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Dissection of the molecular mechanism of hop inhibition in *Lactobacillus brevis*

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Vorwort

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Abbreviations

°C	degree Celsius
2D	two-dimensional
3-MPA	3-mercaptopropionic acid
A	ampere
a	atto (10^{-18})
ACC	acetyl-CoA carboxylase
AcN	acetonitrile
ACP	acyl carrier protein
ADI	arginine deiminase
ADP	adenosine diphosphate
AmBic	ammonium bicarbonate
APS	ammonium persulfate
ATP	adenosine triphosphate
β-CD	β-cyclodextrin
BLM	bilayer lipid membrane
Bromophenol Blue	3′,3″,5′,5″-tetrabromophenolsulfophthalein sodium salt
cal BC	calibrated years before Christ
Calcein-AM	O′-diacetate tetrakis(acetoxymethyl) ester
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
cDNA	copyDNA
cEt	crude ethanol hop extract
cHex	crude hexane hop extract
CID	collision induced dissociation
CoA	coenzyme A
CP	crossing point
cTHF	crude tetrahydrofuran hop extract

Da	Dalton
DEE	diethyl ether
df	hop draff
dH_2O	deionised water
DMS	dimethyl sulphate
DNA	desoxyribonucleic acid
dNTP	desoxy nucleoside triphosphate
ΔΨ	membrane potential
dr	hop δ-resin
ds	double-stranded
DTT	1,4-dithio-D,L-threitol
EDTA	ethylenediaminetetraacetic acid
EF-Tu	elongation factor thermo unstable
em	emission
er	hop ε-resin
ETD	electron transfer dissociation
EtOH	ethanol
ex	excitation
f	femto (10^{-15})
F01-11	hop ε-resin subfraction 1 – 11
FA	fatty acid, formic acid
FAS	fatty acid synthase
Fig.	figure
FMOC	9-fluorenylmethoxycarbonylchloride
g	gram
GABA	γ-aminobutyric acid
GAD (system)	glutamic acid decarboxylase (system)

GadB	glutamic acid decarboxylase
GadC	Glu/GABA antiporter
GC	gas chromatograph(y)
gh	green/fresh hops
Glc	glucose
Glu	glutamic acid
h	hour, hecto (10^2)
ha	hectare (10,000 m^2)
hr	hop hard resin
k	kilo (10^3)
λ	wavelength
L	litre
L.	*Lactobacillus*
LAB	lactic acid bacteria
LC-ESI MS/MS	liquid chromatography coupled to tandem mass spectrometry with electrospray ionisation
LC-MS	liquid chromatography-mass spectrometry
lmw	low molecular weight
µ	micro (10^{-6})
m	milli (10^{-3}), meter
M	Mol, molar, mega (10^6), hop variety HALLERTAUER MAGNUM
m/z	mass to charge ratio
MALDI-TOF MS	matrix-assisted laser desorption ionisation time of flight mass spectrometry
MEBAK	Mitteleuropäische Brautechnische Analysenkommision
MeOH	methanol
MES	2-[N-morpholino]ethanesulfonic acid
mgf	mascot generic format

MIC	minimum inhibitory concentration
min	minute
mio	million
MPI	message passing interface
mRNA	messenger ribonucleic acid
MRS	DE MAN, ROGOSA and SHARPE
Mw	molecular weight
n	nano (10^{-9})
NCBI	National Center for Biotechnology Information
Nig	nigericin
NMR	nuclear magnetic resonance spectroscopy
o.n.	over night
OD	optical density
OPA	o-phthalaldehyde
p	pico (10^{-12})
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
Pe	hop variety PERLE
$pH_{in/ex}$	intracellular / extracellular pH-value
pl	hop pellets
pmf	proton motive force
ppm	parts per million
qRT-PCR	quantitative real-time polymerase chain reaction
RFU	relative fluorescence unit
rh	raw hops
RNA	ribonucleic acid
RP-HPLC	reversed phase high performance liquid chromatography

s	second
sd	standard deviation
SDS	sodium n-dodecyl sulphate
sr	hop soft resin
ss	single-stranded
Tab.	table
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylendiamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TMW	Technische Mikrobiologie Weihenstephan
Tr	hop variety HALLERTAUER TRADITION
tr	hop total resin
Tricine	N-[tris(hydroxymethyl)methyl]glycine
Tris	tris(hydroxymethyl)aminomethane
Ts	hop variety HALLERTAUER TAURUS
TUM	Technische Universität München
UV	ultra violet
V	volt
v/v	volume / volume
Val	valinomycin
w/v	mass / volume
w/w	mass / mass
ZfP	Zentrallabor für Proteinanalytik, München

Contents

1 Introduction

1.1 Beer and the use of hops (*Humulus lupulus*) - a brief history

Consumption of alcoholic beverages is firmly anchored in western cultures as part of meals or social meetings. In most countries of the European Union, beer nowadays is the most prevalent alcoholic drink (Bloomfield et al. 2003; Popova et al. 2007; OECD 2012). On a global scale, following water and tea, beer even ranks three in the most consumed beverages (Nelson 2005). In Germany, approximately 81 mio hL beer was consumed in 2012 equalling a per capita consumption of about 100 L (Statistisches Bundesamt 2013).

The consumption of beer and the brewing process itself have a long and exciting history. Analyses of beer residua found in vessels in an excavation site in upper Egypt suggest that first beers have been brewed 3500 - 3400 cal BC (Maksoud et al. 1994). Presumably, the taste of the ancient Egyptian brews did not have much in common with today's beers as hops were not on the list of ingredients. At that time, the so-called Nekhen-Hoffmann beer was probably primarily only made of water, wheat (*Triticum dicoccum*) and barley (*Hordeum vulgare*). Dates (or date juice) along with grapes were for the most part used as flavouring components (Maksoud et al. 1994; Hornsey 2003). It is reported that the first use of female inflorescences of the hop plant (*Humulus lupulus*) as spices in brewing can be dated back only to the sixth century BC (Sakamoto 2002). Yet, for a long time hops were not the first choice to season beer in Europe. During the Middle Ages, sugar and honey were added to beer as sweeteners and a mixture of different herbs and other plants (e.g. wild rosemary, ginger, laurel leaves and above all sweet gale (*Myrica gale*)) was used to add flavour to beers, which became known under the name *gruit beer* (Verzele & De Keukeleire 1991; Behre 1998; Hornsey 2003). However, the use of hops became more and more established because hops comprised several advantages over the *gruit* mixture, not least the price of the spice. Following the enactment of the German *Reinheitsgebot* (purity law) in 1516 by the Bavarian duke Wilhelm IV, Bavarian brewers were obligated to exclusively use water, barley malt and hops. Subsequently, the

use of hops successively spread also outside of Bavaria (Verzele & De Keukeleire 1991; Behre 1998). The increased use of hops did however not only coin the comfortable and desired flavour and bitter taste of beer, but also rendered it less susceptible to microbial spoilage since several antimicrobial hop components and hop derivatives are known to protect beer from spoilage (Simpson & Fernandez 1992; Garetz 1994; Sakamoto 2002; Sakamoto & Konings 2003; Haseleu et al. 2009; Intelmann & Hofmann 2010). Most likely, this fortunate coincidence was the determining factor for the continuing use of hops and cemented its role in brewing until today.

