsequently, sample was overlaid with 1.0 µl matrix. Finally, preparation was finished, if sample and matrix were co-crystallized.

2.3.4 Cleaning of MALDI target

To avoid peaks as result of contamination in the MALDI spectra, the target was cleaned as described below according to manufactures protocol:

- incubation of target in 70 % ethanol for 5 minutes
- rinsing with hot water and subsequent wiping with 70 % ethanol
- overlaying with 100 µl 80 % trifluoroacetic acid and wiping
- cleaning with de-ionized water and drying

3 RESULTS

3.1 Bacterial growth in beer and NBB

To determine the constitutive beer-spoiling potential of non-adapted *L. brevis* strains to hop compounds, „forcing tests" in beer were performed in microtiter plates. Growth of non-adapted cells was indicated by the presence of a cell layer at the bottom of the microtiter plate or by colour changes of pH indicator, due to acidification. The type strain TMW 1.6 exhibited growth only in non-alcoholic wheat beer, whereas the isolates TMW 1.1369, 1.1370, and 1.1371, which were originally derived from plant fermentation, grew in alcoholic wheat beer. None of these strains grew in lager beer. The isolate from the brewery environment, TMW 1.302, was only able to spoil non-alcoholic and alcoholic wheat beer, whereas the other brewery isolates possessed strong spoilage potential and could grow also in stronger hopped beer like lager and pilsner beer (Table 14). All strains were tested positive after 48 h of incubation in NBB-B. Isolates that could spoil wheat and lager or also pilsner beer in a non-adapted state, were categorized as constitutive beer-spoilers. Strains, which possessed only the ability to grow in wheat beer (alcoholic or non-alcoholic) were classified into the category of non-spoilers (Table 15).

Table 14. Bacterial growth of non-adapted constitutive beer-spoiling strains in different kinds of beer

	alcoholic beer					non-alcoholic beer		
Strain	wheat	lager	pilsner I	pilsner II	pilsner III	wheat	lager	pilsner I
L. brevis TMW 1.313	+	+	+	+	+	+	+	+
L. brevis TMW 1.465	+	+	+	+	+	+	+	+
L. brevis TMW 1.317	+	+	+	+	+	+	+	+
L. brevis TMW 1.485	+	+	+	+	+	+	+	+
L. brevis TMW 1.230	+	+	+	+	+	+	+	+
L. brevis TMW 1.315	+	+	+	+	+	+	+	+
L. brevis TMW 1.240	+	+	-	-	+	+	+	+

Table 15. Bacterial growth of non-adapted non-spoiling strains in different kinds of beer

	alcoholic beer					non-alcoholic beer		
Strain	wheat	lager	pilsner I	pilsner II	pilsner III	wheat	lager	pilsner I
L. brevis TMW 1.302	+	-	-	-	-	+	-	-
L. brevis TMW 1.6	-	-	-	-	-	+	-	-
L. brevis TMW 1.1369	+	-	-	-	-	+	-	-
L. brevis TMW 1.1370	+	-	-	-	-	+	-	-
L. brevis TMW 1.1371	+	-	-	-	-	+	-	-
L. brevis TMW 1.939	+	-	-	-	-	+	-	-
L. brevis TMW 1.841	+	-	-	-	-	+	-	-
L. brevis TMW 1.100	+	-	-	-	-	+	-	-
L. brevis TMW 1.1205	+	-	-	-	-	+	-	-

3.2 Utilisation of glucose, fructose and maltose

The ability of different *L. brevis* strains to ferment glucose, fructose and maltose was investigated in order to identify differences in sugar fermentation profile between constitutive beer-spoiling and non-spoiling strains. A closer look at the utilization of glucose, fructose and maltose revealed that within the species of *L. brevis* three types can be differentiated concerning their sugar utilization. It is notable that all strains isolated from other origins than beer were able to ferment every tested sugar. All these strains showed exponential growth (Figure 4). In contrast within the beer isolates there are some strains, which were not able to ferment maltose or fructose. In presence of glucose growth is worse than in medium containing fructose or maltose (Figure 5). In case of a decreased growth in fructose or maltose medium, screening was performed for the presence of key enzymes in the sugar metabolism. It was found that the necessary genes can be detected by PCR (maltose phosphorylase, mannitol dehydrogenase).

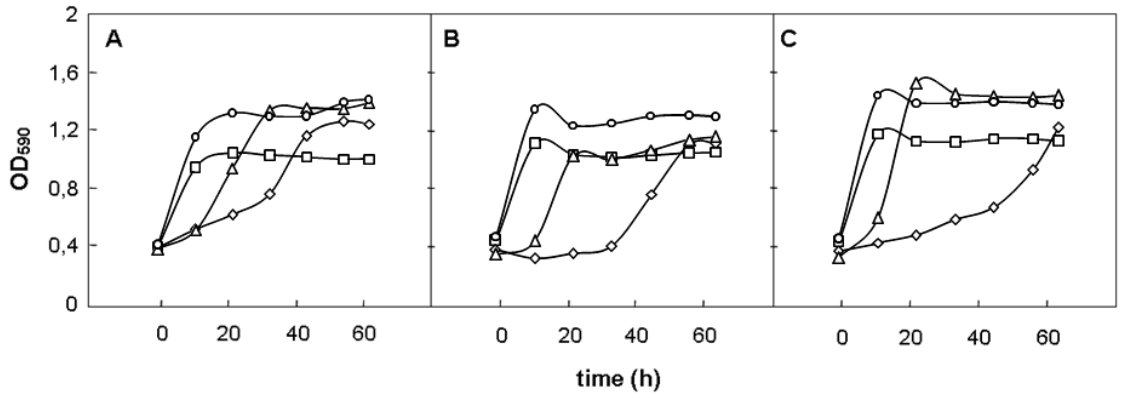


Figure 4. Sugar fermentation profile of three different non-spoiling strains (A) *L. brevis* TMW 1.6^{T}, (B) *L. brevis* TMW 1.1369, (C) *L. brevis* TMW 1.1370 (ring = glucose/fructose/maltose; square = fructose; triangle = maltose, rhombus = glucose)

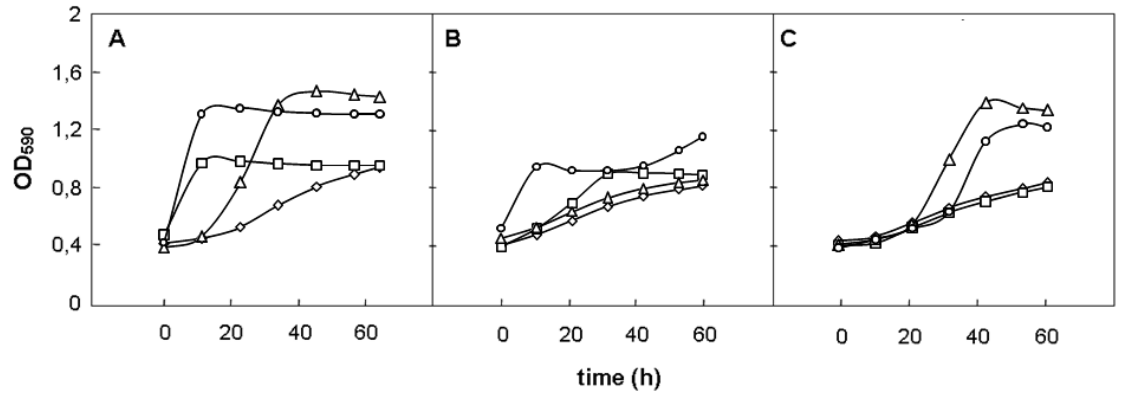


Figure 5. Sugar fermentation profile of three different beer-spoiling strains (A) *L. brevis* TMW 1.313, (B) *L. brevis* TMW 1.317, (C) *L. brevis* TMW 1.485 (ring = glucose/fructose/maltose; square = fructose; triangle = maltose, rhombus = glucose)

3.3 Occurrence and mobility of hop tolerance associated genes

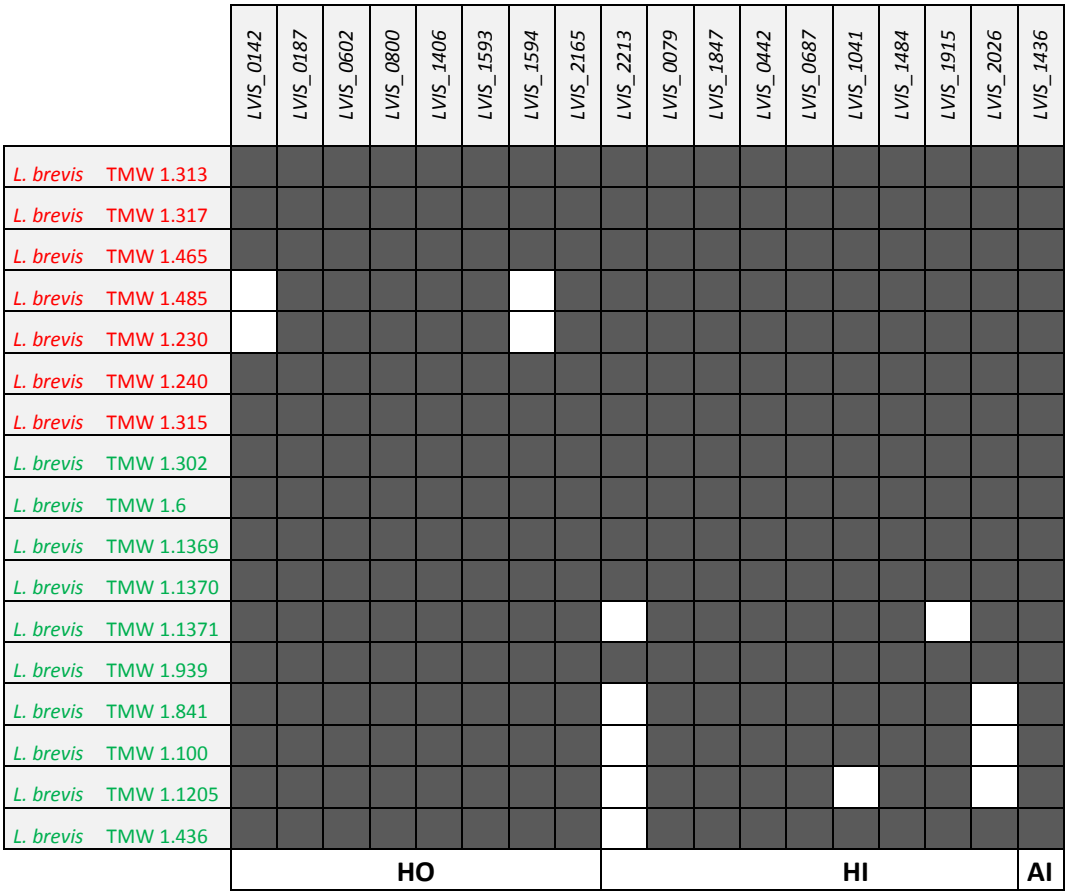
A set of 17 different strains of *L. brevis* were tested for the occurrence of genes, whose proteins were induced or up-regulated under hop- and acid stress according to BEHR et al. (2008). Table 16 gives an overview on the function, characteristics and locus tag of the screened genes.

Table 16. Overview about function, characteristic and locus tag of screened genes (HO: hop overexpressed, HI: hop induced, AI: acid induced)

Function	characteristic	locus tag
2-hydroxyacid dehydrogenase	HO	LVIS_0142
Acetoin dehydrogenase	HO	LVIS_0187
RecR	HO	LVIS_0602 (recR)
ATP-dependent protease	HO	LVIS_0800
Malate/lactate dehydrogenase	HO	LVIS_1406
purine-nucleosid phosphorylase	HO	LVIS_1593 (deoD)
phosphopentomutase	HO	LVIS_1594 (deoB)
Glycerol dehydrogenase	HO	LVIS_2165
Glutamate decarboxylase	HI	LVIS_2213
Glutamate decarboxylase (II)	unknown	LVIS_0079
Glutamate decarboxylase (III)	unknown	LVIS_1847
3-hexulose-6-phosphate synthase related protein	HI	LVIS_0442
glutamine-fructose-6-phosphate transaminase	HI	LVIS_0687
formamidopyrimidine-DNA glycolase	HI	LVIS_1041
peptidylpropyl isomerase	HI	LVIS_1484 (prsA)
phosphoenolpyruvate carboxykinase	HI	LVIS_1915
ornithine carbamoyltransferase	HI	LVIS_2026
Cysteine sulfinate desulfinase/cysteine desulfurase related enzyme	AI	LVIS_1436
Mn2+ and Fe2+ transporter of the NRAMP family	Mn transport	LVIS_0225
Mn2+ and Fe2+ transporter of the NRAMP family	Mn transport	LVIS_0331
Mn2+ and Fe2+ transporter of the NRAMP family	Mn transport	LVIS_0423
ABC-type Mn2+/Zn2+ transport system	Mn transport	LVIS_0471

Thereby, only small differences between several strains could be found. Differences could only be detected in the occurrence of genes of glutamate decarboxlyase (I, LVIS_2213), 2-hydroxyacid dehydrogenase (LVIS_0142), phosphopentomutase (LVIS_1594), and formamidopyrimidine-DNA glycolase (LVIS_1041) (Table 17). PCR reaction of the three Mn^{2+} and Fe^{2+} transporter of the NRAMP family (LVIS_0225, LVIS_0331 and LVIS_0423) and the ABC-type Mn^{2+}/Zn^{2+} transport system (LVIS_0471) were positive in all tested *L. brevis* strains.

Table 17. Presence/absence screening of hop- or acid stress induced genes in constitutive beer-spoiling (red) and non-spoiling (green) strains of *L. brevis*. (grey fields represents positive PCR reaction, white fields represents negative PCR reaction) (HO: hop overexpressed, HI: hop induced, AI: acid induced).



3.4 Genes of ADI pathway

The presence or absence of all genes included in arginine deiminase pathway was tested, because arginine deiminase was constitutive expressed in the group of the beer-spoiling strains. In addition, the presence of an arginine repressor was investigated. All isolates from breweries with a beer-spoiling potential possessed all genes for the arginine deiminase pathway. Common for all tested strains was the occurrence of the arginine repressor and the arginine deiminase gene. Differences could be detected in the occurrence of the arginine/ornithine antiporter, ornithine carbamoyl transferase and carbamate kinase genes, which were absent in the strains TMW 1.841, 1.100, 1.1205 (Table 18).

Table 18. Absence or presence of genes involved in arginine deiminase pathway in constitutive beer-spoiling (red) and non-spoiling (green) strains of *L. brevis* (grey fields represents positive PCR reaction, white fields represents negative PCR reaction).

	repR	*arcD*	*arcA*	*arcB*	*arcC (I)*	*arC (II)*		*repR*	*arcD*	*arcA*	*arcB*	*arcC (I)*	*arC (II)*
L. brevis TMW 1.313	■	■	■	■	■	■	*L. brevis* TMW 1.6	■	■	■	■	■	■
L. brevis TMW 1.317	■	■	■	■	■	■	*L. brevis* TMW 1.1369	■	■	■	■	■	■
L. brevis TMW 1.465	■	■	■	■	■	■	*L. brevis* TMW 1.1370	■	■	■	■	■	■
L. brevis TMW 1.485	■	■	■	■	■	■	*L. brevis* TMW 1.1371	■	■	■	■	■	■
L. brevis TMW 1.230	■	■	■	■	■	■	*L. brevis* TMW 1.939	■	■	■	■	■	■
L. brevis TMW 1.240	■	■	■	■	■	■	*L. brevis* TMW 1.1326	■	■	■	■	■	■
L. brevis TMW 1.315	■	■	■	■	■	■	*L. brevis* TMW 1.100	■		■			
L. brevis TMW 1.1282	■	■	■	■	■	■	*L. brevis* TMW 1.841	■		■			
L. brevis TMW 1.302	■	■	■	■	■	■	*L. brevis* TMW 1.1205	■		■			

For some selected beer-spoiling and non-spoiling strains, utilization of arginine was tested by detection of ammonia in arginine containing growth media. After 24 h incubation, 4 of 5 beer-spoiling strains were positive for ammonia in arginine-supplemented growth media detected by Neßler reagents. Both non-spoiling strains were negative after 24 h (Figure 6). After 2 days of incubation, the test was positive for all strains.

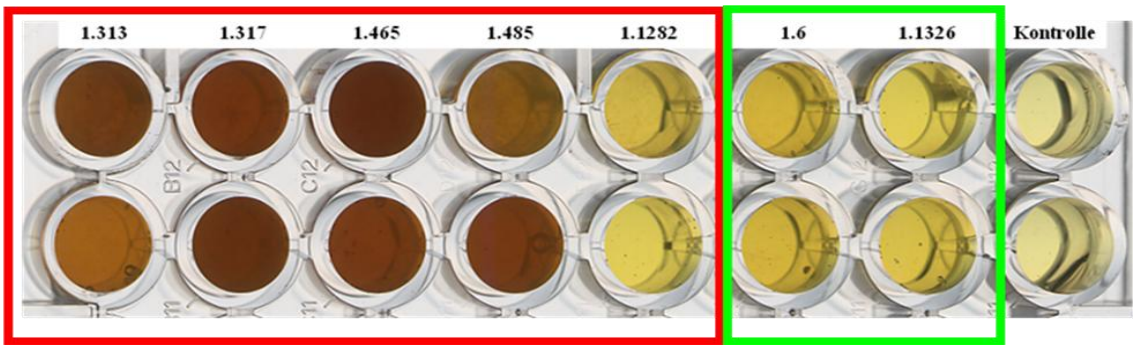


Figure 6. Detection of ammonia in arginine-containing growth medium detected by Neßler reagent (red frame = constitutive beer-spoiling strains, green frame = non-spoiling strains); positive reaction is indicated at brown or orange colour, yellow colour means negative reaction

3.5 Contribution of *hitA*, *horC* and *horA* on minimal inhibition concentration

To determine the contribution of *hitA, horC* and *horA*, 34 strains were screened on absence or presence of hop resistance associated genes. Strains, which possessed only one gene of *hitA, horA* or *horC* were used for the experiment to evaluate the contribution of these genes on basal hop tolerance level (Table 19).

Table 19. Absence or presence of hop-resistant associated genes (grey fields represents positive PCR reaction, white fields represents negative PCR reaction). Constitutive beer-spoilers are labeled red and non-spoilers are labeled green.

	hitA	*horA*	*horC*		*hitA*	*horA*	*horC*
L. brevis TMW 1.313	■	■	■	*L. brevis* TMW 1.305	■		■
L. brevis TMW 1.317	■	■	■	*L. brevis* TMW 1.302		■	
L. brevis TMW 1.465	■	■	■	*L. brevis* TMW 1.473		■	
L. brevis TMW 1.485		■	■	*L. brevis* TMW 1.507		■	
L. brevis TMW 1.230		■	■	*L. brevis* TMW 1.1326			
L. brevis TMW 1.240	■	■		*L. brevis* TMW 1.1216			
L. brevis TMW 1.315	■		■	*L. brevis* TMW 1.1284			
L. brevis TMW 1.310	■			*L. brevis* TMW 1.1421			
L. brevis TMW 1.1282		■	■	*L. brevis* TMW 1.6			
L. brevis TMW 1.1283			■	*L. brevis* TMW 1.1369			
L. brevis TMW 1.337	■	■	■	*L. brevis* TMW 1.1370			
L. brevis TMW 1.362	■	■	■	*L. brevis* TMW 1.1371			
L. brevis TMW 1.474			■	*L. brevis* TMW 1.939			
L. brevis TMW 1.476	■			*L. brevis* TMW 1.841			
L. brevis TMW 1.483	■	■	■	*L. brevis* TMW 1.100			
L. brevis TMW 1.316		■	■	*L. brevis* TMW 1.1205			
L. brevis TMW 1.336	■		■	*L. brevis* TMW 1.436			

Four categories of different *L. brevis* strains were defined and every group included different strains. Group 1 (*L. brevis* TMW 1.476 and TMW 1.310) was only positive for *hitA*, Group 2 (*L. brevis* TMW 1.474 and TMW 1.1283) only positive for *horC* and Group 3 (*L. brevis* TMW 1.507 and TMW 1.302) was positive for *horA*. Group 4 (*L. brevis* TMW 1.1396 and TMW 1.1370) was negative for all three tested genes. The performed MIC test exhibited, that the hop resistance level in the presence of *horA* was similar to that in the absence

of all genes. In contrast, *horC* and *hitA* positive strains exhibited highest minimal inhibition concentration (Figure 7).

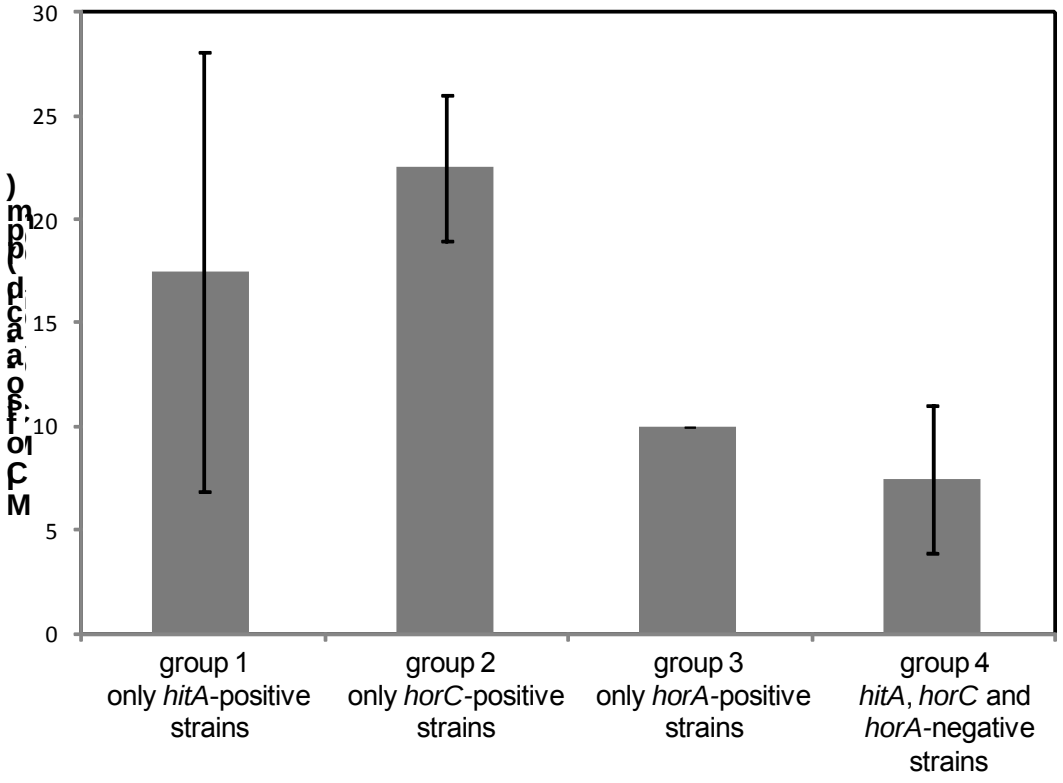


Figure 7. Minimal inhibitory concentration of iso-α-acid depending on absence or presence of *hitA, horA* or *horC*

In order to verify, that HorA is functional, the gene sequence of *L. brevis* TMW 1.507 was determined. Therefore, nucleotide sequence was transcribed by a special online tool (http://www.ebi.ac.uk/Tools/emboss/transeq/). Subsequently, an alignment was performed by the use of clustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). In comparison to the published sequence of HorA (NCBI accession no. BAA21552), five mutations in nucleotide sequence led to a change in amino acid sequence, whereby one of them was located in the second transmembrane domain and a second was located in Walker motif A (Figure 8).