1.2 The hop plant, hop products and hop compounds

Humulus represents a genus of the plant family *Cannabinaceae*. The two known hop species are *Humulus japonicus* and *Humulus lupulus*. Because of the resin content, exclusively the latter is used in brewing and all hops cultivated today are varieties of *Humulus lupulus*. However, its young shoots can also be eaten and are regarded an asparagus alike culinary delicacy. *Humulus japonicus* is merely grown as an ornamental plant (Verzele & De Keukeleire 1991; Garetz 1994).

The transition from the vegetative phase of growth to the generative phase in hops is determined by prevailing lighting conditions. A minimum of 16 - 18 h length of day and subsequent shorter days are prerequisite to reach the generative phase. These limiting conditions are only met between 35 and 55 degrees latitude. Therefore, the main production areas are located in the northern hemisphere in Central Europe (Germany, Czech Republic, Poland, Republic of Slovenia, England, France), USA (Washington, Oregon, Idaho) and northeast China. In 2010, Germany was the biggest hop producer with 18,386 ha cultivated area, followed by the USA (12,647 ha) (Verzele & De Keukeleire 1991; Biendl et al. 2012). Besides the mentioned length of day, the hop plant further requires fertile soils and distinct amounts of rain and groundwater and can hence be considered a rather difficult crop plant to cultivate (Verzele & De Keukeleire 1991).

The perennial climbing plant today is cultivated in commercial hop yards, where its annual bines use strongly hooked hair to grow clockwise along climbing support strings of 5.5 - 7.5 m length. The female inflorescences of the dioecious plant grow on lateral branches of the vine together with hop leaves and are referred to as hop cones. Only the cones contain substances which are of interest to brewers. Hop cones consist of a central stem (strig), outer bracts, and inner bracteoles holding the lupulin glands (cf. Fig. 1) (Verzele & De Keukeleire 1991; Garetz 1994; Behre 1998; Hornsey 2003; Biendl et al. 2012).

Fig. 1: Green hop cones on the day of harvest. Left: intact cone. Right: cutaway. 1, bract; 2, strig; 3, bracteole; 4, lupulin glands.

Depending on the hop variety, weather conditions, the location of the cultivated area and several commercial considerations, hops are harvested from the end of August until the beginning of October in the Northern hemisphere (Verzele & De Keukeleire 1991; Biendl et al. 2012). For most subsequent purposes, immediately after the harvest, fresh hop cones (containing approx. 80 % water) are separated from the bine and leaves, and promptly dried at ideally 62 - 65 °C, to reach a final water concentration of

9 - 10 % (Münsterer 2006; Biendl et al. 2012). After drying, the cones contain about 24 % bitter substances, 5 % polyphenols, 1 % essential oil, 10 % water and 60 % other substances (cellulose, lignin, protein, amino acids, minerals, lipids, carbohydrates and pectin) (values represent averages from several hop varieties according to BIENDL et al. (Biendl et al. 2012)).

Nowadays a broad range of different hop varieties is available on the market and the quest for new cultivars featuring desired characteristics (e.g. variety depending concentrations of constituents, resistances against pests and diseases) by hop cross breeding is proceeding continuously. Hop varieties are classified as aroma hops or bittering hops by respective responsible regulatory authorities. It is generally agreed though, that a categorisation of hops as aroma and bittering hop varieties along their constituents is not feasible because aroma hops contain bitter substances and vice versa (Verzele & De Keukeleire 1991; Biendl et al. 2012). Nevertheless, hop varieties with mild and pleasant aroma, increased polyphenol content and α-acids contents below 10 % are generally referred to as aroma hops (Nance & Setzer 2011; Biendl et al. 2012). Additionally, there are further variety categories in use, such as dual-purpose (bittering and aroma contributing) hops and some which were labelled high-alpha or super high-alpha varieties (alpha-acids content of approx. 15 %). Furthermore, also the descriptions noble hops and noble aroma hops are common. The classification system must hence rather be regarded to aim at a grouping of hops which are mainly used to add bitterness and hops mainly used to add the typical hoppy aroma to beer. Some of the most widely used hops are PERLE, HALLERTAUER TRADITION, SPALTER SELECT, CASCADE, WILLAMETTE and AURORA (aroma hops) and HALLERTAUER MAGNUM, HALLERTAUER TAURUS, NUGGET and GALENA (bittering hops) (Biendl et al. 2012).

Accordingly, the typical hoppy aroma and bitterness of beer is brought about by a plethora of different hop derived compounds from numerous different hop varieties. During beer maturation and subsequent storage and aging, a part of these compounds undergoes further chemical transformations involving further augmentation of the variety of aroma active substances (Peacock et al. 1980; Eyres et al. 2007; Intelmann et al. 2011).

Until the twentieth century, hops were primarily added to beer as dried hop cones (raw hops). Over the years, the use of numerous different hop products gained more importance. In 2010, raw hops accounted for only 2 % of the worlds hop market. The reasons for this include, inter alia, the difficult handling, a short storage life and the constitutional heterogeneity of raw hops. Nowadays, unprocessed hop pellets (49 %) and (ethanol- or CO_2-) hop extracts (28 %) are the most used hop products. Pre-isomerised products account for another 21 % of the global hop market (Garetz 1994; Biendl et al. 2012).

Hop secondary metabolites with relevance in brewing can be assigned to three groups, namely hop resins, hop essential oils and polyphenols.

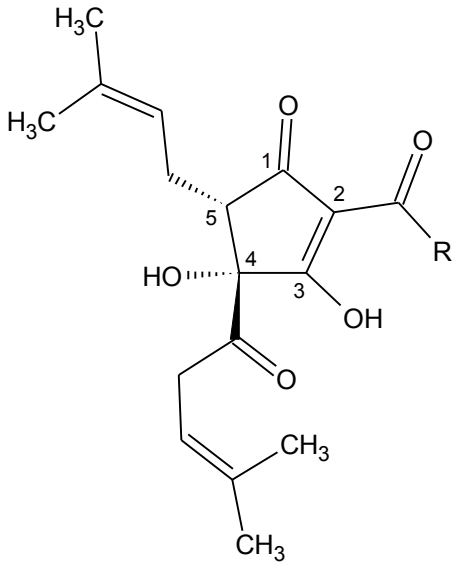
In the bracts and bracteoles of hop cones, mainly polyphenols, comprising flavanols (e.g. catechin, epicatechin, gallocatechin), flavonols (e.g. glycosidically bound kaempferol or quercetin) and phenolic carboxylic acids (e.g. resveratrol, ferulic acid, caffeic acid, coumaric acid) can be found (Stevens 1966; McMurrough et al. 1982; Taylor et al. 2003; Biendl et al. 2012).

In the lupulin glands of the bracteoles, the so-called lupulin, which consists of essential oils and hop resins, is produced. The volatile essential oils include monoterpenes (e.g. myrcene, pinene, limonene), sesquiterpenes (e.g. farnesene, humulene, caryophyllene, selinene) and oxygenated compounds (e.g. linalool, geraniol, nerol, humulene epoxide) (Stevens 1966; Eri et al. 2000; Eyres et al. 2007; Biendl et al. 2012).

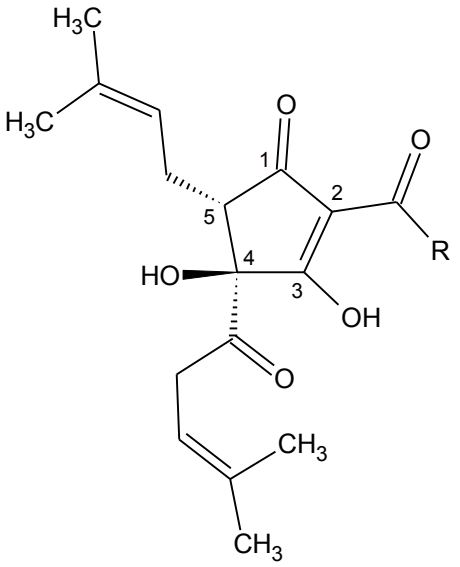
The third group of hop compounds, i.e. the resinous part of lupulin can be subdivided into a hard resin and soft resin fraction on basis of their solubility (Stevens 1966; Pfenninger 1997). The principle prenylated flavanoid of hops, xanthohumol, accounts for a major part of the hard resin. Besides in hops however, it has so far only been found in a Chinese medicinal plant (*Sophora flavescens*), which makes it a fairly extraordinary substance. Other than xanthohumol, the hard resin fraction also contains further prenylated flavanoids such as 8-prenylnaringenine and flavanoids with a geranyl moiety. Yet uncharacterised substances form another part of the hard resin fraction (Milligan et al. 1999; Biendl et al. 2012). The soft resin's two main

compounds are humulones (mainly cohumulone, n-humulone and adhumulone) and lupulones (mainly colupulone, n-lupulone and adlupulone) and were also termed α-acids and β-acids, respectively (Stevens 1966; Biendl et al. 2012).

Although, the soft resin fraction is defined as hexane soluble, and therefore only minor parts of it can be found in beer, the soft resin fraction nonetheless plays a major role in brewing. During the wort boiling process α-acids are converted into iso-α-acids. For each of the three iso-α-acid congeners, a cis and trans diastereoisomeric form was found. The cis and trans conformation refers to the position of the tertiary hydroxyl group at C-4 and the (3-methyl-2-butenyl) alkenyl side chain at C-5. Both groups are located on the same side of the plane of the central five membered homocyclic structure in the cis form, whereas they are located on opposite sides of the ring in the trans form. (De Keukeleire & Verzele 1971). The isomerisation products exhibit significantly increased solubility and antimicrobial properties (Simpson & Smith 1992; García-Villalba et al. 2006). Since iso-α-acids moreover represent the main bittering component in beer (Jaskula et al. 2008; Intelmann & Hofmann 2010; Intelmann et al. 2011), it can be concluded that they play a role of utmost importance in brewing. A depiction of the molecular structure of cis- and trans-iso-α-acids is shown in Fig. 2.



cis-iso-α-acid



trans-iso-α-acid

R	cis-iso-α-acid	trans-iso-α-acid
$CH(CH_3)_2$	cis-iso-cohumulone	trans-iso-cohumulone
$CH_2CH(CH_3)_2$	cis-iso-n-humulone	trans-iso-n-humulone
$CH(CH_3)_2C_2H_5$	cis-iso-adhumulone	trans-iso-adhumulone

Fig. 2: Molecular structure of cis- and trans-iso-α-acids (De Keukeleire & Verzele 1971)

1.3 Spoilage susceptibility of beer and beer-spoiling microorganisms

Maintenance of cellular pH and redox homoeostasis is of prime importance for the viability of microorganisms. Especially in foods, environmental conditions like extreme pH values, aridity, osmotic stress or the presence of weak acid preservatives can endanger the survival of microbes and thus protect the products from microbial spoilage. Beyond that, detrimental temperatures during the production process and subsequent storage can impair microorganisms (Russell et al. 1995; Krämer 2002).

Compared to many other foods and drinks, beer represents a very hostile ambience for microorganisms. Beer offers only scarce nutrient content regarding amino acids and low molecular weight carbohydrates and its oxygen concentration (< 0.1 ppm) and carbon dioxide concentration (approx. 0.5 w/w) make it almost anaerobic with redox potential values ranging

from -42 to -83 mV. These harsh conditions are further aggravated by acidic conditions with pH values between pH 3.8 and pH 4.8 and the presence of ethanol at levels of 0.5 - 10 % (Back 1994; Sakamoto & Konings 2003). Beyond that, the addition of hops during the brewing process imposes another hurdle for microbial growth as several hop derived substances were shown to be antimicrobially active (Shimwell 1937b; Shimwell 1937a; Teuber & Schmalreck 1973; Mizobuchi & Sato 1984; Mizobuchi & Sato 1985; Simpson & Fernandez 1992; Simpson & Smith 1992; Fernandez & Simpson 1993; Simpson 1993a; Simpson 1993b; Schurr et al. 2013).