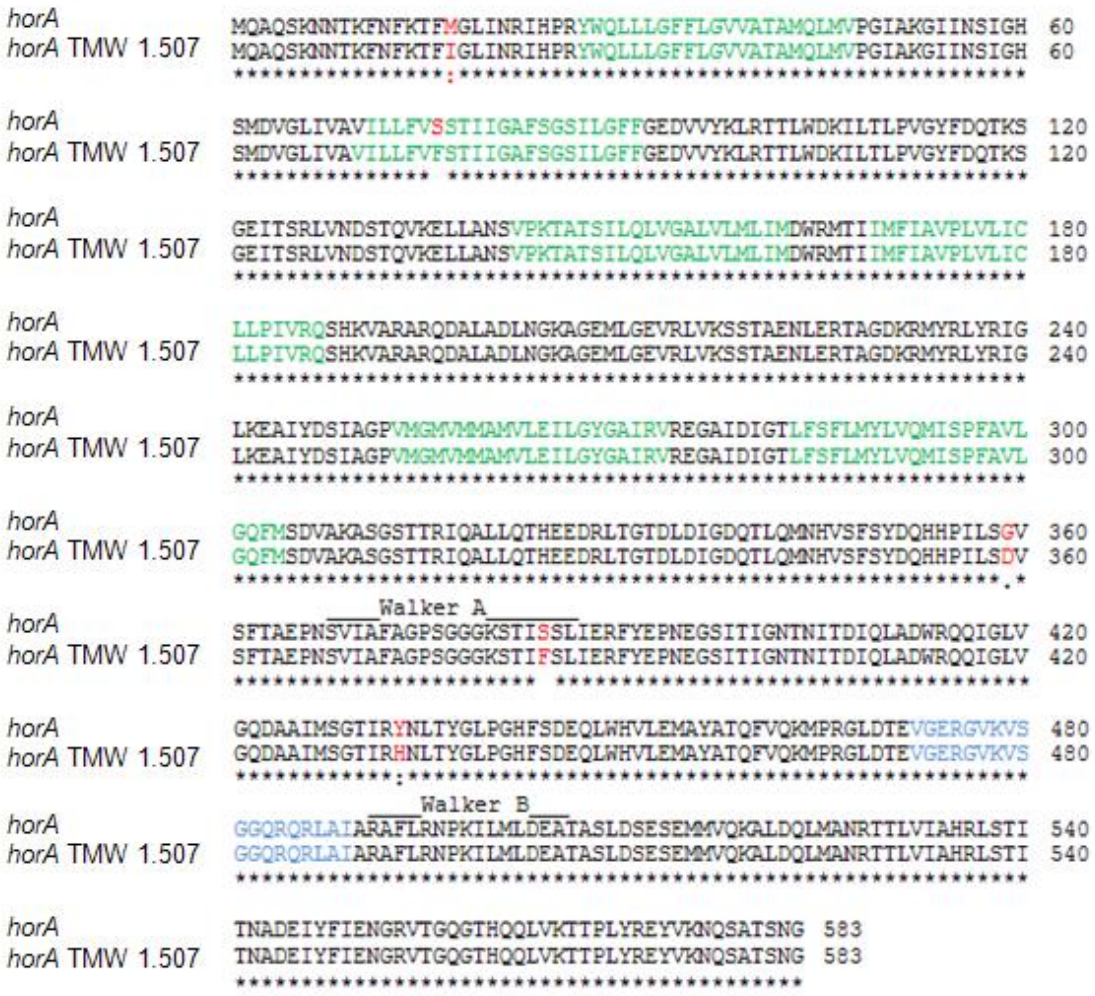


Figure 8. Alignment of published HorA sequence (NCBI accession no. BAA21552) with *L. brevis* TMW 1.507 (red = amino acid exchange; green = transmembrane domain; blue = ATP-binding site; TEICHERT, 2008)

3.6 Sequence analysis of *hitA* and *horC*

TEICHERT (2008) figured out that half of the beer-spoiling strains possess a malfunctioning *horA* gene. For that reason, functionality of other beer-spoiling associated genes (*hitA* and *horC*) was checked. The *horC* genes of TMW 1.465 and 1.485 were identical to the original sequence (NCBI accession no. AB118106). In TMW 1.313, a point mutation led to a change in amino acid sequence at position 333 (threonine -> alanine). Thus, a hydrophilic amino acid was substituted by a hydrophobic amino acid. For *horC*, no assignment to functional motifs was found and influence of this mutation on protein function could not be predicted. In TMW 1.317 *horC* gene possessed a 27 bp gap (Figure 9). This resulted in a lack of 9 amino acids in protein sequence.

```
1.1282          SLKAQIISQKLTTLQKKEKLPDSAKSKLLQAKFKSQLLQAKFVTQKPVHPAQIKISINQG 165
1.485           SLKAQIISQKLTTLQKKEKLPDSAKSKLLQAKFKSQLLQAKFVTQKPVHPAQIKISINQG 165
1.465           SLKAQIISQKLTTLQKKEKLPDSAKSKLLQAKFKSQLLQAKFVTQKPVHPAQIKISINQG 165
horC_brevis     SLKAQIISQKLTTLQKKEKLPDSAKSKLLQAKFKSQLLQAKFVTQKPVHPAQIKISINQG 165
1.313           SLKAQIISQKLTTLQKKEKLPDSAKSKLLQAKFKSQLLQAKFVTQKPVHPAQIKISINQG 165
1.230           SLKAQIISQKLTTLQKKEKLPDSAKSKLLQAKFKSQLLQAKFVTQKPVHPAQIKISINQG 165
1.317           SLKAQIISQKLTTLQKKEELPDSAKS---------QLLQAKFVTQKPVHPAQIKISINQG 156
horC_backi      SLKAQIISQKLTTLQKKEELPDSAKS---------QLLQAKFVTQKPVHPAQIKISINQG 156
horC_lindneri   SLKAQIISQKLTTLQKKEELPDSAKS---------QLLQAKFVTQKPVHPAQIKISINQG 171
                ******************:*******         ************************
```

Figure 9. Alignment of published HorC sequences of *L. brevis* (NCBI accession no, BAD81049), *L. lindneri* (NCBI accession no. BAD97360), and *L. backii* (NCBI accession no BAF56899) with *L. brevis* TMW 1.1282, 1.485, 1.465, 1.313, 1.230, 1.317.

Sequencing of *hitA* revealed, that the sequences of the gene from TMW 1.313 and 1.465 coincided with the original sequence (NCBI accession no. AB035808). The sequence of *hitA* TMW 1.317 contained a point mutation at position 26 (A -> G), leading to a changed amino acid at position 9 (glycine instead of glutamic acid). Both amino acids are hydrophilic. The side chain of glycine is only one proton and is much shorter. The range of amino acid 1 to 47 is the first intracellular domain annotated (HAYASHI et al., 2000). The NRAMP domain, which is responsible for the transporter function of the protein, is annotated by amino acid 71-435. An impairment of the function is unlikely.

3.7 Metabolism in wheat beer, lager beer and pilsner beer

Due to slow visible growth of beer-spoiling bacteria, faster methods were proven for their suitability to detect microbial activity in beer.

3.7.1 Determination of metabolic activity in beer by tetrazolium

The method is based on the reduction of water-soluble tetrazolium salt by NADH deydrogenase activity. Thereby, the colorless tetrazolium is reduced by electron transfer to red formazan. In this reaction, PMS (methylphenaziniummethylsulfat) works as electron transporter from intracellular to extracellular.

All tested strains of *L. brevis* were able to reduce tetrazolium salt to formazan in alcohol free German wheat beer, including the most hop sensitive type strain TMW 1.6. Contrarily, wheat beer with alcohol inhibited growth of the type strain. Furthermore, the reduction of tetrazolium to formazan of the strains TMW 1.302

and TMW 1.1369 was decreased. In moderate hopped lager beer (16 ppm iso-α-acids) the TMW 1.313 strain showed metabolic activity within 20 h. Constitutive beer-spoiling strains could be detected within 80 h, while non-spoiling strains showed no metabolic activity over a range of 200 h (Figure 10). In strong hopped pilsner beers I, II, III (hop contents in the range from 25 to 36 ppm) strain TMW 1.313 exhibited the shortest lag phase.

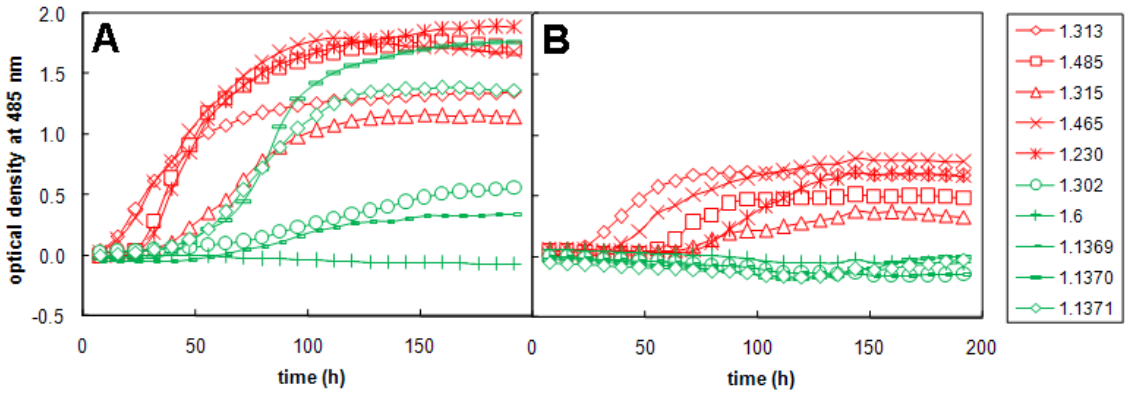


Figure 10. Reduction of tetratzolium salt depending of strain and beer (A) wheat beer; (B) lager beer of constitutive beer-spoiling (red) and non-spoiling strains (green)

3.7.2 Determination of metabolic activity in beer by resazurin

The method is using the principle of irreversible reduction of blue resazurin to pink resorufin or reversible reduction of pink resorufin to dihydroxyresorufin by $NADH_2$. $NADH_2$ is used as cofactor by many cellular dehydrogenases and can be applied as an indicator for microbial metabolic activity (Figure 11).

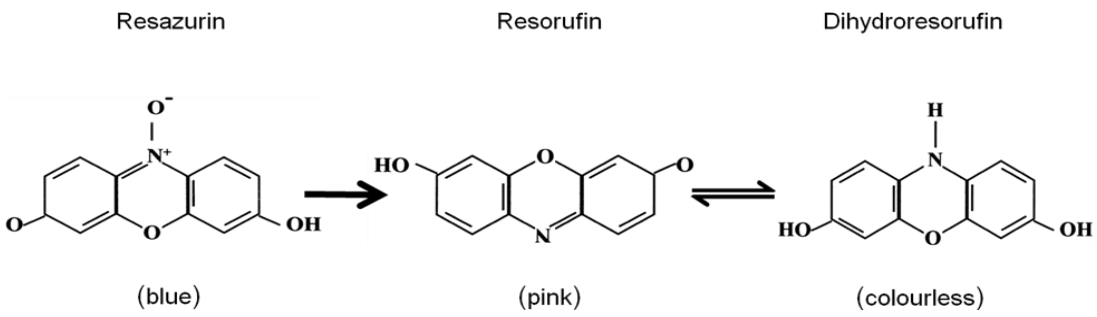


Figure 11. Gradual reduction of resazurin to resorufin (irreversible) and dihydroresorufin (reversible) by $NADH_2$ activity (TWIGG, 1945)

Non-adapted stationary phase cells of the strains TMW 1.313, 1.465, 1.317, 1.485, 1.230, 1.315 and 1.240 exhibited metabolic activity after 48 h incubation in lager beer, detected by a subsequent 1 h incubation with resazurin at pH 7.0 (Figure 12).

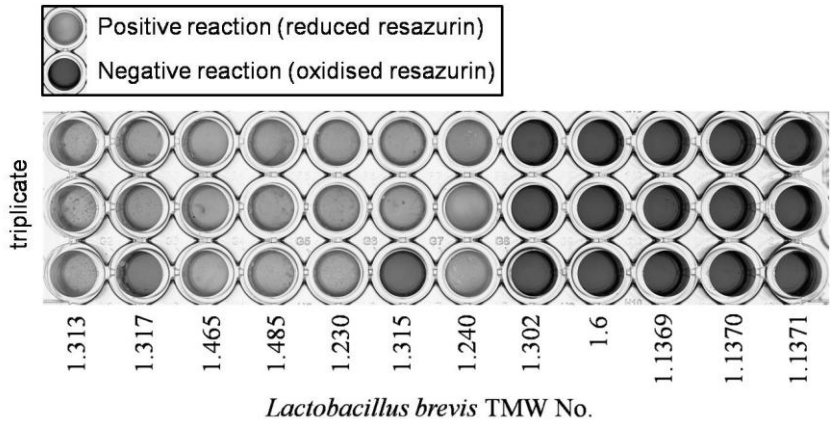


Figure 12. Color change of resazurin indicator after 48 h in a lager beer and 1 h incubationwith resazurin buffer

These strains were able to grow in wheat, lager or pilsner beer. Non-adapted cells of the strains TMW 1.302, 1.1369, 1.1370, 1.1371 and 1.6, which only grew in wheat beer, were negative in this test. Further resazurin incubation for 24 h led to positive results also for the low spoiling strains TMW 1.302, 1.1369, 1.1370 and 1.1371. Sterile controls and TMW 1.6 were negative after 24 h resazurin incubation (Figure 13).

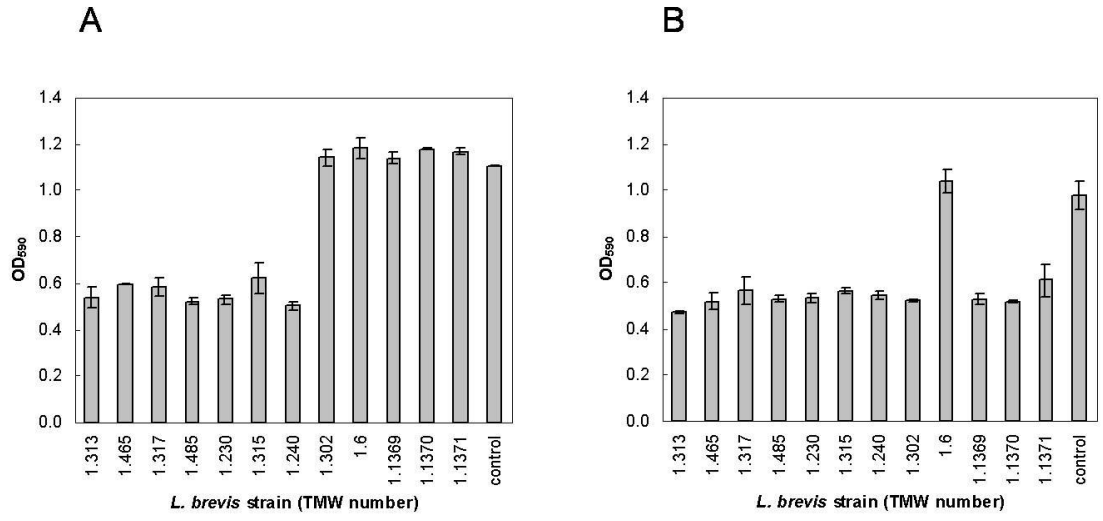


Figure 13. Optical density at 590 nm after 48 h in lager beer; (A) 1 h resazurin incubation and (B) 24 h resazurin incubation

The tests were performed in quintuplicate with different batches of beer. In some cases spoiling strains exhibited no metabolic activity within a given time. Figure 14 shows the summarized occurrence of positive results in five different batches of a respective beer.

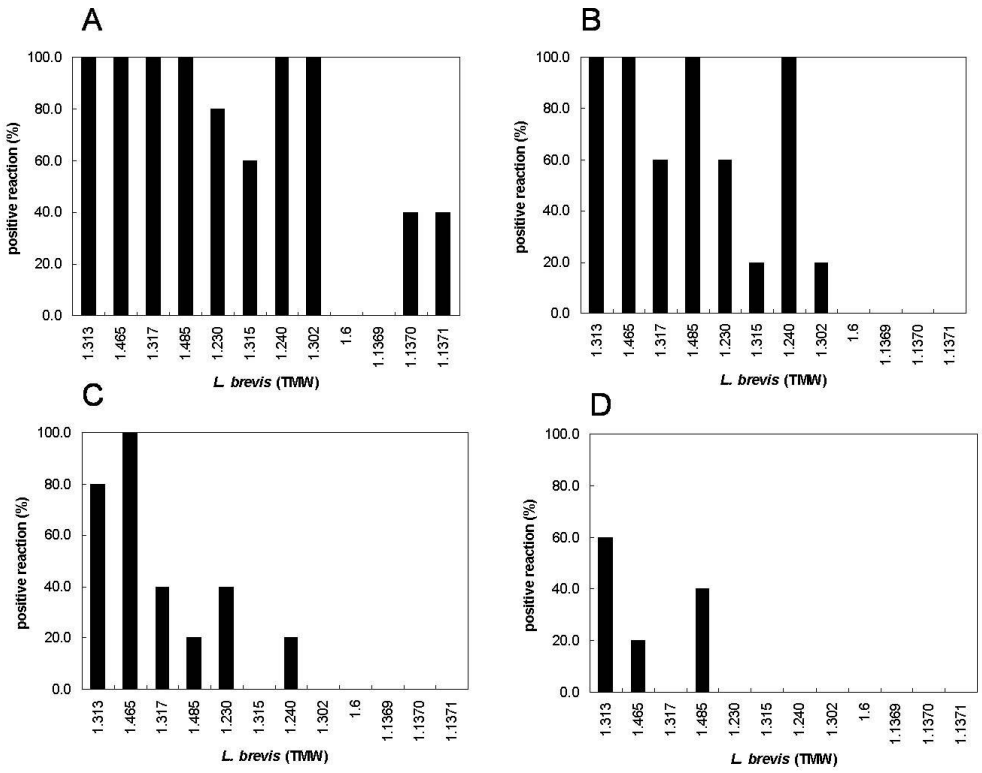


Figure 14. Positive reactions in different beers: A - wheat beer, B - lager beer, C - Pilsner beer III, D - Pilsner beer I.

3.8 RAPD-PCR , clustering and fragment identification

According to HAYASHI et al, (2001), genetic differences within constitutive beer-spoiling and non-spoiling *L. brevis* strains could be detected by unspecific RAPD-PCR. The clustering of RAPD-pattern (Figure 15) resulted in two clusters. Cluster A included seven isolates from beverage industry and one isolate from Belladonna fermentation. The isolate from softdrink TMW 1.465 exhibited strong beer spoiling ability, whereas the beer isolate TMW 1.302 possessed limited tolerance to hop. Cluster B included only non-beer isolates, except TMW 1.240.

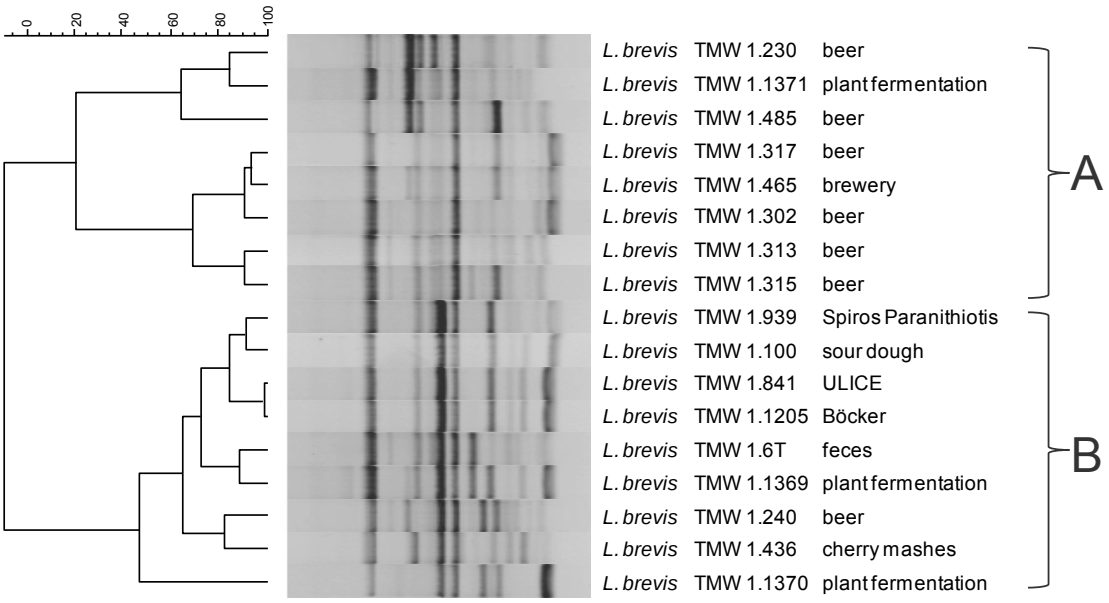


Figure 15. Ward cluster analysis of RAPD fingerprints pattern of different *L. brevis* strains isolated from different origins

In cluster B an 1100 bp fragment was detected (Figure 16). The fragment was sequenced and identified as methionine adenosyltransferase. On the basis of the sequence, a specific primer couple was constructed and the gene determined by PCR reaction. All strains were tested positive by PCR.

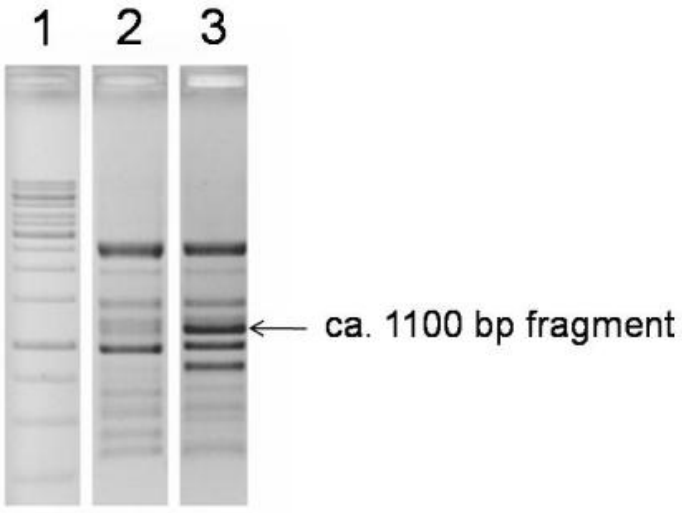


Figure 16. 1 = 1 kb DNA ladder; 2 = *Lactobacillus brevis* TMW 1.313; 3 = *Lactobacillus brevis* TMW 1.6

3.9 Differential proteome analysis of exponential cells

For identification of characteristic traits within the categories of constitutive beer-spoiling and non-spoiling strains, comparative 2D gel electrophoresis was performed. Therefore, whole-cell protein was isolated from constitutive beer-spoiling strains *L. brevis* TMW 1.313, 1.465, 1.485, 1.230, 1.317, 1.315, 1.240 and compared with whole cell protein from non-spoiling strains *L. brevis* TMW 1.302 (non-spoiling strain but beer isolate) 1.6, 1.1369, 1.1370 and 1.1371 in exponential growth phase ($OD_{590\ nm}$ = 0.5). In order to identify general mechanisms of constitutive hop resistance in exponential phase, the beer-spoiling strains were pooled into a group of beer-spoilers and compared with the group of non-spoiling strains. Proteins were identified by LC-MS/MS (Table 20). No protein could be identified, which was expressed in all investigated beer-spoiling strains. Spot 1 could be identified as Zn-dependent alcohol dehydrogenase, which was expressed in 6 of 7 beer-spoiling strains and in none of the 5 strains in the non-spoiling group (Figure 17). Spot 2, an ATP-dependent ClpL protease could be detected in 4 of 7 beer-spoiling strains and in none of 5 non-spoiling strains (Figure 18). Spot 3, an arginine deiminase, could be detected in 3 of 7 beer-spoiling and in 1 of 5 non-spoiling strains (Figure 19). Spot 4, identified as maltose phosphorylase, was expressed in the strong beer-spoiling strains TMW 1.313 in glucose-containing medium (Figure 20).

Table 20. Identified proteins, which were expressed of beer-spoiling strains in exponential growth phase

spot	Mw (kDA)	PI	Accession nr.	function
1	39	5.20	gi|116333550|	Zn-dependent alcohol dehydrogenase
2	78	5.44	gi|28379892|	ATP-dependent Clp protease
3	46	4.96	gi|116334588|	arginine deiminase
4	88	4.60	gi|116333027|	maltose phosphorylase

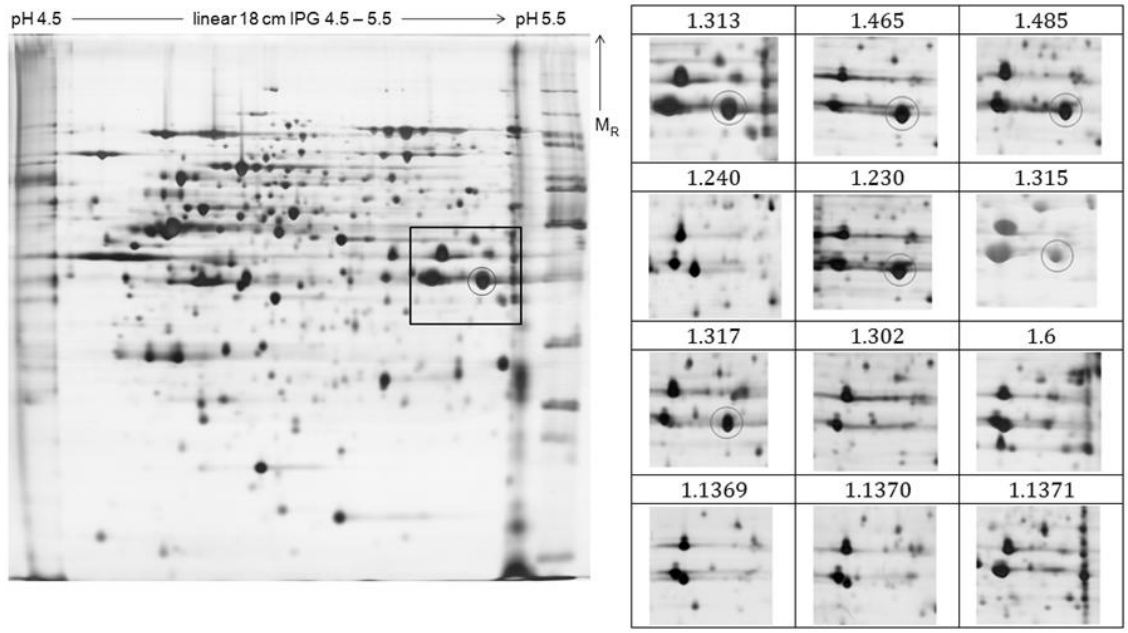


Figure 17. Zn-dependent alcohol dehydrogenase expressed in beer-spoiling strains (TMW 1.313, 1.465, 1.485, 1.230, 1.315 and 1.317). In the beer-spoiling strain 1.240 and the non-spoiling strains TMW 1.302, 1.6, 1.1369, 1.1370 and 1.1371 no expression could be detected.

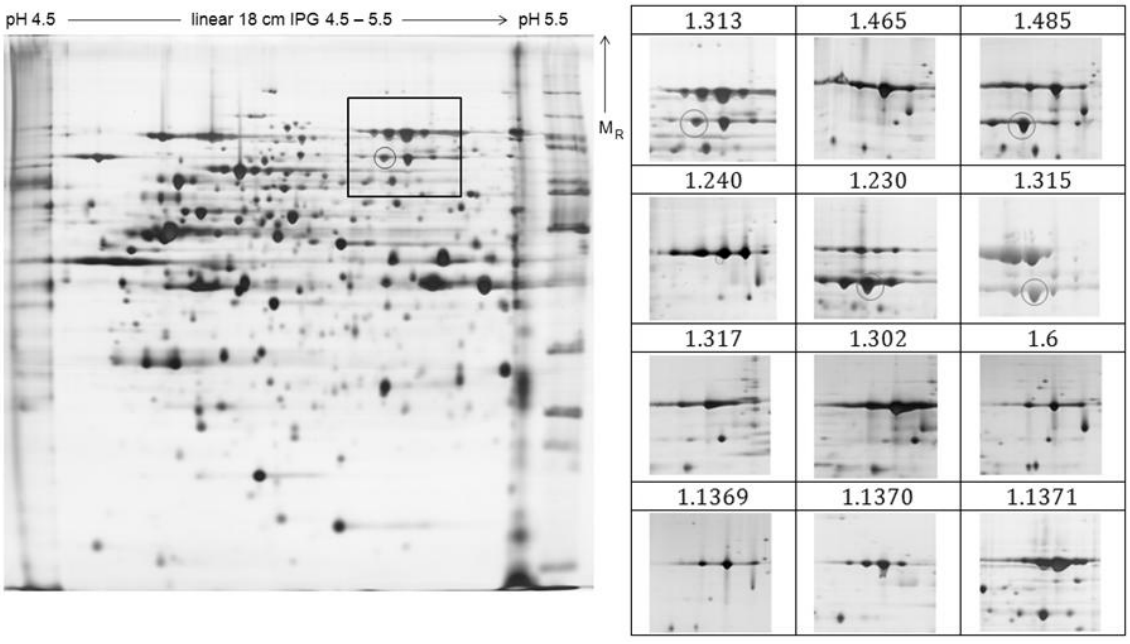


Figure 18. ClpL protease expressed in beer-spoiling strains (TMW 1.313, 1.485, 1.230, and 1.315). In the beer-spoiling strains TMW 1.465, 1.240 and 1.302 and in the non-spoiling strains TMW 1.302, 1.6, 1.1369, 1.1370 and 1.1371 no expression could be detected.

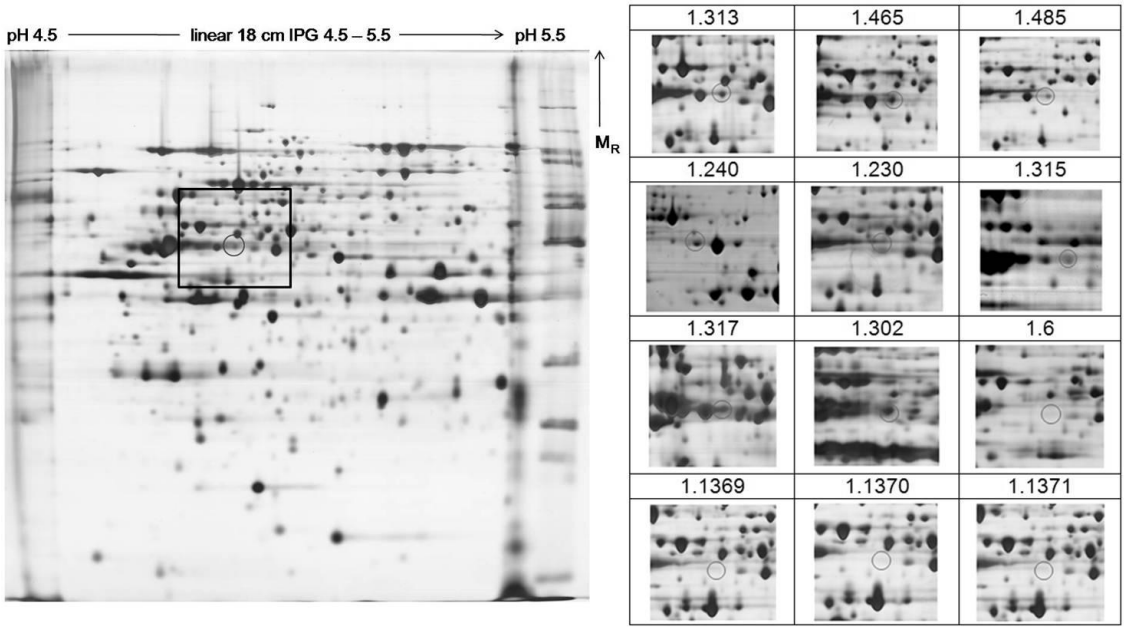


Figure 19. Arginine deiminase expressed in beer-spoiling strains (TMW 1.313, 1.465, 1.485, 1.240, 1.315 and 1.317) and in TMW 1.302. In the beer-spoiling strain 1.230 and the non-spoiling strains TMW 1.6, 1.1369, 1.1370 and 1.1371 no expression could be detected.

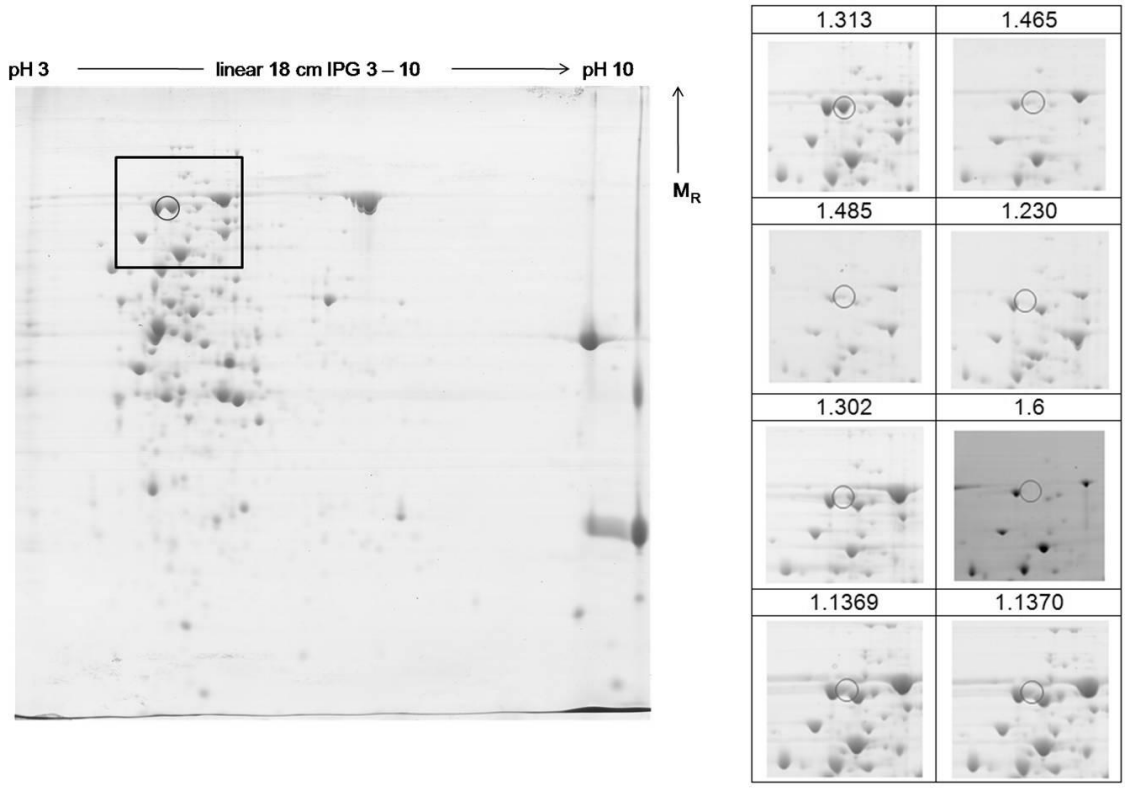


Figure 20. Maltose phosphorylase expression in beer-spoiling strain TMW 1.313. In the beer-spoiling strain 1.465, 1.485, 1.230 and the non-spoiling strains TMW 1.302, 1.6, 1.1369, 1.1370 no expression could be detected.

3.10 Detection of genes contributing to an enhanced beer-spoiling potential from proteomic approach

For validation of results of proteomic approach and development of new markers, PCR analysis was performed of genes, whose proteins were exclusively expressed in the beer-spoiling group. In case of arginine deiminase and maltose phosphorylase, all tested strains were positive. Differences could be detected in occurrence of zinc-dependent alcoholdehydrogenase and ClpL protease (Table 21).

Table 21. Comparison of protein expression vs. presence or absence of genes in beer-spoiling (red) and non-spoiling (green) *L. brevis* strains. In proteomic approach, grey field represents expression of protein and white field represents no expression. In genetic approach, grey fields represents occurrence of gene and white field absence of genes. (* not analyzed)

Proteomic approach	*Zn ADH*	*ClpL*	*ArcA*	*MP*	Genetic approach	*adh*	*clpL*	*arcA*	*MP*
L. brevis TMW 1.313	■	■	■	■	*L. brevis* TMW 1.313	■	■	■	■
L. brevis TMW 1.317	■			*	*L. brevis* TMW 1.317	■		■	■
L. brevis TMW 1.465	■		■		*L. brevis* TMW 1.465	■		■	■
L. brevis TMW 1.485	■	■			*L. brevis* TMW 1.485	■	■	■	■
L. brevis TMW 1.230	■	■			*L. brevis* TMW 1.230	■	■	■	■
L. brevis TMW 1.240				*	*L. brevis* TMW 1.240			■	■
L. brevis TMW 1.315	■	■	■	*	*L. brevis* TMW 1.315	■	■	■	■
L. brevis TMW 1.302					*L. brevis* TMW 1.302	■		■	■
L. brevis TMW 1.6					*L. brevis* TMW 1.6	■		■	■
L. brevis TMW 1.1369					*L. brevis* TMW 1.1369	■		■	■
L. brevis TMW 1.1370					*L. brevis* TMW 1.1370	■		■	■
L. brevis TMW 1.1371				*	*L. brevis* TMW 1.1371			■	■

3.11 Differential proteome analysis of stationary cells

To get a closer view into cellular mechanisms of constitutive beer-spoilers, whole-cell protein from non-adapted beer-spoiling strains *L. brevis* TMW 1.313, 1.465 and 1.485 was isolated and compared with whole cell protein from non-spoiling strains *L. brevis* TMW 1.6, 1.1369 and 1.1370 in stationary growth phase (Table 22).

Table 22. Maximum optical density in stationary phase at growth in MRS4 medium

strain	$OD_{590\ nm}$
L. brevis TMW 1.313	3.69 ± 0.06
L. brevis TMW 1.465	4.14 ± 0.02
L. brevis TMW 1.485	2.98 ± 0.04
L. brevis TMW 1.6	2.13 ± 0.24
L. brevis TMW 1.1369	3.20 ± 0.06
L. brevis TMW 1.1370	4.38 ± 0.01

High-resolution IPGs with 24 cm separation distances in the range from pH 4.5 to 5.5 were used in the first dimension. The second dimension exhibited a separation range from 10 to 250 kDa. In order to identify general mechanisms of constitutive hop resistance in stationary phase, the beer-spoiling strains were pooled into the group of beer-spoilers and compared with the group of non-spoiling strains. In total, five proteins (eID 0003, 0885, 1713, 1782 and 2035; Figure 21), which were only expressed or overexpressed in the beer-spoiling group, could be detected.

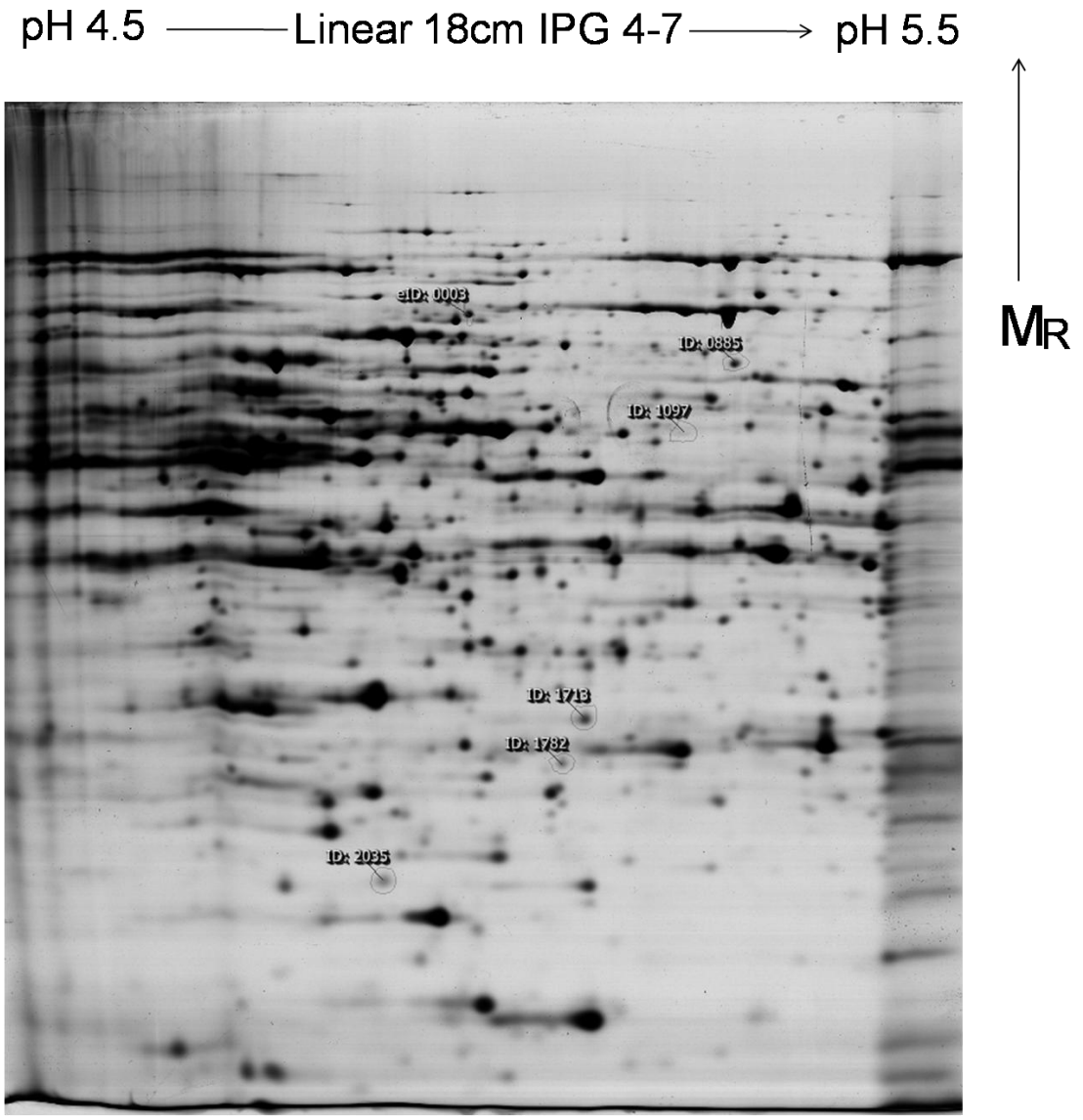


Figure 21. Two-dimensional electrophoretic analysis of silver stained total protein from cells of *L. brevis* TMW 1.313

Furthermore, one protein (eID 1097) was only expressed in the group of nonspoiling strains. The expression profiles of all detected group-specific proteins are depicted in Figure 22 – Figure 27.