Only a fairly small group of ingenious specialists are capable to overcome all these growth limiting factors and grow in beer. Among them, there are a number of yeasts (Loureiro & Querol 1999; Usbeck et al. 2013). Most spoilage incidents (about 90 %) however, are caused by only four bacterial genera, namely *Lactobacillus*, *Pediococcus*, *Pectinatus* and *Megasphaera*. *Lactobacillus* (*L.*) *brevis* can be regarded the most prominent representative of this group, because it is the most frequently occurring beer-spoiling bacterium accounting for approximately 35 % of all cases (Back 1994; Sakamoto & Konings 2003; Thelen et al. 2006; Suzuki 2011).

L. brevis strains have been isolated from multifaceted sources including (fermented) vegetables, cheese, sourdough, spoiled beverages (beer, wine and soft drinks) and the intestinal tract (Bottazzi 1988; Back 1994; Li & Cao 2010).

L. brevis is a Gram-positive, (short) rod-shaped, non-sporeforming, non-motile, (catalase-negative, oxidase-negative) facultative anaerobic bacterium with an optimal growth temperature of 30 °C and optimal growth pH ranging from pH 5.5 to pH 6.0. Its average guanine-cytosine (GC) content is 45 - 47 %. As an obligate heterofermentative saccharolytic microorganism, it uses mainly carbohydrates as energy source (Kandler 1983; Bottazzi 1988; Schlegel 1992; Back 1994; Krämer 2002). *L. brevis* utilises the pentose phosphate pathway (PPP) and phosphoketolase pathway (PKP) to metabolise hexoses like glucose or fructose to generate adenosine triphosphate (ATP). Pentoses can be funnelled into the catabolic pathway at the level of xylulose-5-phosphate. Besides ATP generation, lactic acid,

carbon dioxide, ethanol and acidic acid are yielded (Kandler 1983; Schlegel 1992; Krämer 2002; Berg et al. 2003).

Microbial growth affiliated with the described kind of metabolism and its catabolic end products can lead to altered beer flavour profiles and possibly even formation of haze, i.e. beer-spoilage.

1.4 Antimicrobial mode of action of hop compounds

From the onset of the twentieth century, the antimicrobial activity of hop compounds and hop derivatives has been in the focus of scientists (Shimwell 1937a). The discovered antimicrobial effects of hops are diverse. Iso-α-acids can decrease the cellular content of ATP of resting cells, reduce L-leucin uptake and deplete L-leucine preloaded cells of *L. brevis* (Simpson 1993b). In *Bacillus* (*B.*) *subtilis*, hop derivatives caused the generation of membrane leakages, which led to glucose efflux, impaired respiratory chain dehydrogenase activity and synthesis of proteins, ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) (Teuber & Schmalreck 1973). Bilayer lipid membrane (BLM) technique-based, as well as fluorescence- and radioactivity-based experiments could furthermore verify that iso-α-acids with their acidic hydroxyl group in the C-3 position (cf. Fig. 2) can act as potent ionophores. They can thus impair or even abrogate the cells' proton motive force (pmf) in acidic environments. By this means, the transmembrane proton gradient is dissipated and the intracellular pH (pH_{in}) decreases in acidic environments like beer (Simpson 1993a; Simpson 1993b; Behr & Vogel 2009; Schurr et al. 2013). The loss of pmf leads to impeded nutrient uptake, and low pH_{in} impairs the cellular metabolism as enzyme activity is decreased (Kashket 1987; Simpson & Smith 1992) or completely abolished (e.g. glycolysis minimum pH = 4) (Kashket 1987). The ionophore activity of iso-α-acids has been attributed to the substances' power to act as a weak acid. As such, they can permeate the cell membrane in their undissociated form and release a proton in the cytosol due to higher pH in the intracellular space (Simpson & Smith 1992; Sakamoto et al. 2002; Blanco et al. 2006).

The antimicrobial activity of hops is strongly dependent on environmental or experimental conditions like the pH-value, temperature and the presence of various cations. SIMPSON and SMITH demonstrated that the minimum inhibitory concentration (MIC) of colupulone, n-humulone, trans-iso-n-humulone and trans-humulinic acid is lower under acidic conditions. Low MIC values reflect high antimicrobial activity. The group further showed that the MIC of trans-iso-humulone is increased in the presence of monovalent cations (K^+, Li^+, Rb^+, Na^+) but decreased by divalent cations (Mg^{2+}, Ca^{2+}) (Simpson & Smith 1992; Simpson 1993b) and that the ionophore activity of trans-iso-humulone was decreased at lower temperatures (Simpson 1993a). BEHR and VOGEL showed that the redox-reactivity of iso-α-acids is dependent on pH and the presence of Mn^{2+} (Behr & Vogel 2010). Manganese induced UV-spectral changes have led to the conclusion, that the chromophore β-triketone group (cf. Fig. 2) is associated with manganese binding (Simpson & Hughes 1993). However, to the best of my knowledge, no attempts have been made to further elucidate the manganese/hop interaction site.

1.5 Hop stress tolerance mechanisms

Despite the detrimental effects of hops and additional adverse conditions, microbial growth can occur in beer. Microorganisms capable of overcoming the multitude of hurdles described in 1.3 must therefore be equipped with a set of (hop) stress tolerance mechanisms.

The membrane bound multi drug resistance (MDR) transporter termed HorA has been described first by SAMI et al. HorA has been shown to confer hop tolerance by ATP-dependent export of hop compounds (Sami et al. 1997; Sami et al. 1998; Sakamoto et al. 2001) and is present in various *L. brevis* strains (Preissler et al. 2010; Preissler 2011). Similarly, the MDR transporter HorC, which can also be found in numerous *L. brevis* strains (Suzuki, Kazumaru Iijima, et al. 2005; Preissler et al. 2010; Preissler 2011) is described as a pmf-dependent hop compound extruding protein (Suzuki et al. 2002; Iijima et al. 2006).

Another transport system associated with hop tolerance in beer-spoiling bacteria is a putative divalent cation transporter termed HitA (hop inducible cation transporter). HitA was suggested to mediate the import of ions such as Mn^{2+} into cells which are devoid of transmembrane proton gradient (Hayashi et al. 2001). BEHR et al. demonstrated HitA overexpression in *L. brevis* TMW 1.465 upon adaptation to iso-α-acids (Behr et al. 2006). A screening of a broad spectrum of *L. brevis* strains showed that HitA can be found in many strong beer-spoiling strains (Preissler et al. 2010; Preissler 2011).