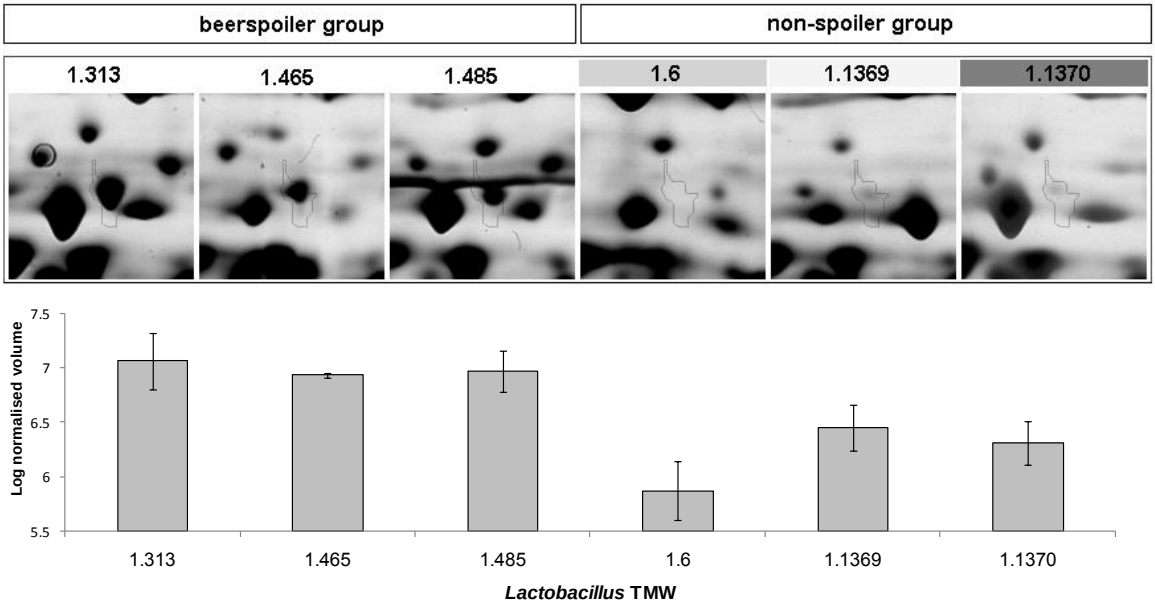


Figure 22. Differential expression analysis of protein 0003 (NAD dependent ligase) depicted as logarithmic normalized spot volume of beer-spoiler and non-spoiler group

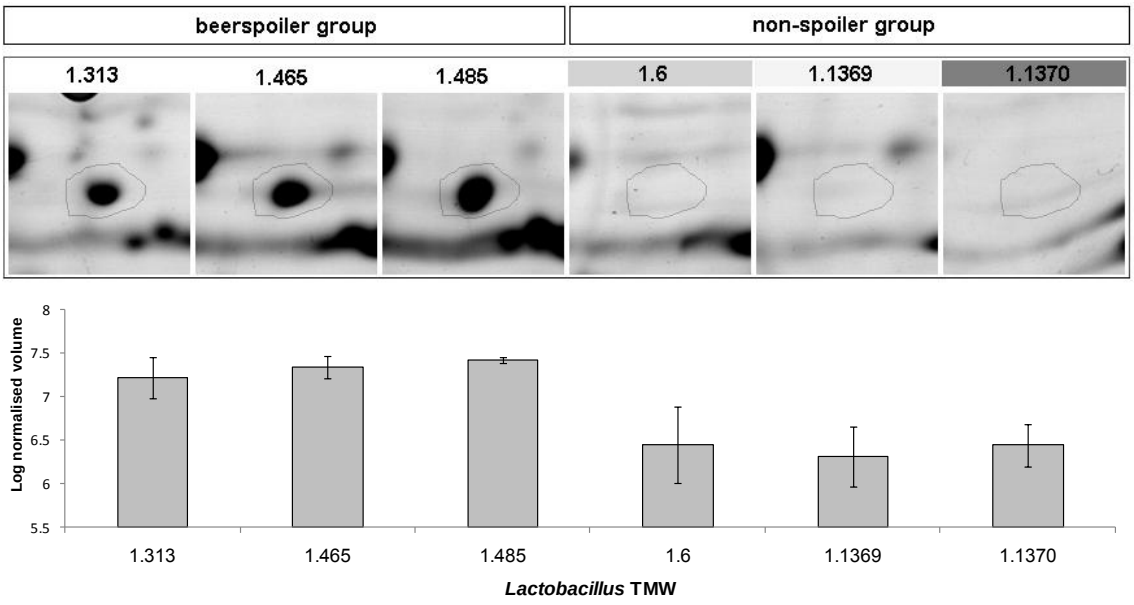


Figure 23. Differential expression analysis of protein 0885 (Putative multicopper oxidase) depicted as logarithmic normalized spot volume of beer-spoiler and non-spoiler group

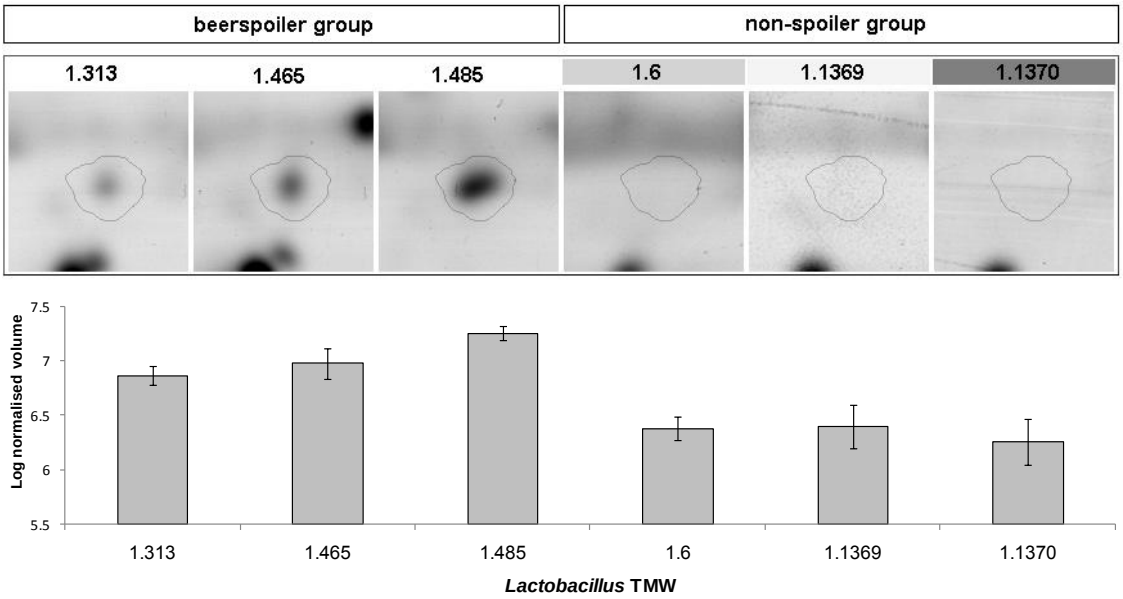


Figure 24. Differential expression analysis of protein 1713 (3-hydroxyisobutyrate dehydrogenase) depicted as logarithmic normalized spot volume of beer-spoiler and non-spoiler group

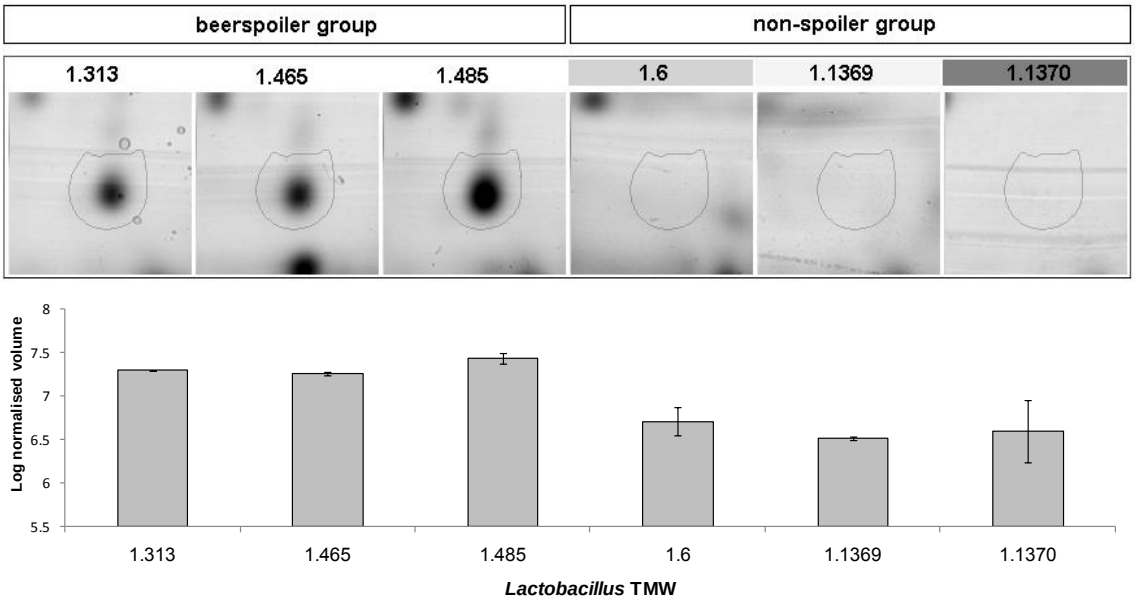


Figure 25. Differential expression analysis of protein 1713 (3-hydroxyisobutyrate dehydrogenase) depicted as logarithmic normalized spot volume of beer-spoiler and non-spoiler group

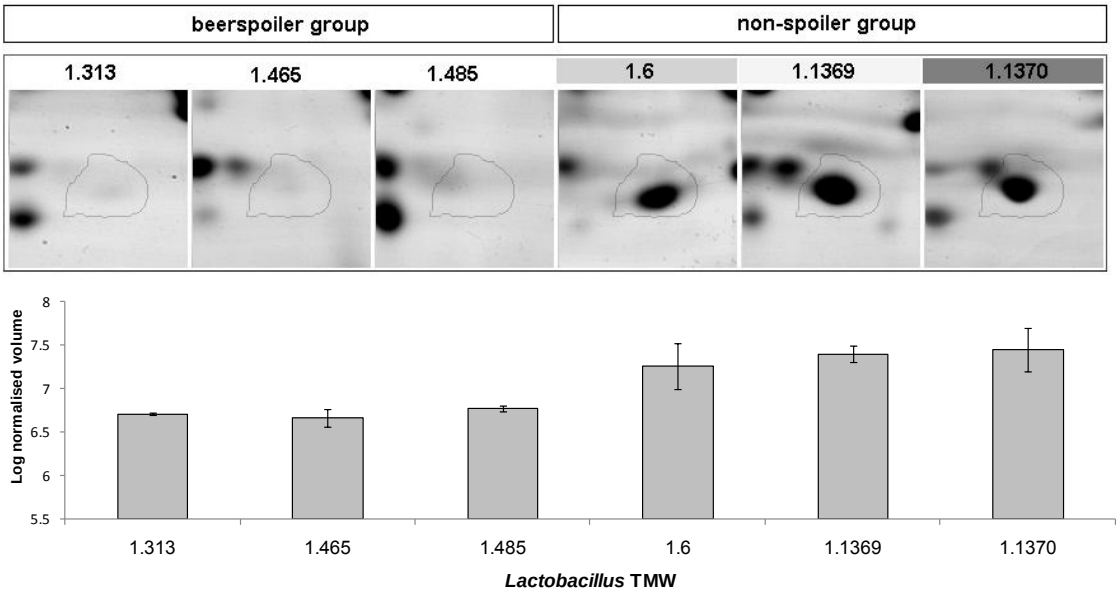


Figure 26. Differential expression analysis of protein 1079 depicted as logarithmic normalized spot volume of beer-spoiler and non-spoiler group

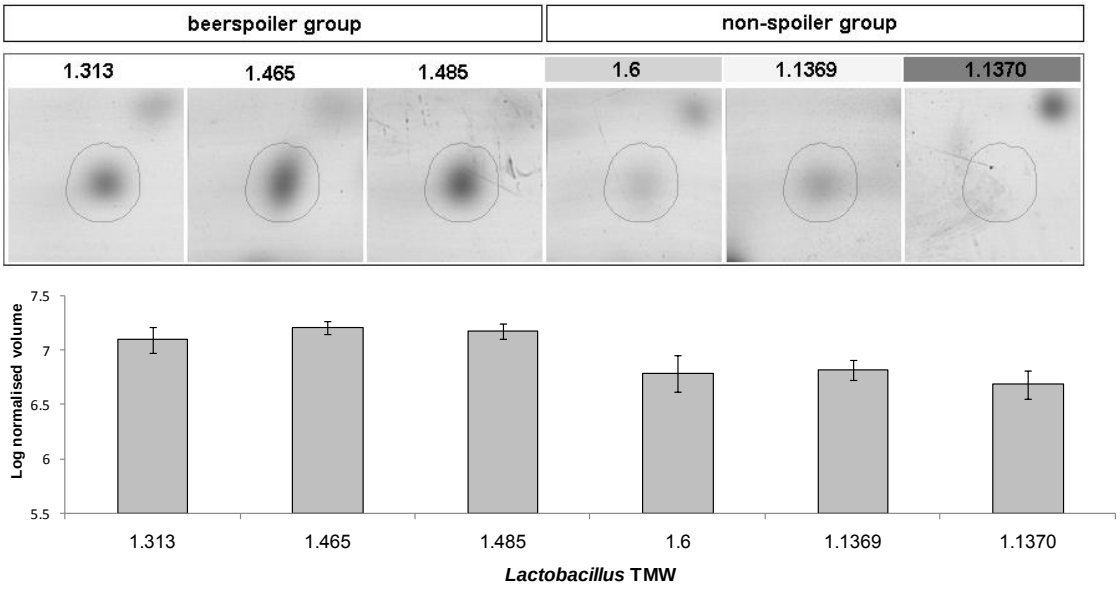


Figure 27. Differential expression analysis of protein 2035 depicted as logarithmic normalized spot volume of beer-spoiler and non-spoiler group

3.12 Protein identification

Protein analysis was performed by mass spectrometry. All differentially expressed proteins in the beer-spoiler group were analysed by LC-ESI MS/MS analysis and mass spectra were interpreted by the Matrix Science Mascot software. Five of six proteins could be identified according to Table 23.

Table 23. Proteins present in stationary phase of beer-spoiling strains in comparison to nonspoiling strains identified by LC-ESI MS/MS

eID	Mw (kDA)	PI	Accession nr.	function
0003	73	5.00	gi|116334213	NAD-dependent DNA ligase
0885	57	5.13	gi|116098407	Putative multicopper oxidase
1713	31	5.17	gi|116334365	3-hydroxyisobutyrate dehydrogenase
1782	27	5.21	gi|116333053	peptide ABC transporter ATPase
2035	19	4.86	gi|116098215	Predicted acetyltransferase

3.13 Analysis of genes contributing to beer-spoiling ability in stationary growth phase

The presence or absence of genes, which are constitutively expressed, but differentially present in beer-spoiling strains in comparison to non-spoiling strains, were checked by specific PCR reaction. All strains were positive for the genes of acetyltransferase (LVIS_0198), peptide ABC transporter ATPase (LVIS_0387), 3-hydroxyisobutyrate dehydrogenase (LVIS_1796), putative multicopper oxidase (LVIS_0394) and NAD-dependent DNA ligase (LVIS_1633) (Table 24). A differentiation of beer-spoiling and non-spoiling strains on genetic level using the genes of overexpressed proteins as marker genes was not realizable.

Table 24. Comparison of protein expression vs. presence or absence of genes in beer-spoiling (red) and non-spoiling (green) *L. brevis* strains. In proteomic approach, grey field represents expression of protein and white field represents no expression. In genetic approach, grey fields represent occurrence of gene and white field absence of genes.

Proteomic approach	*LVIS_1633*	*LVIS_0394*	*LVIS_1796*	*LVIS_0387*	*LVIS_0198*	Genetic approach	*LVIS_1633*	*LVIS_0394*	*LVIS_1796*	*LVIS_0387*	*LVIS_0198*
L. brevis TMW 1.313	■	■	■	■	■	*L. brevis* TMW 1.313	■	■	■	■	■
L. brevis TMW 1.465	■	■	■	■	■	*L. brevis* TMW 1.465	■	■	■	■	■
L. brevis TMW 1.485	■	■	■	■	■	*L. brevis* TMW 1.485	■	■	■	■	■
L. brevis TMW 1.6						*L. brevis* TMW 1.6	■	■	■	■	■
L. brevis TMW 1.1369						*L. brevis* TMW 1.1369	■	■	■	■	■
L. brevis TMW 1.1370						*L. brevis* TMW 1.1370	■	■	■	■	■

3.14 Bacterial growth depending on manganese content in growth media

Manganese is an essential trace element for lactic acid bacteria and influences the inhibitory effects of hop (BEHR and VOGEL, 2009/2010). To investigate the effect of manganese on constitutive beer-spoiling and non-spoiling strains, growth under manganese deficiency (0.16 mg/l) and reference conditions (30 mg/l) was recorded. Growth after 60 h of incubation, measured via optical density, was decreased under manganese deficiency in the range between 12.79 and 49.02 % (Table 25) in comparison to the reference condition.

Table 25. Comparison of maximum optical density depending on manganese content in growth medium

***L. brevis* strain (TMW)**	**Control (OD_{590})**	**Manganese deficiency (OD_{590})**	**(%)**
1.485	1.54 ± 0.05	0.79 ± 0.09	50.98
1.313	1.68 ± 0.02	0.95 ± 0.10	56.57
1.465	1.69 ± 0.03	1.04 ± 0.04	61.54
1.317	1.38 ± 0.35	0.91 ± 0.09	66.10
1.230	1.56 ± 0.02	1.03 ± 0.11	66.27
1.315	1.43 ± 0.15	1.19 ± 0.14	83.62
1.240	1.73 ± 0.05	1.50 ± 0.15	87.21

The growth behavior of strain TMW 1.240 was nearly unaffected of manganese content in the medium. In contrast to that, the growth of strain TMW 1.313 was strongly decreased under manganese deficiency conditions (Figure 28).

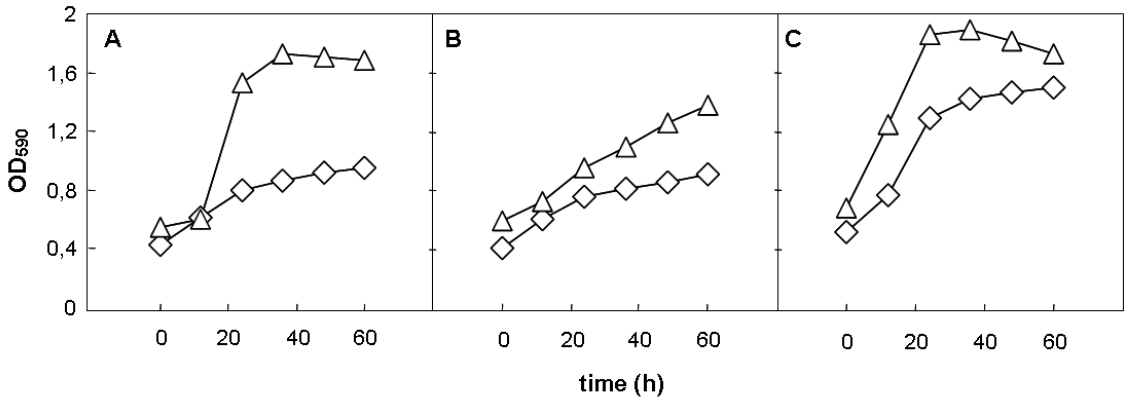


Figure 28. Differences in growth behavior of three different strains (A – TMW 1.313, B – TMW 1.317 and C - TMW 1.240) depending on manganese in growth medium. Triangle = 30 mg/L manganese; rhombus = 0.16 mg/l.

3.15 Cellular manganese content depending on manganese content in growth media

The method is using the principle of calcein fluorescence quenching (HASINOFF 2003; TOMITA et al. 2008), whereas increasing concentrations of manganese result in decreasing fluorescence measures of calcein. For releasing intracellular manganese, cell membranes were destroyed by cooking. Released manganese was detected in the supernatant and correlated to intracellular manganese level. Thereby, a comparison of intracellular manganese depending on growth conditions is possible. Cells grown under manganese-starvation exhibited decreased manganese levels in the range of 0.23 to 1.32 µM (manganese measured as manganese release of cells in 1 ml detection buffer; cell density was set to OD_{590}=1). Under reference conditions (30 mg manganese per liter) manganese release of cells to detection buffer was between 13.75 to 62.01 µM (Figure 29).

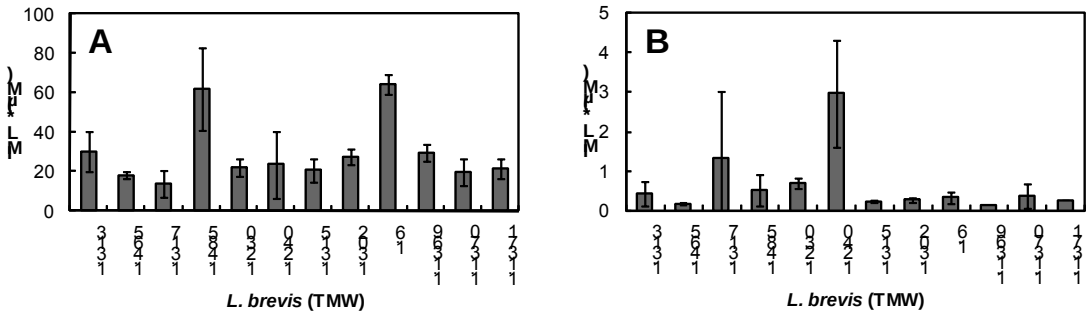


Figure 29. Dependence of manganese content in growth media on intracellular manganese level (IML) for (A), 30 mg/l manganese and (B) 0.16 mg/l manganese (*release of cells set to OD_{590} = 1.0 in one ml of detection buffer)

3.16 Effect of intracellular manganese level on beer-spoiling ability

Manganese-starved cells exhibited, in comparison to reference conditions, better growth in stronger hopped beer (pilsner beer). This was indicated by metabolic activity (Figure 30). For lower hopped beer (wheat beer, lager beer) as well as for long-term incubation experiments (4 weeks) in the respective beers, this effect was not observed (Table 26).

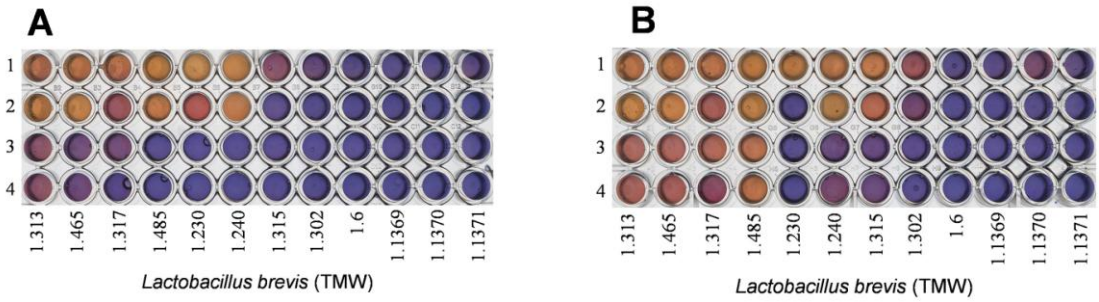


Figure 30. Metabolic activity in different beers (1), wheat beer, (2) lager beer, (3) pilsner beer I and (4), pilsner beer II after precultivation in mMRS4 (A), 30 mg/l manganese and (B) 0.16 mg/l manganese (blue = negativ reaction)

Table 26. Summarized counts of metabolic activity lacking ~~-s~~poiling" strains inoculated in different beers depending on preculture medium after (A) 3 days and (B) 28 days

	Mn⊕ (A)	Mn- (A)	Mn⊕ (B)	Mn- (B)
Wheat beer	1	0	1	1
Lager beer	2	2	1	2
Pilsner beer I	14	7	5	4
Pilsner beer II	16	7	4	4
total	**33**	**16**	**11**	**11**

3.17 Differential proteome analysis of manganese-starved cells in exponential growth phase

Differential proteomic should be used to understand the influence on hop tolerance level of manganese-starved cells. Therefore, whole-cell proteins were isolated from *L. brevis* TMW 1.317, 1.485 and 1.240 under reference and manganese-starvation conditions. High-resolution IPGs with 24-cm separation distances in the range from pH 4 to 7 were used in the first dimension. The second dimension exhibited a separation range from 10 to 250 kDa. In order to identify general manganese-starvation overexpressed proteins in *L. brevis*, the experimental data of all three strains were joined in a reference conditions group and a manganese deficiency group for 2D gel image analysis. Under these settings no proteins could be identified, which were overexpressed by all three strains in a similar manner. When strain TMW 1.240 was excluded from the expression analysis, five proteins were identified to be overexpressed as a result of manganese-starvation in TMW 1.485 as well as in TMW 1.317. The expression values of hop-overexpressed proteins are depicted in Table 27. Finally, the proteome of TMW 1.240 under reference condition and manganese-starvation was analysed separately and no significant differences in expression profile could be detected.

Table 27. Proteins expressed under manganese-starvation identified by LC-ESI MS/MS

Protein	Anova (p)	Fold	Function and accession no.
13	0.04	10.2	carbamate kinase (YP_796288)
22	0.03	7.8	not determined
30	0.03	6.9	Asp-tRNA-Asn/Glu-tRNA-Gln amidotransferase A subunit (YP_795737)
102	0.04	3.4	6-phosphogluconate dehydrogenase (YP_794296)
245	0.05	13.5	2-hydroxyacid dehydrogenase (ABJ63312)

3.18 Differential proteome analysis of manganese-starved cells in stationary growth phase of TMW 1.240

In exponential growth phase, manganese-starved cells of *L. brevis* TMW 1.240 exhibited no differences in 2D pattern in comparison to reference conditions. In contrast to that, cells in stationary phase grown under reference conditions showed differences in expression pattern of proteins. Thereby, in manganese-starved cells in stationary phase, four proteins could be detected as overexpressed (Figure 31). In contrast to that, three over-expressed proteins in reference proteome could be detected and identified (Table 28).

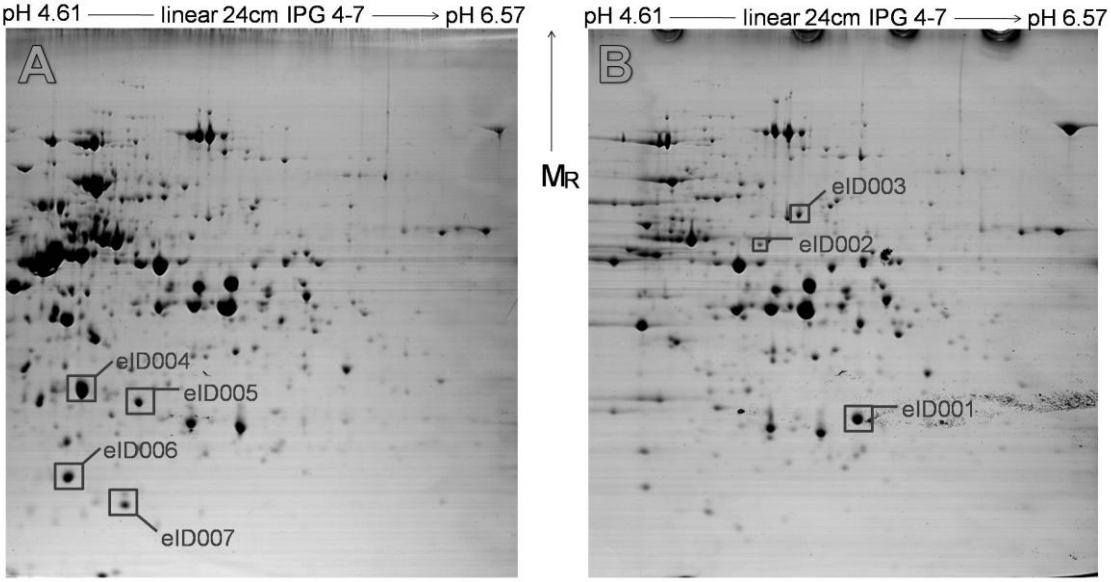


Figure 31. Two-dimensional electrophoretic analysis of silver stained total protein from cells of *L. brevis* TMW 1.240 under (A), low intracellular manganese content (B) and high intracellular mangenese content

Table 28. Differently expressed proteins under low intracellular manganese content and high intracellular mangenese content of L. brevis TMW 1.240

eID	Mw (kDA)	PI	Accession nr.	function
0001	27	5.44	gi|30749782	Chain A, R-Alcohol dehydrogenase
0002	47	5.14	gi|116332897	adenylosuccinate synthase
0003	57	5.12	gi|116334166	gluconate kinase
0004	27	4.86	gi|116332857	acetoin reductase
0005	32	5.13	gi|116334718	transcriptional regulator and fructokinase
0007	22	5.03	gi|116333275	ribosome-associated protein Y

3.19 Influence of low manganese and beer environment on intracellular trace elements

As described previously, low content of manganese in growth media (0.16 mg/l) resulted in low intracellular manganese concentrations (8386 mg/kg to 149 mg/kg DW). To determine, whether there is an effect of manganese-starvation also on other trace elements, intracellular concentrations of aluminum, calcium, magnesium, sodium, potassium, iron, copper and zinc were determined. Thereby, the low manganese content in growth medium resulted in a decreased intracellular concentration of calcium (299 mg/kg to 195 mg/kg DW), potassium (21425 mg/kg to 4890 mg/kg DW) and zinc (83 mg/kg to 38 mg/kg DW). In contrast to that, intracellular concentrations of sodium (196 mg/kg to 331 mg/kg DW), iron (15 mg/kg to 34 mg/kg DM) and copper (17 mg/kg to 46 mg/kg DW) were increased (Figure 32).

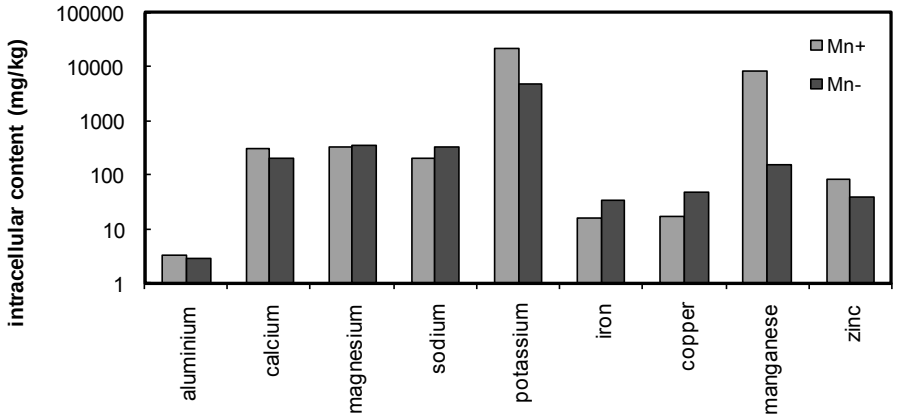


Figure 32. Content of intracellular trace elements in *L. brevis* TMW 1.313 depending on manganese concentration in growth medium (Mn+ = 30 mg/l, Mn- = 0.16 mg/l)

Subsequently, the effect of beer incubation on change of intracellular trace elements was investigated. Therefore, cells were precultured either in medium I (30 mg/l manganese) or medium II (0.16 mg/l manganese) and afterwards incubated in pilsner beer with 25 ppm iso-α-acids. For cells, which are pre-cultured in medium I, the concentrations of calcium (299 mg/kg to 1071 mg/kg DW), magnesium (333 mg/kg to 2892 mg/kg DW) and iron (15 mg/kg to 34 mg/kg DW) were increased. Furthermore, the beer incubation resulted in a

decrease of intracellular sodium (196 mg/kg to 2 mg/kg DW), potassium (21425 mg/kg to 123 mg/kg DW) and zinc (83 mg/kg to 8 mg/kg DW) (Figure 33).

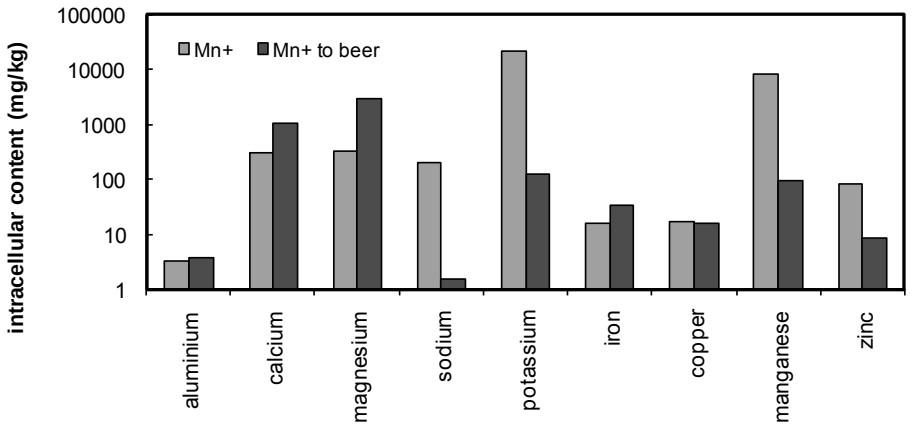


Figure 33. Change of intracellular trace elements in *L. brevis* TMW 1.313 due to incubation in pilsner beer (25 ppm iso-α-acids) after precultivation in manganese-rich medium (30 mg/l)

Same effects could be observed in manganese-starved cells, which were first cultured in medium II (Mn-) and subsequently incubated in beer. Also, the concentrations of calcium (195 mg/kg to 933 mg/kg DW), magnesium (358 mg/kg to 2308 mg/kg DW) and iron (34 mg/kg to 75 mg/kg DW) were increased. The contents of sodium (331 mg/kg to 10 mg/kg DW), potassium (4890 mg/kg to 103 mg/kg DW) and zinc (38 mg/kg to 4 mg/kg DW) were decreased (Figure 34). Interestingly, manganese-starved cells exhibited another decrease of intracellular manganese after beer incubation (149 mg/kg to 10 mg/kg DW).

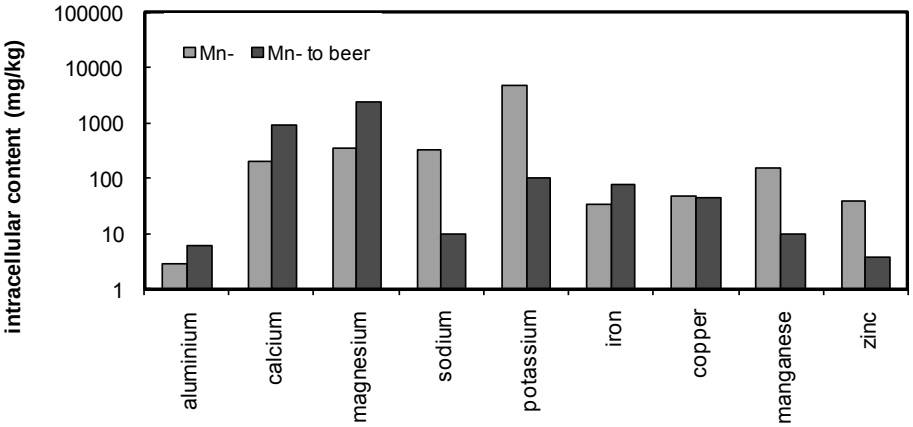


Figure 34. Change of intracellular trace elements in *L. brevis* TMW 1.313 due to incubation in pilsner beer (25 ppm iso-α-acids) after precultivation in medium with low manganese content (0.16 mg/l)

3.20 Influence of low manganese and beer environment on fatty acid composition in cell membrane

Manganese-starved cells exhibited increased tolerance against hop compounds. Therefore, the influence of manganese-starvation and beer incubation on fatty acid composition of the cell membrane was investigated. The comparison of cells, incubated in medium I (30 mg/l manganese) and medium II (16 mg/l manganese), exhibited that the fatty acids 12:0, 16:0 3 OH were detectable only in medium I. An increased percentage of the fatty acids 17:0 cyclo, 18:0, 19:0 cyclo w8c and 20:2 w6,9c could be determined after incubation in beer, whereas the percentage of 18:1 w9c was decreased (Figure 35).

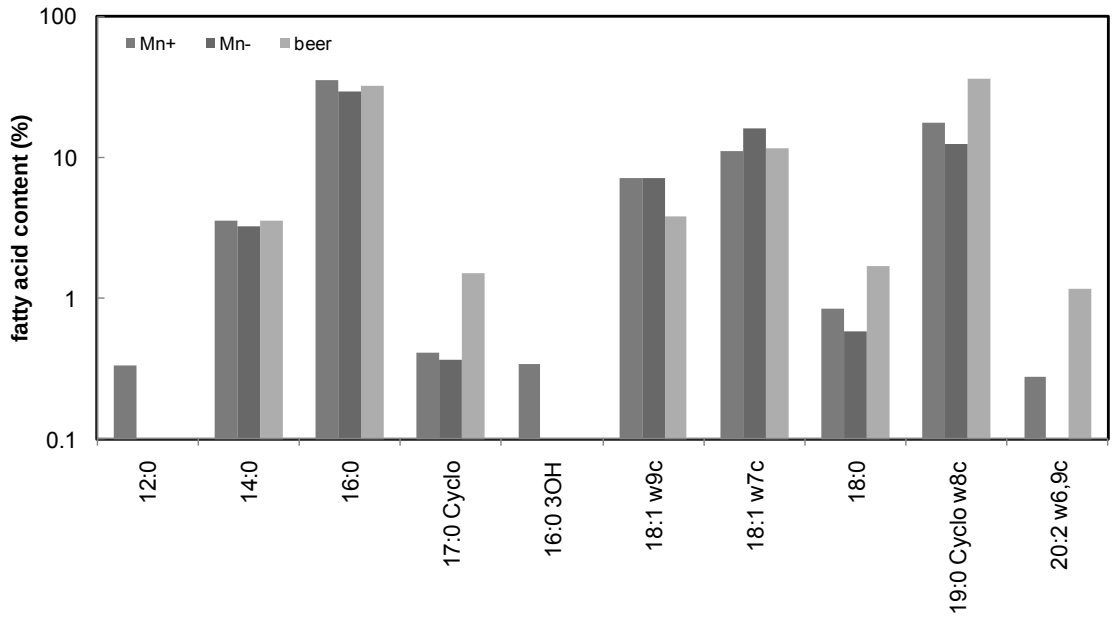


Figure 35. Change in fatty acid composition in *L. brevis* TMW 1.313 depending on growth medium after 2 days at 30 °C

3.21 Comparison of fatty acid composition of beer-spoiling and non-spoiling strains

The fatty acid compositions of beer-spoiling and non-spoiling *L. brevis* strains were analyzed to investigate group-specific differences in this both ecotypes. For this purpose, two beer-spoiling strains (*L. brevis* TMW 1.314 and 1.465) and two non-spoiling strains (*L. brevis* TMW 1.6^T and 1.1369) were grouped and compared. Differences in fatty acid composition could be detected in the increased amount of 14:0, 17:0 Cyclo and 19:0 iso (Figure 36).

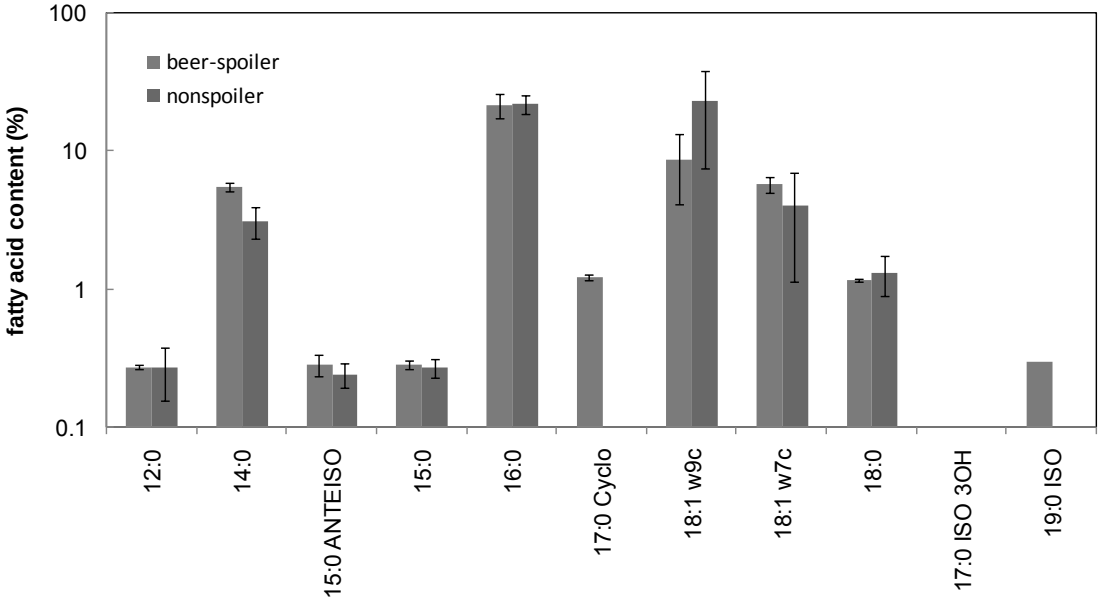


Figure 36. Fatty acid composition of beer-spoiling and non-spoiling *L. brevis* strains after growth in manganese-rich medium (30 mg/l) for 2 days at 30 °C

3.22 Strainlevel differentiation MALDI TOF-MS spectra versus RAPD-PCR

Due to the detection limit of minimal 10 kDa large proteins by 2D gel electrophoresis, further characteristic traits of constitutive beer-spoiling and non-spoiling *L. brevis* strains were investigated by MALDI-TOF MS. Furthermore, the ability of MALDI to separate different *L. brevis* strains was examined. Therefore, from three independent colonies of every strain, three mean spectra summarized from 240 single spectra were generated and clustered in BioNumerics (Pearson, UPGMA). A strain specific clustering was given, when all three mean spectra coincided in the same cluster. This was the case for *L. brevis* TMW 1.1370, 1.1371, 1.6, 1.302, 1.12161 1.1369 and 1.313 (Figure 37).

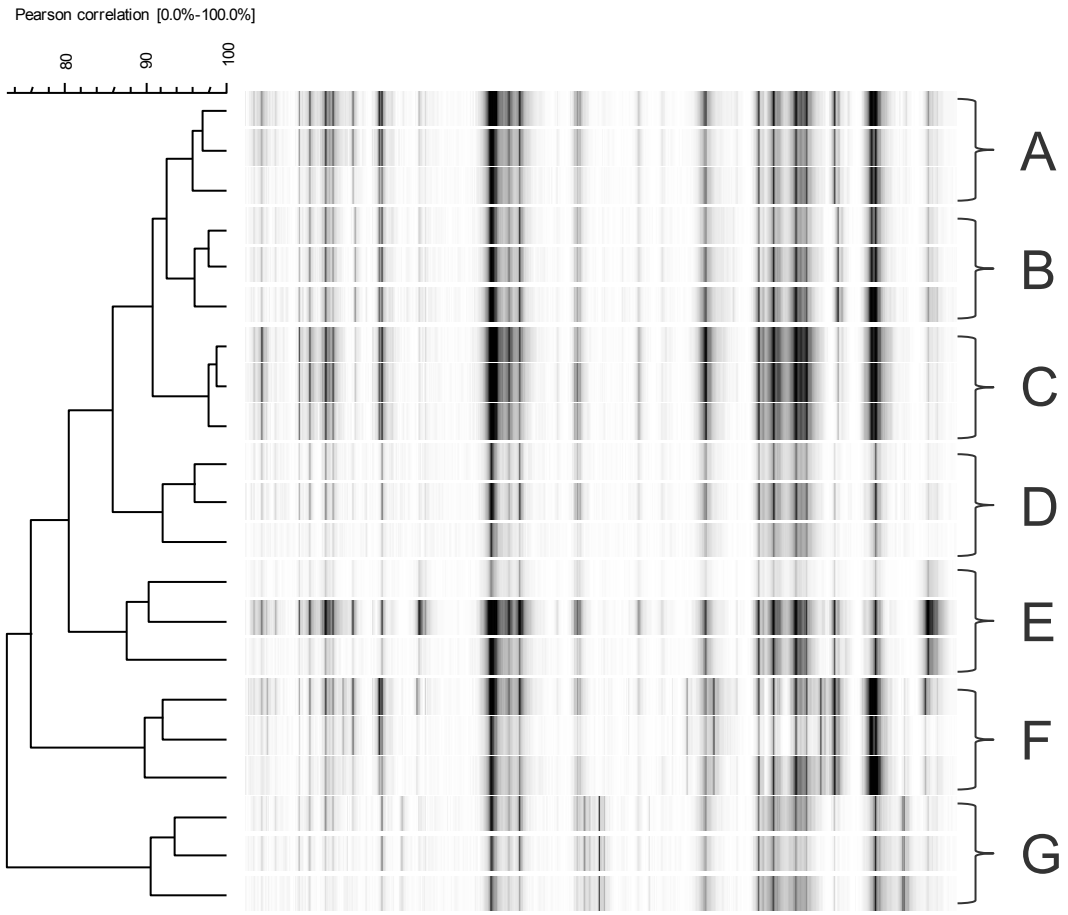


Figure 37. UPGMA cluster analysis of MALDI-TOF MS spectra of different *L. brevis* strains isolated from different origins (A) TMW 1.1370, (B) TMW 1.1371, (C) TMW 1.6, (D) TMW 1.302, (E) TMW 1.1216, (F) TMW 1.1369 (G) TMW 1.313

In contrast to that, the strains TMW 1.485 and 1.230 as well as the strains TMW 1.317 and 1.315 could not be differentiated by MALDI-TOF and subsequent clustering of mean spectra (Figure 38).