The presence of the genes encoding for the described membrane transport systems alone, however, is not a decisive factor for hop tolerance (Preissler 2011). It was furthermore found that HorA was not expressed during an iso-α-acids adaptation process, although the *L. brevis* strain (TMW 1.465) met the genetic prerequisites (Behr et al. 2006). On the other hand, a strong positive correlation of beer-spoiling ability of *L. brevis* and the presence of genes (*horB*, *horC*, ORF5, *horA*) encoding for HorA and HorC could be demonstrated for a set of 46 strains (Suzuki, Kazumaru Iijima, et al. 2005). These partly deviating results may be a consequence of the use of different media or beers and hop-adapted or non-adapted microorganisms in the determination of the spoilage potential (Preissler 2011).

Beer-spoiling *L. brevis* ABBC45 was demonstrated to increase its F_OF_1-ATPase[1] activity and expression during adaptation to hops (iso-α-acids) to counteract the aforementioned ionophore activity of hop compounds and thereby maintain pmf and pH_{in} (Sakamoto et al. 2002). Similarly, hop resistant lactic acid bacteria were reported to maintain higher ATP pools in comparison to hop sensitive organisms (Simpson 1993b). In line with these findings, overexpression of enzymes of the arginine deiminase pathway (ADI)

[1] According to the "Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB)", the correct name of the enzyme F_OF_1-ATPase (EC 3.6.3.14) is: ATP phosphohydrolase (H^+-transporting).

However, also other names have been used for the enzyme: ATP synthase; F_1-ATPase; F_OF_1-ATPase; H^+-transporting ATPase; mitochondrial ATPase; coupling factors (F_0, F_1 and CF_1); chloroplast ATPase.

Further, although incorrect, the term F_0F_1-ATPase is frequently used. The "O" in F_O refers to the inhibitory effect of oligomycin. The frequent incorrect use might be due to the visual similarity of O and 0.

Reference: (NC-IUBMB 2000)

contributing to energy generation and pmf maintenance could also be demonstrated in hop adapted *L. brevis* TMW 1.465 (Behr et al. 2006).

BEHR et al. were able to show an increase of lipoteichoic acids (LTA) incorporated in the cell wall of *L. brevis* after adaptation to hop stress (Behr et al. 2006). LTA form part of a polyanionic network which is described as a polyelectrolyte gel. With its ion-exchange character, this network is also involved in the homeostasis of metal ions (Neuhaus & Baddiley 2003) and supplies a permanent pool of divalent cations (Hughes et al. 1973). Considering the above mentioned role of cations in the inhibitory effect of hops (cf. 1.4) and the outstanding role of Mn^{2+} in the survival of lactobacilli (Archibald & Duong 1984; Archibald 1986), the increase of LTA in the cell wall can be interpreted as a protective shield. Similarly, YASUI and YODA found a high correlation between the beer-spoilage potential of *L. brevis* strains and the presence of a so-called SABSL (specific antigen to beer-spoiling *L. brevis* strains) antigen in the cell wall and supposed that SABSL might serve as a selective iso-humulone trap (Yasui & Yoda 1997).

Beyond changes of the cell wall, also the cell membrane can be subject to modifications as response to hop stress. Iso-α-acids mediated hop stress induced alterations of the composition and concomitant fluidity of the cell membrane which are associated with decreased membrane permeability (Behr et al. 2006). The role of cytoplasmic membranes in microorganisms is described in more detail in 1.6.

In proteomic investigations, BEHR et al. could furthermore show, that a hop adapted *L. brevis* strain was capable to upregulate expression of manganese binding proteins and to adjust the expression of energy generation related enzymes and enzymes involved in DNA repair mechanism and protein degeneration (Behr et al. 2007; Behr 2008).

1.6 Hop stress and cytoplasmic membranes

Cytoplasmic membranes consist of two antipodal asymmetrical (glycero-) phospholipid layers (often called leaflets) and numerous embedded (integral) proteins serving various functions. The hydrophobic ends of phospholipids and triglycerides are orientated to the inner side, while hydrophilic head

groups form the outer part of the bilayer (Singer & Nicolson 1972; Schlegel 1992; Berg et al. 2003).

In bacteria, cytoplasmic membrane phospholipids, which account for the biggest share of membrane lipids, are mainly composed of glycerol, a phosphate group and two fatty acid chains. This type of phospholipid, in which fatty acids are linked to glycerol 3-phosphate by ester bonds in the C-1 and C-2 position, is referred to as glycerophospholipids (Berg et al. 2003; Zhang & Rock 2008).

Due to their composition and structure, cytoplasmic membranes are described as semipermeable or selectively permeable. Accordingly, as such they are permeable for hydrophobic non-polar molecules (e.g. CH_4, O_2) and small uncharged polar molecules (e.g. H_2O, glycerol, CO_2). On the contrary, they are impermeable for bigger, charged and uncharged polar molecules (e.g. glucose, nucleic acids, amino acids) and ions (Cl^-, HCO_3^-, Na^+, H^+, Mn^{2+}, Mg^{2+}) (Schlegel 1992; Berg et al. 2003).

The illustrated characteristics, together with membrane embedded energy dependent ion pumps, allow the generation of transmembrane ion gradients (Berg et al. 2003). These electrochemical gradients consist of two components, namely an electrical component (membrane potential, $\Delta\Psi$) and a chemical component (transmembrane concentration gradient of charged species e.g. ΔpH, ΔNa^+). The electrochemical H^+ gradient, as it can be found, for instance, in lactic acid bacteria (LAB) is called proton motive force (pmf) and is characterised by higher extracellular H^+ concentration. It therefore features inside negative $\Delta\Psi$ and its ΔpH is outside acidic (Krulwich et al. 2005).

Pmf can drive as vital mechanisms as ATP generation (F_OF_1-ATPase, (Boyer 1993; Dimroth et al. 2006)), nutrient uptake (glucose/H^+ symport, (Keevil et al. 1984; Ye et al. 1994)) or export of toxic substances (MDR transporters, (Suzuki, Kazumaru Iijima, et al. 2005; Nikasa et al. 2013)). The maintenance of pmf as an attribute of the cytoplasmic membrane is therefore crucial for the viability of bacteria. However, it can be subject to various perturbations.

BEHR and VOGEL showed that hop ionophore iso-α-acids interfered with the membrane potential and characterised iso-α-acids as pH-dependent uncouplers of class I or II, which are capable of transmembrane proton transport at various pH-values. Another effect of iso-α-acids on biological membranes was found to be the facilitation of transmembrane charge transport at low pH and in presence of manganese. The increased conductivity of otherwise outstandingly well insulating BLMs and concomitant redox reaction can hence cause oxidative stress (Behr & Vogel 2009; Behr & Vogel 2010).