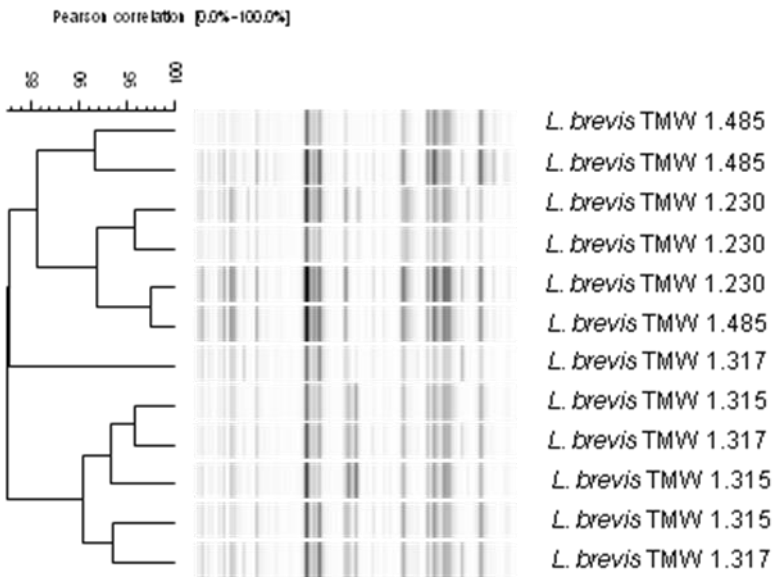


Figure 38. UPGMA cluster analysis of MALDI-TOF MS spectra of four different *L. brevis* strains

For these four strains, a RAPD-PCR was performed in triplicate with M13 primers. The UPGMA clustering results in two big clusters, whereas one cluster included the strains TMW 1.315 and 1.230 and the other cluster included the strains TMW 1.485 and 1.317. Within the big clusters, triplicate pattern of every strain could be separate from other strains in the sub-clusters (Figure 39).

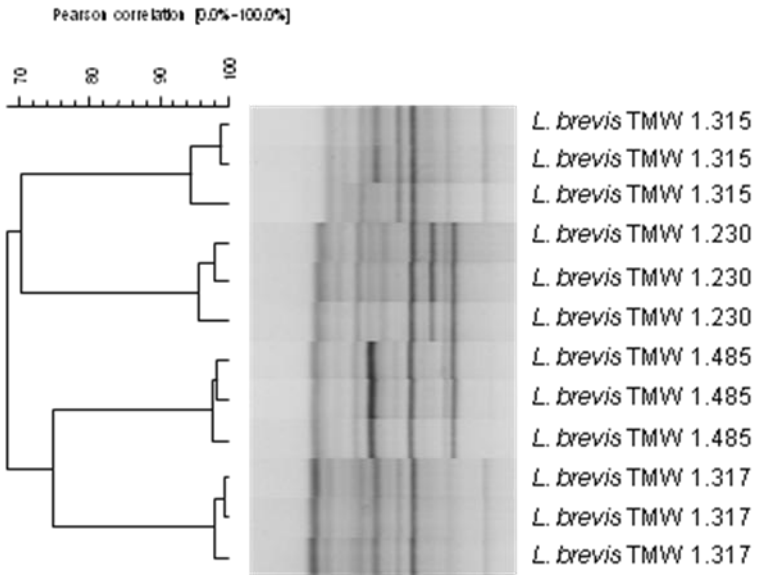


Figure 39. UPGMA cluster analysis of RAPD pattern of four different *L. brevis* strains

3.23 Differentiation of strains isolated from different origin by MALDI TOF/MS

In a second experiment, all spectra were clustered together and with one exception, two big cluster could be differentiate. Cluster A (*L. brevis* TMW 1.302, 1.485, 1.1216, 1.230, 1.315, 1.317, 1.313 and 1.465) was mainly formed by beer-isolates with one exception of *L. brevis* TMW 1.1216 (Figure 40). Cluster B was formed only by isolates from non-brewery environment (*L. brevis* TMW 1.1370, 1.1371, 1.6 and 1.1369).

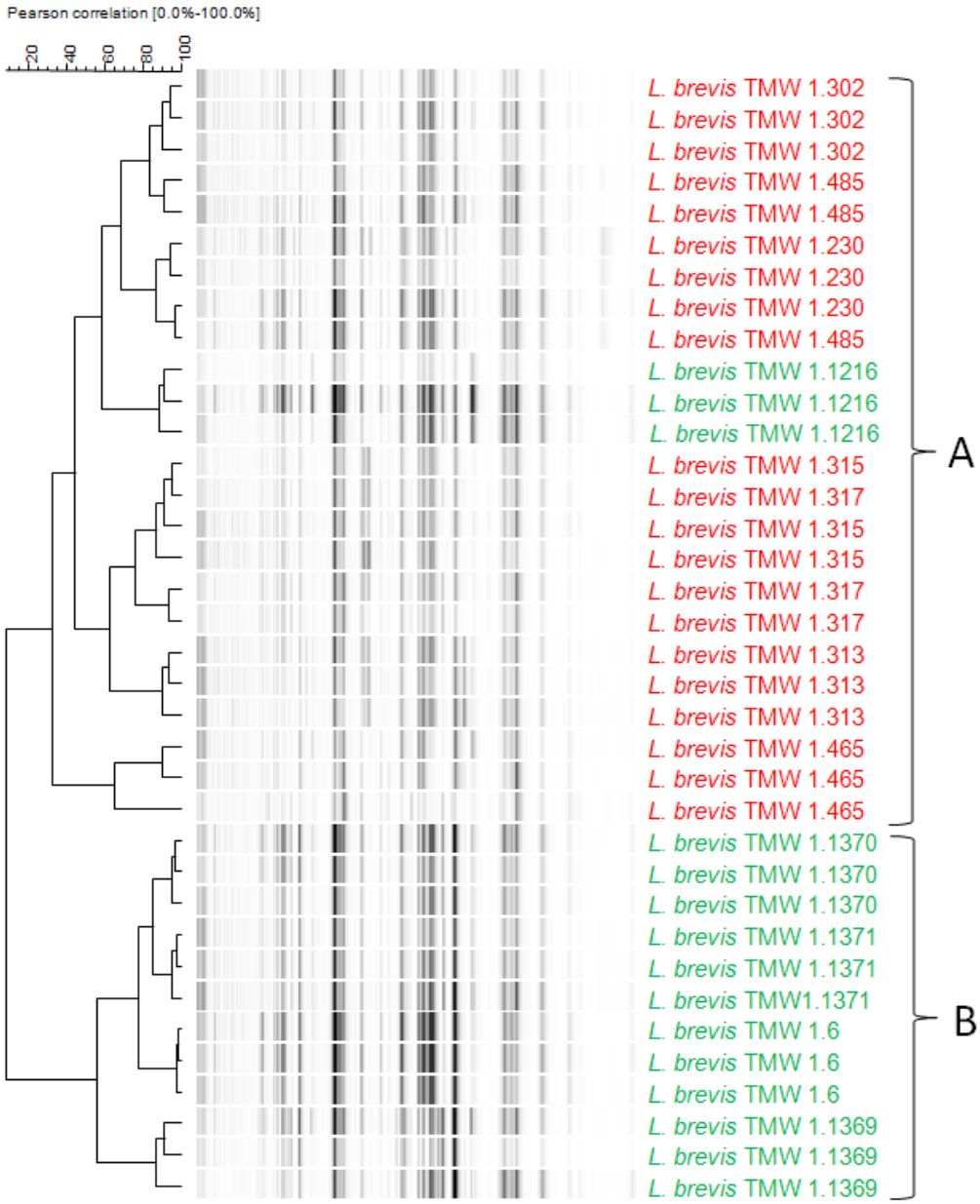


Figure 40. WARD cluster analysis of MALDI-TOF MS spectra different *L. brevis* isolates. Green = non-brewery isolates; red = brewery isolates.

Subsequently, three mean spectra of every strain were summarized to one spectrum and all spectra were clustered also with BioNumerics. This resulted also in two clusters A and B. Cluster A contained only isolated strains from brewery. With the exception of *L. brevis* TMW 1.302, all tested beer isolates exhibited strong beer-spoiling ability. Cluster B included only strains isolated from non-brewery environment (*L. brevis* TMW 1.1371, 1.1369, 1.6, 1.1216 and 1.1370) (Figure 41).

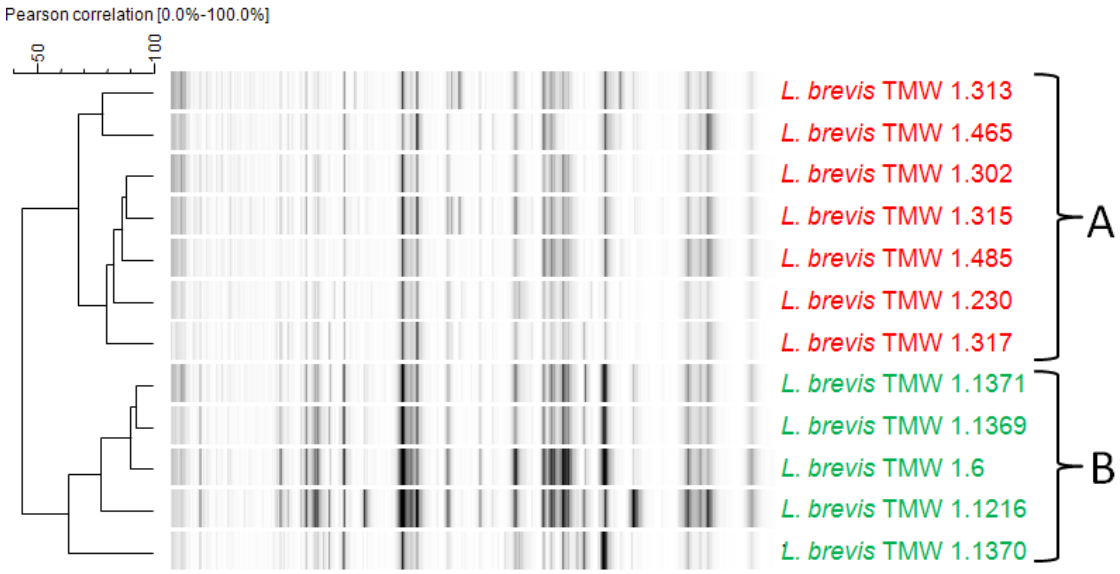


Figure 41. WARD cluster analysis of MALDI-TOF MS spectra different *L. brevis* isolates. Green = non-brewery isolates; red = brewery isolates.

4 DISCUSSION

The ability of a bacterium to grow in beer presumes a tolerance to hop acids. While Gram-negative bacteria are almost resistant toward hop compounds because of their outer membrane, the cell wall and cytosolic membrane of Gram-positive bacteria are more permeable for hop acids and thus they are prone to inhibition by hop compounds (BACK, 1981). In an acidic environment, intrusion of hop acids into cell entails insertion of protons and lowering of intracellular pH. Bacteria are able to extrude hop acids by MDR transport systems. Thereby, two different kinds of MDR transports can be differentiated. Type I is depending on ATP energy, whereas type II is independent of ATP (KONINGS and POELARENDS, 2002) and can use proton motive force (BOLHUIS et al., 1994). It seems to be very unlikely that the low energy amount, which is available in beer is used to extrude hop compounds in an endless cycle. On the other hand, hop acids cause a drop of intracellular pH in hop-sensitive cells. YANSANJAV et al. (2004) demonstrated that hop tolerant cells of *L. brevis* maintain or increase their intracellular pH to counteract collapse of porton motive force (pmf). This ensures that pmf-dependent transporters are able to continue its work. However, extrusion of protons to maintain constant intracellular pH via H^+-ATPases is an ATP-consuming process. Therefore, removing of hop acids by pmf-dependent transporters like HorC is indirectly ATP-dependent. Some of the known mechanisms of hop tolerance such as HitA are only expressed under hop stress conditions (HAYASHI et al., 2005), whereas in non-adapted cells, this protective mechanism is not activated. Therefore, true hop tolerant cells must possess a basal level of tolerance. In this work, different isolates of *L. brevis* were set in a uniform, physiological state by propagating ten sub-cultures on MRS agar plates. In particular, it was important to wean hop-adapted beer isolates from hop compounds and characterize the fundamental level of hop tolerance and beer-spoiling ability. The so classified hop-sensitive strains with low beer-spoiling ability and the hop tolerant strains with constitutive and high ability to spoil beer were the basis of determination of basal expressed mechanisms that confer hop tolerance. Therefore, constitutive

beer-spoiling strains were compared to non-spoiling strains in terms of utilization of glucose, amino acids, presence of known resistance mechanisms, expressed proteome in exponential and stationary growth phase and influence of intracellular manganese content on the hop tolerance.

4.1 Differences in sugar fermentation profile

The ability to grow in beer relays on the fact, that sugars, which are present in beer can be utilizes and metabolized by beer-spoiling bacteria. Carbohydrates included in beer are mainly products from the degradation of cereal starch, such as dextrins (14.4 g/l), maltotriose (2.03 g/l), maltose (2.19 g/l) and glucose (1.38 g/l). Also, some beers have low concentrations of fructose (0.29 g/l) (FERNANDEZ and SIMPSON, 1995). Therefore, the ability of different strains of *L. brevis* to metabolize sugars present in beer was investigated. A simple API is not really suitable, because weakly positive results after serveral days are difficult to interpret. For this reason, in this study the growth behaviour was investigated over a period of 60 hours by the measurment of optical density. It was shown that exponential growth, which indicates good fermentability of a sugar could not be detected in any strain/sugar combination. In the case of the strains *L. brevis* TMW 1.230 and TMW 1.485, the increase of the optical density in the medium containing fructose as a single carbon source was only linear in contrast to the exponential growth in the medium containing maltose. These two strains seem to be strongly adapted to their natural environment beer, where maltose is the predominant carbon source and fructose content is very low. In contrast to that, the beer-spoiling strain 1.317 exhibited only linear growth in maltose-containing medium and exponential growth in fructose-containing medium, although the content of fructose in beer is limited. Interestingly, SUZUKI et al. (2005) investigated the utilization of different substrates in beer and found, that the concentration of maltose after growth of three different beer-spoiling bacteria (*L. brevis*, *L. lindneri* and *L. paracollinoides*) was constant in comparison to the control beer. Nevertheless, differences in fermentation profile of the three tested sugars could only be exhibited in the beer-spoiling strains. Within the ecotype of beer-spoilers, three different types were differentiated: Type I (e.g. *L. brevis* TMW 1.313) which is able to metabolize all tested sugars

(glucose, fructose, maltose), type II which is able to ferment fructose and glucose (e.g. *L. brevis* TMW 1.317) whereas type III grows well only on maltose and glucose. In contrast to that, all tested non-spoiling strains exhibited exponential growth on glucose as well as on maltose and fructose.

4.2 Occurrence and mobility of hop tolerance associated genes and enzymes of ADI pathway

The species of *L. brevis* includes strains, which are tolerant to hop acids and also strains, which were killed or inhibited in growth by hop compounds (SUZUKI et al., 2002, PREISSLER et al., 2010). The occurrence of genes of proteins, which are up-regulated under hop- and acid stress (according to BEHR et al., 2007) were checked and compared. Only small differences between beer-spoiling and non-spoiling strains could be detected and the major part of genes were present independently of the beer-spoiling potential. In the strains TMW 1.485 and 1.230, which possess a long lag phases and were unable to ferment fructose, the genes of the proteins 2-hydroxyacid dehydrogenase and phosphopentomutase could not be detected. The conversion of oxalacetate to malate by 2-hydroxyacid dehydrogenase regenerate NAD from $NADH_2$, which doubles the ATP yield and growth in heterofermentative lactic acid bacteria (ARAI et al. 2001). On the other hand, the degradation of nucleotides by phosphopentomutase results in additional energy production. The lack of this enzyme in the strains 1.485 and 1.230 could explain the long lag phases of these strains, since additional pathways of energy production are missing. Apart from that, slow growth in a nutrient-limited growth medium like beer, could be a mechanism of adaptation to survive. Furthermore, differences were found in the presence of genes involved in amino acid metabolism. The gene for glutamate decarboxylase was found in all beer-spoiling strains and also in strains isolated from faeces and plant fermentation. Interestingly, in the tested isolates originating from sourdough, this gene was not present. HIGUSHI et al. (1997) reported, that decarboxylation of glutamate to GABA can be coupled with energy production by removing protons from the cytoplasm to stabilize intracellular pH. The availability of utilizable nutrients in beer is very low and the amounts of glutamate and asparagine are in the range of 25 - 610 µmol/l

(FERNANDEZ and SIMPSONS, 1995). In *L. brevis* more than one gene is annotated in function as glutamate decarboxylase. The sequences of glutamate decarboxylase II and III, which are present in all strains, are very similar. In contrast to that, the sequence of glutamate decarboxylase I is different. Interestingly, the blast of glutamate decarboxylase I resulted also in a hit for gene, which is annotated as tyrosine decarboxylase. For tyrosine decarboxlases it is described, that within one species the occurrence of this gene is strain-dependent (LUCAS et al., 2003). This coincides with the observations in this thesis. Furthermore, due to the decarboxylation of tyrosine additional energy can be produced and a supplementary pathway for regulation of intracellular pH is available (MOLENAAR et al., 1993; KONINGS et al., 1997). Differences were also found in the presence of genes of the ADI pathway. All tested strains possessed the arginine deiminase and the arginine repressor, but some non-spoiling strains exhibited no positive results for the gene presence of OTC, CK and arginine/ornithine permease. The role of ADI pathway is described in the next chapter.

4.3 Role of ADI pathway

Previous studies revealed that OTC, an enzyme of the arginine deiminase (ADI) pathway was up-regulated under hop- and acid stress conditions (BEHR et al., 2006). Depending on strain and species, several factors were identified, which activate the ADI pathway. It is described for *L. sanfranciscensis* that the ADI pathway is initiated by arginine and unaffected in the presence of glucose in the growth medium (ANGELIS et al., 2002). In contrast to that, *L. sakei* exhibited inversed behaviour in comparison to *L. sanfranciscensis* (MONTEL and CHAMPOMIER, 1987). This study showed for all the tested *L. brevis* strains, that arginine was metabolized within 24 to 48 h also in presence of glucose. Thereby, strain-specific differences in the speed of fermentation could be observed, whereby beer-spoiling strains fermented arginine within shorter time. This is in agreement with the results of proteomic analysis, where arginine deiminase was expressed in the exponential phase in glucose-containing growth medium in most beer-spoiling strains. The screening on genes of ADI operon showed that all tested strains possess genes for the arginine deiminase

and arginine repressor. Nevertheless, BACK et al. (2004) reported, that 20 % of all *L. brevis* strains are not able to cleave arginine. If strains are able to cleave arginine by deamination, citrulline and ammonia is produced, which contribute to alkalization of beer. The modulation of the pH value influences the effect of hop acids, which are less active at higher pH (SIMPSON and SMITH, 1992; BEHR et al., 2010). If the operon of ADI pathway includes all genes for complete arginine degradation, the cleavage of citrulline to ornithine and carbamylphosphate by ornithine carbamoyltransferase and subsequent reaction of carbamylphosphate by carbamate kinase produce additional ATP energy, ammonia and CO_2. It has been shown, that only supplementation of arginine to strong hopped growth medium (86 µM iso-α at pH 4.0) allows a non-adapted strain of *L. brevis* to grow (BEHR et al., 2006). Other researchers investigated utilized substrates in beer and found out, that the amount of arginine was reduced from 14.0 mg/l to 0.0 mg/l (SUZUKI et al., 2005). This clearly demonstrates the contribution of the arginine deiminase pathway in the hop tolerance. However, different beers vary in their content of arginine in the range of 0.1 – 81.8 mg/l (mean = 34.8 mg/l) (FERNANDEZ and SIMPSON, 1995). For this reason, assessment of a beer is depending on arginine content in beer and specific properties of different beer-spoiling strains.

4.4 Contribution of *hitA*, *horC* and *horA* on minimal inhibition concentration

The contribution of the presence of *horA* and *horC* on hop tolerance has already been tested at pH 5.5 (SUZUKI et al, 2005). At this pH value the effectiveness of hop acids is very limited, so the authors measured high minimum inhibitory concentrations (MICs). In this work, a high number of different *L. brevis* strains were checked for the presence of known beer-spoiling associated genes (*hitA*, *horA* and *horC*). MICs were determined for those strains, which had only one of the three genes. It seems, that the contribution of *horA* on hop tolerance in non-adapted strains was only limited, because in comparison to strains, without *horA, horC* or *hitA*, the minimal inhibition concentration was almost identical. In contrast to that, carriers of *hitA* or *horC* exhibited higher levels of hop tolerance. Furthermore, MICs in the study of SUZUKI (2005) were determined with pre-

adapted strains. In this thesis, non-adapted strains were used to determine the basal level of hop resistance considering the presence of *hitA* or *horA* or *horC*. This could explain differences in MIC due to different expression of *horA, hitA* and *horC*. Investigations carried out in this thesis confirmed, that carriers of *horA* as single genetic beer-spoiling associated marker possess only low beer-spoiling potential. Furthermore, TEICHERT (2008) found out, that half of beer-spoiling strains possess mutated and malfunctioned *horA* genes. In contrast to the marker *hitA*, which is mostly found in beer-spoiling *L. brevis*. The mechanism, how HitA confers tolerance to hop is still unclear. HitA is homologous to NRAMP (natural resistance-associated macrophage proteins) with sequence coverage of 67 % (KEHRES et al., 2000; HAYASHI et al., 2001). Also hop-sensitive strains possess the genes of NRAMP, but they show only limited tolerance to hop acids. However, NRAMPs are described as Mn^{2+}/H^{+} symporter whereby protons were transported coupled with manganese. In contrast to that, it has been demonstrated that exclusion of manganese is induced by hop compounds (VOGEL et al. 2010). It is conceivable that HitA regulate the influx of only small amounts of manganese to ensure basic functions of the cellular metabolism. Similarly, the function of HorC has not been fully clarified until today, but in contrast to HorA it seems, that HorC is a pmf-driven multidrug transporter (IIJIMA et al., 2006). Judging from the generated results, it seems that HorC excludes hop acids more effective than HorA, because HorC uses pmf as energy source. However, hop compounds are responsible for the decrease of intracellular pH. To counteract this H^{+}-ATPases are up-regulated under hop stress to extrude protones and maintain intracellular pH and pmf (SAKAMOTO et al., 2002). The extrusion of protons against a concentration gradient is ATP-dependent. Thus, pmf-depended transporters like HorC are indirect dependent on ATP energy. How far it is useful to extrude hop compound by energy-depended transport system in a medium with low resources of energy retains questionable.