In bacteria and other organisms, cytoplasmic membranes fulfil several vital functions, such as, the separation of the cytoplasm and the extracellular space, selective nutrient uptake, export of various substances and the aforementioned maintenance of pmf (Berg et al. 2003). It can therefore be regarded an indispensable necessity for microorganisms to preserve cell membrane functionality. It has been shown that different stress factors (e.g. acidity, temperature) can induce changes in the fatty acid (FA) composition of cell membranes (Castro et al. 1997; Quivey et al. 2000; Fozo & Quivey 2004; Wang et al. 2005). A more detailed review discussing further stress factors, like osmotic stress, high pressure, xenobiotics etc., is given by MYKYTCZUK et al. (Mykytczuk et al. 2007).

Bacteria can manage changes of the cell membrane fatty acid composition either by modification of membrane glycerolipids or by de novo synthesis of fatty acids. The most important acyl chain modifications comprise cyclisation, desaturation and cis-trans isomerisation (Cronan 2002; Zhang & Rock 2008). The mechanism of greater importance, however, is the fatty acid biosynthesis and subsequent glycerolipid synthesis (Zhang & Rock 2008).

Bacteria employ the fatty acid synthase II (FAS II) pathway consisting of a set of enzymes, which are encoded for by highly conserved genes (Zhang & Rock 2008). The FAS II pathway is illustrated in Fig. 3.

In the initiating step of the FAS II pathway, the carboxylation of acetyl-coenzyme A (CoA) to malonyl-CoA is catalysed by the biotin cofactor dependent acetyl-CoA carboxylase (ACC) Then, malonyl-CoA is attached to a low molecular weight (lmw) protein by malonyl-CoA-ACP transacylase. This

lmw protein is termed acyl carrier protein (ACP). Thereafter, β-ketoacyl-ACP synthase mediates the condensation of acyl-CoA and malonyl-ACP forming a β-ketoacyl-ACP intermediate (Jackowski & Rock 1987).

ACP plays a role of outstanding importance in FA biosynthesis. In subsequent reactions, ACP serves as a shuttle protein, transferring the pathway's intermediates to FAS II subunits during the FA elongation process. The intermediates are covalently bound to the 4'-phosphopantetheine prosthetic group of ACP as thioesters (Sabaitis & Powell 1976; Heath et al. 2002; Berg et al. 2003). This prosthetic group is transferred by the enzyme ACP-synthase to inactive apo-ACP forming the active holo-ACP. As practically the entire ACP pool is present in the holo form *in vivo*, availability of prosthetic groups cannot be a limiting factor of FA and consequently also of glycerophospholipid biosynthesis (Heath et al. 2002).

The follow-up FA-elongation process involves four iterative reactions (Heath & Rock 1995). Firstly, the β-ketoacyl-ACP intermediate is reduced to β-hydroxyacyl-ACP (β-ketoacyl-ACP reductase). Then, trans-2-enoyl-ACP is formed by dehydration. This reaction can be catalysed by at least two isoforms of β-hydroxyacyl-ACP dehydratase (Heath & Rock 1996). Enoyl-ACP reductases catalyse another reduction forming acyl-ACP (Heath & Rock 1995), before the reaction cycle can commence again with an elongation condensation reaction mediated by ketoacyl-ACP synthase, finally forming a new β-ketoacyl-ACP intermediate (Morgan-Kiss & Cronan 2008; Zhang & Rock 2008). Ultimately, the synthesised FAs are sequentially bound to glycerol-3-phosphate in a two-stage process, forming phosphatidic acid, the main precursor of glycerophospholipids with differently modified polar head groups attached to the C-3 bound phosphate (Zhang & Rock 2008; Lu et al. 2006). The two enzymes in charge of this two-stage process are accordingly called glycerol phosphate acyltransferase and 1-acylglycerol phosphate acyltransferase (Heath et al. 2002).

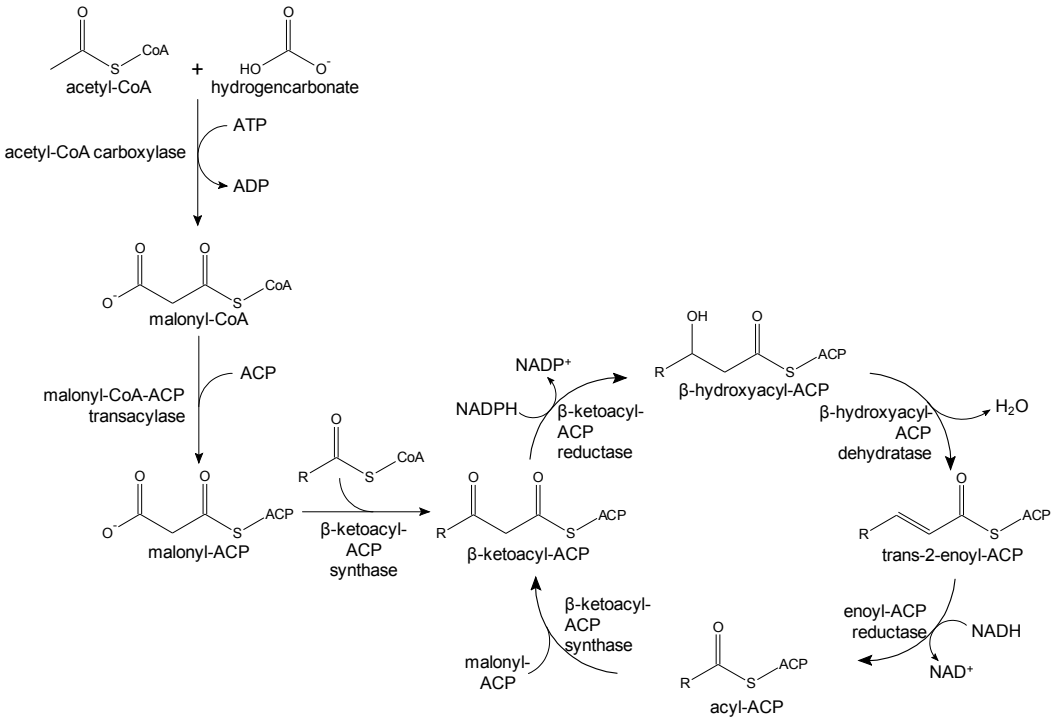


Fig. 3: FAS II pathway. Adapted from (Heath et al. 2002) and (Zhang & Rock 2008). Acyl carrier protein (ACP) serves as a shuttle transferring fatty acid biosynthesis intermediates between enzymatic subunits.

In addition to the aforementioned stress factors, yet another trigger for cell membrane composition shifts can be hop compounds. In line with the above-mentioned influence on pmf of BLMs, BEHR et al. were able to show hop induced alterations in the membrane fatty acid composition in a *L. brevis* strain on a physiological level (Behr et al. 2006). Moreover, a proteomic approach elucidated part of the mechanistic background of the observed changes (Behr et al. 2007). However, the proteomic approach chosen exhibits certain immanent limitations, as only proteins between 10 kDa and 250 kDa could be assessed by two-dimensional (2D) gel electrophoresis (Behr 2008; Preissler 2011). Above all, 2D gel electrophoresis is not a practicable tool for screenings, as it is highly laborious and time consuming.

1.7 The glutamic acid decarboxylase (GAD) system

It is axiomatic that LAB must be able to survive and propagate under acidic conditions. COTTER and HILL reviewed acid tolerance mechanisms available

to Gram-positive bacteria and pointed out characteristics of selected microorganisms. Responses of LAB to acid stress comprise a wide range of tolerance mechanisms. Along with others processes, the three most important acid stress alleviating systems are F_OF_1-ATPase proton pumps, the ADI pathway and the glutamic acid decarboxylase (GAD) system (Cotter & Hill 2003). As outlined above (cf. 1.5), the F_OF_1-ATPase and the ADI pathway can also play a role in hop tolerance of *L. brevis*.

The existence and the mode of action of the GAD system were first examined in LAB about 60 years ago (Lagerborg & Clapper 1952). Similar to the ADI pathway, the GAD system is an amino acid dependent stress tolerance mechanism. It is based on the proton-consuming decarboxylation of glutamic acid (Glu) by glutamic acid decarboxylase (GadB) and the subsequent energy-independent antiport of the catalysed γ-aminobutyric acid (GABA) and extracellular Glu by a membrane-bound transporter (GadC). A schematic depiction of the GAD system mechanism is given in Fig. 4.

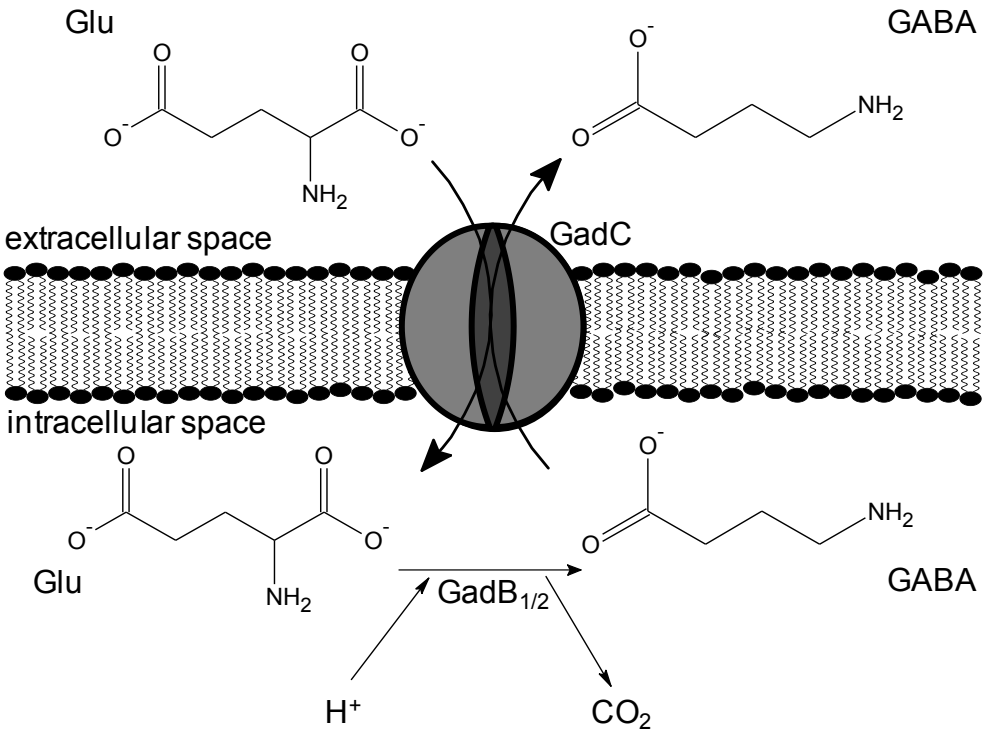


Fig. 4: Schematic depiction of the *L. brevis* GAD system. Glu, glutamic acid; GABA, γ-aminobutyric acid; $GadB_{1/2}$, glutamic acid decarboxylases 1 and 2, respectively; GadC, energy-independent Glu/GABA antiporter.

The net effect of this process is the export of a proton from the cytoplasm resulting in increased pH_{in} and generation of pmf. The threefold export of a synthesised decarboxylation product (like GABA) accounts for sufficient pmf for F_OF_1-ATPase-mediated ATP generation (McCarty 1992; Higuchi et al. 1997; Small & Waterman 1998; Cotter et al. 2001; Cotter & Hill 2003; Ma et al. 2012). Amongst a broad range of reviewed LAB species, a *L. brevis* strain (*L. brevis* NCL912) isolated from paocai (traditional Chinese fermented vegetable dish) proved to be the most potent GABA producer (345.83 mM). The yield of several other assessed species varied considerably. The strains were isolated from such different sources as kimchi, cheese and fermented crucian carps (Li & Cao 2010). It could further been shown that GABA production of *L. brevis* NCL912 was greatly influenced by growth medium composition (Li et al. 2010).

1.8 Objectives

Although research in the field of hop tolerance and antimicrobial mode of action of hops were already first approached at the beginning of the twentieth century (Shimwell 1937a), neither can be regarded as fully understood yet.

The aim of this work was therefore to dissect the mechanisms of antimicrobial hops (*Humulus lupulus*) in *Lactobacillus (L.) brevis* and provide mechanistic insights. In this approach, the mode of action of hop inhibition should be characterised on various levels including the microorganism's viability, physiology, metabolism and electrochemical membrane characteristics.

The antimicrobial potential of a multitude of hop compounds should be assessed by determination of individual MIC values for four *L. brevis* strains. Therefor, compounds obtained from an activity-orientated fractionation of different hop varieties, as well as crude hop extracts, commercial hop products, pure substances and hop derivatives should be used.

The low molecular weight sub-proteome of two *L. brevis* strains under reference acid stress and two different hop stress qualities should be investigated using MALDI-TOF MS as quickly manageable tool for high sample throughput, to allow a comparison of different test conditions in combination with many sampling points over a prolonged period of investigation.