4.5 Mobility and transfer of *horC* within strains and species

The sequences of *hitA* and *horC* were determined in selected strains. For *hitA* and *horC* it was demonstrated, that all genes were functional, whereas in two

strains, they exhibited mutations in the gene of HorA (Behr et al., 2006; Teichert, 2009). In contrast to *hitA*, which is specific for *L. brevis,* plasmids included the sequences of *horA* and/or *horC* can be exchanged between different species via horizontal gene transfer (Suzuki and Yamashita, 2004). Thereby, the *horC* gene of *L. brevis* TMW 1.317 exhibited commonalities in gene sequences, which are typical for other beer-spoiling species such as *L. lindneri*, *L. backii* and *L. paracollinoides* (Table 29).

Table 29. Occurrence of 9 amino acid gap in different beer-spoiling lactic acid bacteria

Species and strain	9 amino acid gap (KLLQAKFKS)
L. brevis ABBC45, TMW 1.313, 1.485, 1.465, 1.230	Negative
L. brevis TMW 1.317	Positive
L. lindneri DSM 20692	Positive
L. backii LA21, LA22	Positive
L. paracollinoides JCM 11969^T	Positive

It can be assumed, that genes are modified and exchanged within different species (IIJIMA et al., 2007). The phylogram indicates (Figure 42) that the *horC* sequence of *L. brevis* TMW 1.317 could be a link to the sequences of *L. backii* and *L. lindneri*.

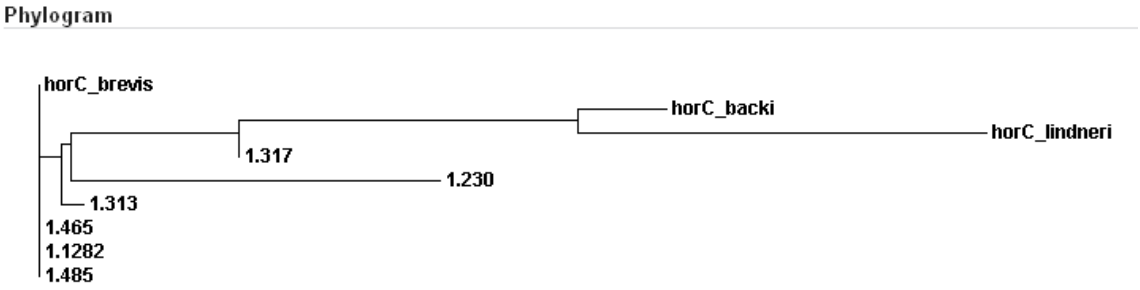


Figure 42. Phylogram of *horC* sequences generated with clustalw2

The detailed analysis of different *horC* gene sequences could not clarify whether duplication or deletion of the 9 amino acid sequence took place. Interestingly, occurrence of duplication or deletion of the 9 amino acid sequence

is not species specific and differences are also detectable in different strains of *L. brevis*.

4.6 Proteome of beer-spoiling strains in comparison to non-spoiling strains in exponential growth phase

Due to the wide range of heterogeneity within the species of *L. brevis* (SOHIER et al., 1999) proteins which are only expressed in all beer-spoiling strains could not be detected, because different pathways can result in the same effect to protect cells under different stress conditions. Some proteins, which could be identified, are involved in general stress response of lactic acid bacteria. Spot 1, a Zn-dependent alcohol dehydrogenase is part of heterofermentative lactic acid fermentation and catalyzes the conversion of acetaldehyde into ethanol, whereby $NADH_2$ is regenerated. Under hop stress in a medium containing maltose, fructose and glucose the formation of ethanol was suppressed and replaced by the formation of acetate, which resulted in an additional ATP yield (BEHR et al., 2007; SUZUKI et al., 2005). However, in the case of acetate formation the redox balance can only be maintained, if an external electron acceptor like fructose or oxygen is available. In beer, the amounts of oxygen are very low and the content of fructose depends on the type of beer. On the other hand, it could be demonstrated, that incubation in beer resulted in a decrease of intracellular zinc. Probably, enzyme-bounded zinc in highly up-regulated Zn-dependent enzymes of beer-spoiling strains in comparison with non-spoiling strains influences homeostasis of free intracellular zinc. In yeast cells, it could be demonstrated that zinc homeostasis contributes to hop tolerance (HAZELWOOD et al., 2010).

The exact function of ATP-dependent ClpL protease (spot 2) is still unclear to date. WALL et al. (2007) could observe increased synthesis of ClpL in *L. reuteri* as response to acid stress. Furthermore, northern blot analysis and realtime PCR at *O. oeni* show that presence of 10 % ethanol induced expression of ClpL, particularly in the stationary growth phase (BELTRAMO et al., 2004). In *L. rhamnosus* two genes of ClpL protease (ClpL1 and ClpL2) are existent, whereby the amino acid sequence of ClpL2 is 99 % identical to the sequence of *L. brevis* (Figure 43).

```
mobile_clpL2      YRATGKLPQNNKTIEVSKDGKQAVKKGGILEKLGTNLTEQARDGLLDPVIGRENEIQETA 60
WCFS1             YRATGKLPQNNKTIEVSKDGKQAVKKGGILEKLGTNLTEQARDGLLDPVIGRENEIQETA 60
TMW_1.313         YRATGKLPQNNKTIEVSKDGKQAFKKGGILEKLGTNLTEQARDGLLDPVIGRENEIQETA 60
                  ***********************.************************************
mobile_clpL2      EILSRRTKNNPILVGDAGVGKTAVVEGLAQAIVAGKVPETLQDKEIYSIDLSSLEAGTQY 120
WCFS1             EILSRRTKNNPILVGDAGVGKTAVVEGLAQAIVAGKVPETIQDKEIYSIDLSSLEAGTQY 120
TMW_1.313         EILSRRTKNNPILVGDAGVGKTAVVEGLAQAIVAGKVPETIQDKEIYSIDLSSLEAGTQY 120
                  ****************************************:*******************
mobile_clpL2      RGSFEENIKQLVKEVKAAGNIILFFDEIHQIIGTGATGGEDGGKGLADIIKPALSRGELT 180
WCFS1             RGSFEENIKQLVKEVKAAGNIILFFDEIHQIIGTGATGGEDGGKGLADIIKPALSRGELT 180
TMW_1.313         RGSFEENIKQLVKEVKAAGNIILFFDEIHQIIGTGATGGEDGGKGLADIIKPALSRGELT 180
                  ************************************************************
mobile_clpL2      VIGATTQDEYRNTILKNAALARRFNEVVINEPTAADTLRILQGVKKLYEKHHHVVLPDDA 240
WCFS1             VIGATTQDEYRNTILKNAALARRFNDVVINEPTAADTLRILQGVKKLYEKHHHVVLPDDV 240
TMW_1.313         VIGATTQDEYRNTILKNAALARRFNDVVINEPTAADTLRILQGVKKLYEKHHHVVLPDDV 240
                  ************************:***********************************.
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Figure 43. Alignment of partial sequence of ClpL protease from *L. brevis* TMW 1.313 with mobile ClpL2 of *L. rhamnosus* (accession no. AY659979.1) and *L. plantarum* WCFS1 (NP_786784.1).

The ClpL2 protease is described as mobile element, which is transferred via horizontal gene transfer from *L. plantarum*. This is indicated by the absence of this gene in other strains of *L. rhamnosus* (SUOKKO et al., 2005). In *L. brevis*, 59.6 % of the beer-isolates (n = 52) are carrier of this gene, whereas only 12.5 % of strains (n = 16), isolated from other origins, possess ClpL2. Gene expression analysis of *clpL1* and *clpL2* revealed, that both genes could be induced by heat shock and only *clpL2* in the presence of H_2O_2. Interestingly, gene expression of *clpL1* was more depending on growth phase as *clpL2* (SUOKKO et al., 2005). The results of this study confirmed that ClpL2 was present in exponential growth phase as well as in stationary growth phase of *L. brevis*. Furthermore, analysis of flanking regions of *clpL2* exhibited no known promoter sequences (SUOKKO et al., 2005). In *L.brevis ssp. gravesensis*, this gene (accession no. NZ_ACGG01000148.1) is also present with a sequence coverage of 99 %. In close vicinity to *clpL2*, a transposase (accession no. NZ_ACGG01000144.1) and a gene, which codes for a hop-inducible cation transporter (accession no. NZ_ACGG01000149.1), indicate, that clpL2 is a mobile element, which contributes to hop tolerance and beer-spoiling potential.

This assumption is supported by the fact that *clpL2* is involved in the intracellular quality control of proteins. ClpL2 is also up-regulated under acid stress conditions similar to the beer environment (SCHIRMER et al. 1996).

While BEHR et al. (2006) demonstrated, that the ornithine transcarbamylase was up-regulated under hop- and acid stress conditions, the proteome analysis exhibited that most of non-adapted beer-spoiling *L. brevis* strains expressed arginine deiminase (spot 3) in the exponential phase. The role of the arginine deiminase pathway has already been discussed. Interestingly, only the non-adapted variant of *L. brevis* required the ADI pathway for survival hop- and acid stress conditions (BEHR et al., 2006), which indicate that expression of arginine deiminase is a prerequisite for survival in beer.

Another important enzyme of carbohydrate metabolism in *L. brevis*, the maltose phosphorylase (spot4) is able to cleave maltose by addition of anorganic phosphate. This resulted in production of glucose and glucose-6-phosphate, whereby glucose-6-phosphate is activated for glycolysis without consumption of ATP. Remaining glucose, that has to be activated by ATP for glycolysis, can be extruded until all maltose is consumed to avoid intracellular osmotic stress. The exclusion of glucose is visible in an increase of glucose in growth medium. At a later time, glucose can again be transported into cell and activated for metabolism. Investigations of SUZUKI et al. (2004) exhibited that hop-sensitive variants of some beer-spoiling strains lost the ability to ferment maltose. The authors concluded that the loss of maltose fermentation ability in some beer-spoiling *L. brevis* strains is correlated with the loss of hop tolerance. Furthermore, FUJII et al. (2005) identified a beer-spoiler associated gene locus, which possesses the genes encoding for maltose phospholyrase, aldose epimerase, and b-phosphoglucomutase. The ability to ferment maltose seems to be important, because maltose is the predominant carbohydrate in beer. So MOORE and RAINBOW (1955) concluded, that the availability of maltose may reflect an adaptation of beer-spoiling lactobacilli to their natural environment beer. In contrast to that is the observation that maltose was not fermented in hop-containing medium or beer (SUZUKI et al., 2005; BEHR et al., 2006)

4.7 Proteome of beer-spoiling strains in comparison to non-spoiling strains in stationary growth phase

To get a closer view on differences in physiology of beer-spoiling and non-spoiling strains, a differential proteomic analysis of stationary grown cells was performed. Five proteins could be detected, which were only expressed in beer-spoiling strains. The proteins, which could be identified, were involved in DNA repair, cation homeostasis, peptide and amino acid metabolism and compound modification.

NAD-dependent DNA ligase (LigA) (eID0003) is a protein, which is involved in DNA replication, DNA repair and recombination because of their ability to join the breaks in double-stranded DNA (TOMKINSON and LEVIN, 1997). Breaks of double-stranded DNA could occur under oxidative stress that is present under hop stress conditions and can be a lethal event (HASSETT and IMLAY 2007, BEHR and VOGEL, 2009/2010). Interestingly, ligation of DNA fragments by LigA requires a divalent cation as cofactor, whereby manganese was identified to be most effective (BLASIUS et al., 2007). In *Staphylococcus aureus* the amounts of LigA were found to be diminished more than 60% in stationary phase under glucose starvation (MICHALIK et al., 2009). The assumption that NAD-dependent DNA ligase influenced NAD turnover could be refuted by other researchers (PARK et al., 1989).

The protein with the eID 0885 was identified as multicopper oxidase and plays an important role in iron metabolism and also in the homeostasis of copper (CRICHTON et al., 2001). Furthermore, multicopper oxidase can catalyze oxidation of Mn^{2+} to Mn_2O_3 that results in an additional energy yield. Moreover, accumulated and oxidized manganese could be used as terminal electron acceptor. Nevertheless, the role of multicopper oxidase in lactobacilli is unclear and it only could be speculated, wheater Mn^{2+} oxidation takes place or not. Fact is that reduction of intracellular Mn^{2+} levels, which could be reached also by manganese oxidation, helps *L. brevis* to survive hop stress.

3-Hydroxyisobutyrate dehydrogenase (eID 1713) is an oxidoreductase and a key enzyme of catabolism of the branched chain amino acids valine, leucine and isoleucine. On the one hand, the degradation of these amino acids results in an additional energy yield and on the other hand, the pathway merges in

synthesis of branched-chain fatty acids via production of propionyl-CoA (SERRAZANETTI et al., 2011). The influence of these fatty acids on cell membrane properties of beer-spoiling strains is discussed later.

The peptide ABC transporter ATPase (eID 1782) is part of the family of ABC transporter, which is characterized by high substrate specificity and consumption of ATP. Lactic acid bacteria cover their requirement for nitrogen mainly by transport of peptides and subsequent digest via pepsidase activity (KUNJI et al., 1996; SMID et al., 1990). Thereby, the intake of peptids and subsequent intracellular digest is energetically favourable than uptake of single amino acids (VERMEULEN, 2006). Furthermore, in the presence of peptides consumption of glucose is decreased (personal communication Quirin Sinz). Additionally, degradation of peptides and amino acids resulted in elevated energy yield. The content of soluble nitrogen in beer which includes proteins, peptides and amino acids (ca. 0.07 g/L) is in average 0.5 g/L (FERNANDEZ and SIMPSON, 1995).

Finally, the protein eID 2035 could be identified as acetyltransferase without closer specification. Different antibiotics, such as chloramphenicol and streptogramin, which effect translation at the ribosome, can be inactivated by acetylation via acetyltransferase (SHAW, 1975, SUGANTINO and RODERICK, 2002). It can only be speculated, weather inactivation of hop compounds in a mechanism similar to chloramphenicol resistance is possible.

Taken together, beer spoiling *L. brevis* express mechanisms, which are involved in pH and zinc homeostasis, utilization of maltose and degradation of mis-folded proteins. In stationary state, constitutive beer-spoilers can be separated from non-spoiling strains along the expression of proteins, which are part of replication and DNA repair, homeostasis of copper or manganese, uptake of peptides, utilization of branched chain amino acids and unspecific acetylation of currently unknown substances or hop compounds.

4.8 Metabolism of beer-spoiling strains in different kinds of beer

In this thesis an inexpensive and rapid method based on the metabolic activity was designed for the detection of strong beer-spoiling *L. brevis* strains. Since the metabolic activity of these strains is the only decisive trait for the brewer, the definition of a clear benchmark for the evaluation of strains requires the separation of intrinsic tolerance from adaptive or acquired tolerance. Once established, the test could also be used to evaluate strains isolated from spoilage cases in terms of their maximal adaptive potential to hops.

The adaptation to hop compounds has an important influence on the hop tolerance level and masks the constitutive spoilage potential of a given strain (BEHR et al. 2006). BEHR et al. (2006) adapted the strain *L. brevis* TMW 1.465 to increasing hop concentrations up to 103.2 µM over 45 days. Upon adaptation, the lag phase and the growth rate of adapted *L. brevis* TMW 1.465 were nearly independent of the iso-α-acids concentration in the growth medium. For determining the constitutive tolerance level and to identify strong beer-spoiling *L. brevis*, the strains have to be in a non-adapted status. We defined non-adapted as a status obtained upon growth in unhopped mMRS4 media over ten subcultures equalling 22 -116 generations depending on the strain. Strains from other environments than brewery exhibited good growth behaviour on both mMRS4 plates and in broth, whereas the *L. brevis* isolates from beer spoilage cases TMW 1.230, 1.485, 1.317 and 1.315 showed extended lag phases and decreased growth rates after subculturing in fresh mMRS4. They were persistently difficult to culture even over ten subcultures on mMRS4. This indicates their limited adaptability to grow in mMRS4 and a generally different metabotype. In contrast, the strains TMW 1.313, 1.465, 1.240 and 1.302 were easy to culture (SUZUKI et al., 2007).

The comparison between forcing tests and MIC tests confirmed that hop tolerance was a prerequisite for beer-spoiling strains. Furthermore, only the strains derived from beverage industry environments (beer, soft drink) possessed at least one of the *horA, horC* and *hitA* genes. For the strains investigated in this work, it was also found that the concomitance of two genes of *horA*, *horC* or *hitA* is necessary to enable a strain to spoil medium hopped

beer (German lager beer, 16 ppm iso-α-acids) or stronger hopped beer (pilsner beer up to 36 ppm iso-α-acids). This supports the hypothesis that hop tolerance is a cumulative trait and cannot be referred to a single marker, but rather to multiple traits, which must be surpassed to actually growth in respective beers. Interestingly, other studies found strains, which also possessed all three genes and exhibited no spoilage ability (FUJII et al., 2005). Moreover, independent of the presence of *horA, horC* and *hitA* non-brewery isolates of *L. brevis* could spoil German wheat beer. To date, genes involved in the tolerance mechanism are not known. Furthermore strains with identical genetical facilities regarding to the existence of *horA, horC* and *hitA* exhibited different growth behaviour. These important differences are not visible in PCR results, but can be seen by analysing metabolism and growth. This further suggests that a general „metabotype" enabling a strain to cope with the hopped beer environment, is based on a group of traits, which can partly replace each other. It confirms that the ability to grow in beer depends on the expression of more genes in addition to *horA, horC* and *hitA*. There may potentially be uncharacterized genes, which might be needed for surviving stressful beer environment. For the identification of such novel genes involved in hop tolerance, physiological tests apart from the genetic background are needed.

In order to improve the characterisation of the physiology of beer-spoiling strains, we followed metabolic activity by detecting reduction of tetrazolium salt to formazan in different beers over two weeks. This method is more sensitive than detecting visible growth, because $NADH_2$ is produced during the metabolism by dehydrogenase activity, before visible growth occurs. The sensitivity of this method allows discrimination of beer-spoiling and non-spoiling strains in German lager beer within five days. Moreover, we could classify two groups within the beer-spoiling strains. One group can grow in several types of beer with short lag phase, whereas the second group needed longer time to adapt to beer environment. On the basis of the determination of dehydrogenase activity, we developed a simplified and rapid method that could differentiate between beer-spoiling and non-spoiling strains within two days using resazurin as indicator for dehydrogenase activity. Several research groups considered that beer is a good starting point to develop an initial selection medium for beer-spoiling strains (HAAKENSEN et al., 2009; SUZUKI et al., 2007). For our

experiment and a rapid discrimination of strong beer-spoiling strains within two days, we used Bavarian wheat beer, German lager and German pilsner beer. The Bavarian wheat beer did not discriminate the beer-spoiler from the non-spoiler because it was not selective enough (at 8 ppm iso-α-acids) to avoid metabolic activity of all the non-spoiling strains. The pilsner beer (36 ppm iso-α-acids) was selective enough, but some beer-spoiling strains were negative within two days. Only German lager beer with moderate hop content (16 ppm iso-α-acids) was considered as differentiation medium because it was selective enough to prevent growth of strains with low spoilage potential. According to the MIC test, the non-spoiling strains possessed a hop tolerance level below 10 ppm iso-α-acids. Consequently, for the latter group it was possible to spoil low hopped Bavarian wheat beer with 8 ppm iso-α-acids, but not higher hopped German lager beer. In comparison with detecting beer-spoiling strains using NBB-B, the new method can discriminate strong beer-spoiling strains from low-spoiling strains, whereas NBB-B is positive for all *L. brevis* strains. NBB-B has been developed in Bavaria/Germany, where the brewing of low hopped wheat beer is typical. Forcing tests in wheat beer showed that within a period of four weeks all tested strains could spoil this kind of beer. Accordingly, NBB-B tests were positive for all *L. brevis*, so they are especially useful for the control of wheat beer production. However, with the latter test a categorization of *L. brevis* strains, which could only spoil wheat beer and strains that could also spoil pilsner beer could not be established. Other researchers designed also beer-based media (SUZUKI et al., 2007) and could differentiate beer-spoiling lactobacilli from non-spoiling between two to five days depending on species and physiological statue. In contrast to that MRS-based agar plates containing 9 BU hop compounds and 5% v/v alcohol (HGA+E) by HAAKENSEN et al. (2009) were positive for 9.1 % of non-beer lactobacilli and 100 % of spoiling lactobacilli. In beer- or MRS-based media false-positive results are possible due to their nutritious composition. Direct testing in beer and final detection with resazurin exclude false-positive results, because the test is only positive for beer-spoiling strains.

Testing metabolic activity in beer establishes advanced information about the effect of hop on different types of hop sensitive strains. One additional day of incubation of the samples in beer-resazurin buffer mix (increased pH,

decreased hop activity) showed positive results also for some non-spoiling strains. This indicates that such strains can survive this reduced hop stress, whereas the cells of other non-spoiling strains were killed. Thus, pH of a beer besides its direct effect on growth of bacteria is critical for its microbiological stability by affecting on the amount of antimicrobial active (protonated) hop components. The knowledge about the type of effect of specific hop compounds can be important for breweries. If the effect of hop compounds is only bacteriostatic, cells can survive in beer. Such strains would have the potential to become beer-spoiling strains if horizontal gene transfer of tolerance genes occurs (SUZUKI et al., 2004).

In conclusion, the resazurin assay in moderate hopped beer allows the detection of beer-spoiling strains within the highly variable and most common beer spoiler *L. brevis*. The use of beer without any modification allows also the detection of strains in hard-to-culture status. Resazurin can be used as a cheap and convenient indicator for detecting metabolic activity of bacteria in beer and may also be useful for the evaluation of other beer-spoiling bacteria.

4.9 Role of intracellular manganese

To understand the role of manganese in beer-spoiling bacteria, intracellular manganese levels were modulated in *L. brevis* as a function of manganese content in growth medium and the effect on beer-spoiling ability was investigated. It is already known that requirements of manganese in hop-sensitive strains do not differ in comparison to hop-tolerant organisms (FERNANDEZ and SIMPSON, 1993). However, differences were observed in their ability to grow on culture medium, due to the fact that beer-spoiling strains are often in a hard-to-culture statue according to adaptation to low nutrient availability environment (HAAKENSEN et al., 2009, SUZUKI et al., 2007). In this thesis, the manganese content was adjusted in growth medium according to manganese content in beer, which related to 0.53 % of manganese concentration under reference conditions. Thereby, intracellular manganese level can be modulated and a reduction of intracellular manganese of about 98 % compared to reference can be enforced. This effect is also described for *L. plantarum* (ARCHIBALD and DUONG, 1983). Low intracellular manganese levels

resulted in decreased maximum growth yield. The influence of manganese on growth yield is strain-dependent. Whereas some strains are strongly influenced under manganese-starvation (TMW 1.485, 1.313), the strain TMW 1.240 is almost unaffected, although this trace element is described to be essential for lactic acid bacteria (ARCHIBALD, 1986). On proteomic level, this strain exhibited no significant differences in expression profile, whereas in TMW 1.485 and 1.317 significant differences could be detected. Interestingly, unusually low intracellular manganese levels increased the ability to spoil beer in shorter time. The decrease of intracellular manganese by putative manganese exclusion transporters seems to be an important adaptation step for development of hop tolerance. In contrast to the assumption that *hitA* balances the hop-induced manganese leakage, low intracellular manganese levels contribute to higher levels of hop tolerance. BLM (Bilayer Lipid Membrane) experiments indicated that oxidized manganese-hop compound complexes inside the bacterium could act as electron acceptors and causing oxidative stress (BEHR and VOGEL, 2010). A low-level intracellular manganese homeostasis could decrease inhibitory effects of hop compounds and indicates that exclusion of manganese could be an important mechanism for tolerance against hop compounds. However, manganese exclusion transporters in *Lactobacillus spec.* are still unknown. In *Streptococcus pneumonia*, ROSCH et al. (2009) identified a cation diffusion facilitator (CDF) protein of unknown substrate specificity that functions as a manganese export system. A blast of the amino acid sequence of this protein resulted in 3 hits in *L. brevis* (Table 30).

Table 30. Blast hits of CDF protein (*Streptococcus pneumonia*) in *L. brevis* (Identities = identical residues in this alignment; Positives = the number of conservative substitutions)

Species	Protein	Identities (%)	Positives (%)
L. brevis ATCC 27305	cation diffusion facilitator	29	55
L. brevis ATCC 367	Co/Zn/Cd cation transporter	30	56
L. brevis ATCC 367	Co/Zn/Cd efflux system	23	48

The proteins are annotated as unspecific cation transporter or Co/Zn/Cd cation transporter. It is known, that manganese transporters can also transport other cations including Fe^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+} and Ni^{2+} (KEHRES et al., 2000; LOW et al., 2003). This could be the reason, why some possible manganese

transporters in the genome of *L. brevis* ATCC 367 are predicted as Co/Zn/Cd cation transporters. The experiments indicate that the physiological state of decreased intracellular manganese is a prerequisite for survival under hop stress conditions. Furthermore, preliminary experiments show that non-spoiling strains release little manganese, whereas beer-spoilers release high amounts upon 3 h hop challenge in beer (VOGEL et al., 2010). On the other hand, cells, which were incubated under manganese-starvation and exhibited low intracellular manganese levels showed increased hop tolerance and decreased lag phase under hop stress conditions. To get a closer view of molecular mechanisms behind these physiologic adaptations, the proteomes of three strains were compared, which showed a strongly altered hop tolerance level upon manganese-starvation.

The investigations indicate that manganese-starvation induces expression of proteins, which are also overexpressed under hop stress condition at high manganese levels. Protein 13 was identified as carbamate kinase, which is part of the arginine deiminase pathway. This enzyme contributes to ATP generation and elevates the extracellular pH to compensate acid stress. It could be demonstrated, that Mn^{2+} is a metal-cofactor of carbamate kinase and in comparison with Mg^{2+} 2-fold more effective, if ADP or ATP is available (PILLAI et al., 1980; NADRA et al., 1986). Up-regulated enzymes of ADI pathway induced by manganese deficiency contribute to survival in beer of non-adapted beer-spoiling strains (BEHR et al, 2006). In beer, the availability of fermentable carbohydrates is very low and the utilization of arginine is an alternative source for energy generation. Depending on used beer, concentration of arginine is in the range of 1 – 470 µmol/L (FERNANDEZ and SIMPSON, 1995). This could explain, why manganese-starved cells exhibited faster growth in beer. Protein 245, which is the 2-hydroxyacid dehydrogenase and catalyzes the conversion form oxaloacetate to malate (ARAI et al., 2001) is also overexpressed under hop stress conditions (BEHR and VOGEL, 2009). These findings enforce the assumption that hop compounds induce manganese deficiency in cells and overproduction of 2-hydroxyacid dehydrogenase is a mechanism to handle the stress of manganese-starvation. Furthermore, the protein 102 could be identified as 6-phosphogluconate dehydrogenase. This enzyme is part of the pentose phosphate pathway, which is responsible for generation of reducing

equivalents (NADPH), production of ribose-5-phosphate for synthesis of nucleotides and production of erythrose-4-phosphate, which is used in the synthesis of aromatic amino acids. In *O. oeni* 6-phosphogluconate is responsible for controlling the channeling of fructose into phosphoketolase pathway by the inhibition of phosphoglucose isomerase (RICHTER et al., 2003). Moreover, 6-phosphogluconate dehydrogenase was up-regulated under acid stress. The authors concluded that 6-phosphogluconate dehydrogenase, which is the rate-limiting enzyme of the pentose phosphate pathway, is responsible for maintaining an optimal redox status for cell growth under low pH (LEE et al., 2008). Experiments in *Aspergillus niger* showed that manganese decreases the activity of the pentose phosphate pathway enzymes, but the influence of manganese on the enzymes of primary metabolism appears to be indirect (KUBICEK and RÖHR, 1977). Another effect of manganese-starvation conditions on manganese-dependent ribonucleotide reductase activity could be monitored in *Brevibacterium ammoniagenes* (WILLING et al., 1988). It is conceivable that other enzymes in translation process like the overexpressed protein 30, an Asp-tRNA-Asn/Glu-tRNA-Gln amidotransferase, involved in translation process are influenced by manganese deficiency. Asp-tRNA-Asn/Glu-tRNA-Gln amidotransferase consists of the three subunits GatA (protein 30), GatB and GatC and is responsible for correct decoding of glutamine codons. As already mentioned, manganese-starved cells are more suitable to survive in beer environment, which was tested by metabolic assays. Beer is a medium that comprises a plurality of hurdles, which complicates growth. Beside hop acids, ethanol, low nutrient and oxygen availability, one of these hurdles is the low pH. In *S. mutans*, acid tolerance resulted in upregulation of GatA and GatB of glutamyl-tRNA amidotransferase, which is responsible for protein biosynthesis. Additionally, GatA is able to produce ammonia by deamination of glutamine and can be used to alkalize growth media.

The strain TMW 1.240 was almost unaffected with regard to manganese availability. In exponential growth, no differences in proteome of manganese-starved cells and cells, which were grown under reference conditions, could be detected. Only in stationary phase, differences on proteomic level could be detected in manganese-starved cells. Interestingly, one protein, which could be identified as acetoin dehydrogenase, was also up-regulated under hop- and

acid stress conditions (BEHR et al., 2007). It is well known that exogenous pyruvate enhances acetoin production (TSAU et al., 1992). However, SUZUKI et al. (2005) reported that beside citrate, malate and arginine also pyruvate was consumed by beer-spoiling LAB strains. This could be an explanation for the increased fitness of manganese-starved cells in beer, because the formation of neutral acetoin prevents additional acidification. Furthermore, conversion of acetoin to diacetyl is NAD^+-depended and in case of reversibility this reaction can take part in redox balance and $NAD^+/NADH_2$ homeostasis (SPECK and FREESE 1973). Furthermore, a Clp protease which contributes to degradation of miss-folded proteins was up-regulated under manganese-starvation. It is already known, that lactobacilli need Mn^{2+} as scavenger of harmful oxygen radicals. Under manganese-starvation this protective mechanism is inactivated and oxidative stress, which is also induced by hop acids, results in inactivated proteins (BEHR and VOGEL, 2009/2010). The latter have to be degraded by Clp protease to avoid disturbance in metabolism by miss-folded proteins. Another protein, up-regulated in manganese-starved cells is the ribosome-associated protein Y (YvyD). YvyD is a member of the ribosomal 30S family and seems to be induced after amino acid and phosphate starvation. Furthermore, this enzyme is also induced by ethanol and acid stress, which are both hurdles, which inhibit bacterial growth in beer (DRZEWIECKI et al., 1998). Manganese-starvation of cells of *L. brevis* increased the fitness with regard to survival in beer and probably, up-regulation of YvyD contributes to tolerance to ethanol stress.

In conclusion, manganese-starved cells exhibited low intracellular Mn^{2+}-levels and an improved capability to grow in pilsner beer. To explain increased fitness comparative proteome analyses were performed and up-regulated proteins were identified under manganese-starvation, which are involved in the arginine deiminase pathway, sugar metabolism, redox homeostasis and translation. These contribute to the fitness of *L. brevis*. Thus, manganese-starvation increases fitness of *L. brevis* and thus tolerance to hop acids, while the inhibitory activity of hop acids is decreased.

4.10 Role of intracellular trace elements

Bacterial cells miss internal compartmentalization and metal ion homeostasis is maintained primarily by regulation of metal cation flux across the cytoplasmic membrane. The incubation in beer induces both influx of calcium, iron and magnesium and release of sodium, potassium, manganese and zinc. Manganese influences the hop inhibitory effects and beer-spoiling lactobacilli decrease intracellular manganese (BEHR et al., 2010). Most commonly, Mn^{2+} and Mg^{2+} are interchangeable on account of the similarities between chelate structures of these ions (JAKUBOVICS and JENKINSON, 2001). This could explain the influx of magnesium, which probably increases hop inhibitory effects to a lower extent and can be used as co-factor for manganese-dependent enzymes. Furthermore, intracellular zinc was also reduced. The physicochemical properties of zinc and manganese are very similar, so it is conceivable that zinc acts in the same way as manganese, which is the reason of efflux.

4.11 Role of fatty acid composition in cell membrane

BEHR et al (2006) measured the membrane fluidity in *L. brevis* TMW 1.465 and noticed that hop and acid stress resulted in decreased membrane fluidity in comparison to reference conditions. In this work, the percentage of cyclic fatty acid in the beer-spoiler group was increased compared with the non-spoiler group. The comparison exhibited that the percentages of saturated fatty acids (14:0, 17:0 cyclo, 19:0 iso) in the cell membrane of beer-spoiling strains were increased. In contrast to that, the unsaturated fatty acid 18:1, which resulted in a higher level of membrane fluidity was decreased. Furthermore, in an independent experiment, where non-adapted cells of *L. brevis* TMW 1.313 were incubated in beer, the amounts of two cyclic fatty acids (17:0 and 19:0) in the cell membrane were increased. Several authors reported, that cyclization of fatty acids is frequently used as a tool to reduce membrane fluidity and decrease permeability (BROWN et al., 1997). For *E. coli* it was reported that cyclic fatty acids strongly decrease the permeability of cell membrane and confer increased tolerance to acidic conditions (SHABALA and ROSS 2008). Therefore, a decrease in membrane fluidity could prevent the penetration of

undesirable molecules like hop acids and thus contribute to hop tolerance and beer-spoiling ability.

4.12 Identification of beer-spoiling strains by MALDI-TOF-MS

As rapid method for determination of bacteria and yeast species, MALDI-TOF/MS was also establish as tool to separate different strains within one species (ARNOLD and REILLY, 1998). For species identification of bacteria the mass range between 2000 to 20000 Da is used, because this is nearly unaffected of different culture conditions (MAIER et al., 2006). In this range very few metabolites are detectable and mainly other high abundance proteins e.g. ribosomal appeared. It is known that hop tolerance is a property, which is conferred by several mechanism of stress response. It is highly probable that in the range of 2000 – 20000 Da mass peaks are present, which are specific for beer-spoiling or non-spoiling strains. This mass peaks can be used as biomarkers for detection of beer-spoiling strains. Thereby, biomarkers do not necessarily have to be associated to the mechanism of hop tolerance but merely correlate with this ability. Due to the high number of data points obtained during measurement of mass spectra, data analysis is only possible by means of bioinformatics. To avoid a loss of data unprocessed mass spectra were analyzed. Only correction of baseline and curve smoothing was performed. The mean of 3 independent spectra of each strain were used for analysis. BioNumerics is a tool to distinguish bacterial mass spectra of different origin by cluster analysis. The assumption that differences in mass spectra of beer-spoiling and non-spoiling strains are present was validated by cluster analysis. Thereby, the concomitant use and analysis of well characterized strains with known origin and beer-spoiling potential as reference give the possibility to identify potential beer-spoiling strains by MALDI. However, it is difficult to get more detailed information about the physiological function behind each peak, because a lot of molecules show identical molecular weights, which currently delineated this method more suitable for practical use.

SUMMARY

The tolerance towards hop acids is one of the most important prerequisites for the survival and growth of bacteria in beer. Furthermore, due to yeast fermentation, the final product beer is poor in nitrogen sources and fermentable sugars like glucose or maltose. Additional stress factors such as alcohol and low pH require advanced mechanisms in stress tolerance. In recent years, new and previously unknown species of beer-spoiling bacteria have been identified, which carry plasmids of already known and beer spoilage-associated genes *horA* and *horC* (HAAKANSEN et al, 2008; EHRMANN et al., 2010). The analysis of flanking regions of different beer-spoiling bacteria suggests the exchange of these genes via horizontal transfer (IIJIMA et al, 2007). To enable plasmid transfer, previously non-spoiling bacteria should express mechanisms of basal hop tolerance. In order to investigate mechanisms of basal hop tolerance, a categorization is necessary. Therefore, different isolates of *L. brevis* (beer, sour dough, plant fermentation) were propagated several times to wean hop-adapted isolates and bring all strains in the same physiological state. Subsequently, all strains were checked for their constitutive beer-spoiling potential and categorized into constitutive beer-spoilers and non-spoilers. This categorization was the basis to identify typical traits within the category of constitutive beer-spoiling strains. By means of identification of these typical traits, biomarkers can be derived for early detection of potentially beer-spoiling bacteria. Based on categorization of beer-spoiling *L. brevis* strains, differential proteomics was performed to identify such mechanisms. It was found, that in exponential growth phase, constitutive beer-spoiling *L. brevis* express mechanisms, which are involved in pH and zinc homeostasis, utilization of maltose and degradation of mis-folded proteins. In stationary state, constitutive beer-spoilers can be separated from non-spoiling strains along the expression of proteins, which are part of replication and DNA repair, homeostasis of copper or manganese, uptake of peptides, utilization of branched chain amino acids and unspecific acetylation of currently unknown substances or hop compounds. It was further investigated, which differences are present in the composition of the cell membrane in both categories. Within the constitutive beer-spoilers, the

percentage of middle-chain, saturated (14:0), cyclic (17:0 cyclo) and unsaturated iso-fatty acids (19:0 iso) was increased. The fatty acid composition influences the fluidity and permeability of cell membranes. This effect is particularly described for cyclic fatty acids, which contribute to increased tolerance to acids and antibiotics (BROWN et al., 1997; SHABALA and ROSS 2008). A differentiation of constitutive beer-spoiling and non-spoiling strains was also possible by the use of mass spectrometry in the range between 2.000 – 20.000 Da.

A further part of the work deals with the influence of divalent manganese on hop tolerance of *L. brevis*. It could be confirmed that the intracellular manganese content depends on the growth mediums manganese content. Low intracellular levels of manganese resulted from lower medium manganese levels. At the same time it could be demonstrated that low intracellular manganese content accelerates growth in strongly hopped beers such as Pilsner beer. On this basis, the proteome was examined under reference conditions and manganese deficiency by comparative 2-D gel electrophoresis and differentially regulated proteins were identified. Depending on the investigated strain, expression of proteins was induced, which are involved in mechanisms of energy gain, acid stress response, redox balance, protein biosynthesis and degradation of mis-folded proteins.

Taken together this thesis categorized for the first time non-adapted *L. brevis* strains along their constitutive potential to spoil beer. In this way, mechanisms of beer-spoiling bacteria, which contribute to a basal level of hop tolerance, could be identified. Additionally, usually non-specific, but typical bacterial stress responses could be induced in the lack of manganese. Thus, the level of basal hop tolerance was increased in constitutively beer-spoiling *L. brevis*.

ZUSAMMENFASSUNG

Die Toleranz gegenüber Hopfensäuren ist eine der wichtigsten Vorrausetzungen für das Überleben und Wachstum von Bakterien in Bier. Aufgrund der Tatsache, dass bei der Herstellung von Bier durch die Hefefermentation ein Großteil an Würzeinhaltsstoffen abgebaut wird, befinden sich im Endprodukt nur noch geringe Mengen an Stickstoffverbindungen und verwertbaren Zuckern. Gleichzeitig erfordern der bei der Hefegärung entstandene Alkohol und der niedrige pH-Wert im Bier eine zusätzliche Stressantwort, um das Überleben zu gewährleisten. In den letzen Jahren sind immer mehr neue, bisher unbekannte Bakterienspezies identifiziert worden, welche Träger von Plasmiden sind, auf denen sich die bereits bekannten und die mit Bierverderb assoziierten Gene *horA* and *horC* befinden (HAAKANSEN et al, 2008; EHRMANN et al., 2010). Die Analyse der flankierenden Regionen unterschiedlicher bierverderbender Bakterienspezies deutet darauf hin, dass diese Gene durch horizontalen Transfer ausgetauscht werden können (IIJIMA et al, 2007). Damit ein solcher Plasmidtransfer stattfinden kann, sollten Bakterien, die bisher kein starkes Bierverderbspotential zeigten, dennoch Mechanismen einer basalen Toleranz gegenüber Hopfensäuren aufweisen. Dazu wurden unterschiedliche Isolate von *L. brevis* (Bier, Sauerteig, Pflanzenfermentation) auf MRS4-Medium mehrmals propagiert, um Hopfen-adaptierte Stämme zu entwöhnen und alle Isolate auf ein einheitlichen Level zu bringen. Dadurch wurde die Voraussetzung geschaffen, die Mechanismen basaler Hopfentoleranz zu untersuchen und eine Kategorisierung hinsichtlich des konstitutiven Verderbspotentials zu realisieren. Dies geschah durch Wachstumstests in verschieden stark gehopften Bieren, wodurch alle Stämme hinsichtlich ihres konstitutiven Bierverderbspotentials überprüft und in konstitutive Bierverderber und Nichtbierverderber kategorisiert werden konnten. Diese Einteilung bildete die Grundlage für die Identifizierung typischer Merkmale innerhalb der Kategorie der konstitutiv bierverderbenden *L. brevis* Stämme. Dabei sollten anhand der Identifizierung charakteristischer Merkmale Marker abgeleitet werden, die der Früherkennung potentiell bierschädlicher Bakterien dienen. Als Werkzeug zur Identifizierung solcher Mechanismen diente die differentielle Proteomik auf Grundlage der vorab

kategorisierten *L. brevis* Stämme. Dabei konnte festgestellt werden, dass konstitutive bierverderbende *L. brevis* in der exponentiellen Wachstumsphase Mechanismen exprimieren, die Bestandteil der pH und Zinkhomöostase, der Verwertung von Maltose und Reparatur sowie Abbau denaturierter oder falsch gefalteter Proteine sind. Im stationären Zustand unterschieden sich konstitutive Bierverderber von nichtbierverderbenden Stämmen durch exprimierte Proteine, die involviert sind in der Replikation und Reparatur von DNA, in der Homöostase von Kupfer- bzw. Mangan, in der Aufnahme von Peptiden und Verwertung verzweigtkettiger Aminosäuren sowie in der unspezifischen Acetylierung intrazellulärer, derzeit unbekannter Substanzen oder auch Hopfeninhaltstoffe. Des Weiteren wurde untersucht, inwieweit Unterschiede in der Zusammensetzung der Zellmembran in Bakterien beider Kategorien vorhanden sind. Dabei konnte gezeigt werden, dass innerhalb der konstitutiven Bierverderber der Anteil an mittelkettigen, gesättigten (14:0), zyklischen (17:0 Cyclo) und gesättigten iso-Fettsäuren (19:0 iso) erhöht war. Die Zusammensetzung der Zellmembran hat dabei Einfluss auf die Fluidität und Permeabilität. Dieser Effekt ist im Besonderen für zyklische Fettsäuren beschrieben und vermittelt erhöhte Säure- und Antibiotikatoleranz (Brown et al., 1997; Shabala and Ross 2008). Zusätzlich konnten beide Kategorien anhand ihrer Massenspektrogramme im Bereich zwischen 2.000 – 20.000 Da differenziert werden. Allerdings war es nicht möglich zu detektieren, welche Funktion hinter den einzelnen Peaks sich verbergen, die zu einer solchen Differenzierung führen.

Ein weiterer Teil dieser Arbeit beschäftigte mit dem Einfluss von zweiwertigem Mangan auf die Hopfentoleranz von *L. brevis*. Dabei konnte für *L. brevis* bestätigt werden, dass der intrazelluläre Mangangehalt von der Mangankonzentration im Wachstumsmedium abhängig ist. Niedrige intrazelluläre Manganlevel waren das Resultat niedriger Konzentrationen an Mangan im Medium. Gleichzeitig konnte gezeigt werden, dass eine geringe Konzentration an intrazellulärem Mangan das Wachstum in stark gehopften Bieren (Pils) beschleunigt. Davon ausgehend wurden das Proteom unter Referenzbedingungen und bei Manganmangel unter Verwendung differentieller Proteomik untersucht und unterschiedlich regulierte Proteine identifiziert. Dabei wurden in Abhängigkeit vom untersuchten Stamm die Expression von Proteinen induziert,

die der zusätzlichen Energieerzeugung dienen, Säurestress vermeiden, für ein optimales Redoxgleichgewicht sorgen, Bestandteil der Proteinbiosynthese sind bzw. falsch gefaltete Proteine degradieren.

Zusammenfassend kann gesagt werden, dass in dieser Arbeit erstmalig unterschiedliche, nicht an Hopfen adaptierte *L. brevis* Stämme durch Wachstumstest in Bier anhand ihres konstitutiven Potentials zum Bierverderb kategorisiert wurden. Dadurch konnten Mechanismen identifiziert werden, die in bierverderbenden Bakterien ein Grundlevel an Hopfentoleranz vermitteln. Gleichzeitig konnten zusätzliche, in der Regel unspezifische und für Bakterien typische Stressantworten durch den Mangel an Mangan induziert werden, die den Level an Hopfentoleranz erhöhen.

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