To gain insight into the GAD system's role in beer-spoilage and to draw a comparison between strains with different beer-spoilage potential, two *L. brevis* strains differing in hop sensitivity should be selected. Metabolic features (GABA production, broth acidification, survival and cytoplasmic pH) were aimed to be studied and a transcriptional analysis of the *L. brevis* GAD system in the presence and absence of hop stress induced by iso-a-acids ought to be performed.

Finally, chemically modified iso-α-acids should be designed and synthesised to enhance the basic understanding of the molecular mechanism of antimicrobial iso-α-acids. The ability and site of divalent cation-binding, the ionophore effect, the influence on membrane potential and membrane

conductivity and the antimicrobial potential of the modified substances should be examined.

2 Material and Methods

2.1 Material

2.1.1 **Devices**

Devices used in this work are listed in Tab. 1

Tab. 1: Devices used in this work

Device	**Model**	**Manufacturer**
1-D gel electrophoresis system	Mini protean® Tetra System	BioRad, München, Germany
Balance	SI-234	Denver Instrument, Bohemia, NY, USA
Balance	SBA 52	Scaltec Instruments, Heiligenstadt, Germany
Balance	SPO 61	Scaltec Instruments, Heiligenstadt, Germany
Centrifuge	Z 216 MK	Hermle Labortechnik GmbH, Wehingen, Germany
Centrifuge	Z382 K	Hermle Labortechnik GmbH, Wehingen, Germany
Centrifuge	Z383 K	Hermle Labortechnik GmbH, Wehingen, Germany
Centrifuge	1-14	Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany
Electrical multichannel pipette	EDP® 3-Adjustable Spacer Pipette, 20-300µL	Rainin Instrument LLC, Oakland CA, USA
Electronically controlled manual dispenser	Multipette® stream	Eppendorf AG, Hamburg, Germany
ESI-QIT mass spectrometer	amaZon ETD ion trap mass spectrometer	Bruker Daltronics, Bremen, Germany
Freeze-dryer	FreeZone Plus 2.5 freeze dry system	Labconco, Kansas City, MO, USA
Gas chromatograph	7890 A	Agilent Technologies, Santa Clara, CA, USA
GC autosampler	CTC CombiPAL	CTC Analytics GmbH, Zwingen, Switzerland
GC triple axis	5975 C	Agilent Technologies, Santa

Device	Model	Manufacturer
quadrupole mass selective detector		Clara, CA, USA
HPLC system	UltiMate 3000	Dionex, Idstein, Germany
HPLC system	1100 HPLC system	Agilent Technologies, Böblingen, Germany
Incubator	Memmert INB series	Memmert GmbH & Co. KG, Schwabach, Germany
Incubator	Vacutherm VT6025	Heraeus Instruments, Hanau, Germany
Laminar airflow clean bench	HERA safe	Heraeus Instruments, Hanau, Germany
Laminar airflow clean bench	Kojair®, Biowizard Golden Line	KOJAIR TECH OY, Vilppula, Finland
Luminescence spectrometer	LS 50B	PerkinElmer, Rodgau, Germany
Magnetic stirrer	AREC heating magnetic stirrer	VELP® Scientifica, Usmate, Italy
MALDI-TOF mass spectrometer	Microflex LT	Bruker Daltronics, Bremen, Germany
Microplate reader	Sunrise	Tecan Deutschland GmbH, Crailsheim, Germany
Microplate reader	SPECTRA Fluor	Tecan Deutschland GmbH, Crailsheim, Germany
Microplate reader	FLUOstar Omega	BMG LABTECH GmbH, Ortenberg, Germany
Microwave oven	intellowave	LG Electronics Deutschland GmbH, Ratingen, Germany
NanoDrop spectrophotometer	NanoDrop 1000	Thermo Scientific, Wilmington, DE, USA
Overhead shaker	Reax 2	Heidolph Instruments GmbH, Schwabach, Germany
pH electrode	InLab® Semi-Micro pH, pH 0-12	Mettler-Toledo GmbH, Gießen, Germany
pH electrode	InLab® 412, pH 0-14	Mettler-Toledo GmbH, Gießen, Germany
pH meter	Knick pH-Meter 761 Calimatic	Knick Elektronische Messgeräte GmbH, Berlin Germany
pH meter	ProLab3000	SI Analytics GmbH, Mainz, Germany
Pipettes	Pipetman (2 µL, 20 µL, 100 µL, 200 µL, 1000 µL)	Gilson International B.V, Deutschland, Limburg-Offheim, Germany

Device	Model	Manufacturer
Pipetting robot	RoboSeq® 4204 S	MWG Biotech AG, Ebersberg, Germany
qRT-PCR device	Light cycler	Roche Diagnostics GmbH, Mannheim, Germany
Redox electrode	autoclavable Pt-Ag/AgCl electrode	SI Analytics GmbH, Mainz, Germany
Rotary evaporator system	VV2000, WB2000	Heidolph Instruments GmbH, Schwabach, Germany
Spectrophotometer	Novaspec II	Pharmacia Biotech, Uppsala, Sweden
Thermal cycler	Mastercycler gradient	Eppendorf AG, Hamburg, Germany
Thermostatic circulator	MultiTempIII	Pharmacia Biotech, Uppsala, Sweden
Thermostatted HPLC column compartment	TCC-100	Dionex, Idstein, Germany
Ultra sonic water bath	Sonorex Super RK103H	Bandelin electronic, Berlin, Germany
UV-visible spectrophotometer	LKB Biochrom 4060	Pharmacia Biotech, Uppsala, Sweden
Vortex mixer	Vortex Genie 2	Scientific Industries Inc., Bohemia, NY, USA
Water bath	MD12 LAUDA	Lauda DR. R. Wobser GmbH & Co. KG, Lauda-Königshofen, Germany

2.1.2 **Chemicals**

Chemicals used in this work are listed in Tab. 2

Tab. 2: Chemicals used in this work

Chemical	Specification	Manufacturer
1,4-Dioxane	anhydrous, ≥99.8 %	SIGMA-ALDRICH, Steinheim, Germany
1,4-Dithio-D,L-threitol	high purity	GERBU Biotechnik GmbH, Heidelberg, Germany
2-Iodoacetamide; C_2H_4INO	≥99 %	AppliChem GmbH, Darmstadt, Germany
2-Propanol	≥99.9 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Chemical	Specification	Manufacturer
3-MPA (3-mercaptopropionic acid)	≥99.0 %	SIGMA-ALDRICH, Steinheim, Germany
Acetic acid	100 %, glacial	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Acetone	≥99.5 %, for synthesis	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Acetonitrile	≥99.9 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Acrylamide/Bis solution	29:1; 30 % (w/v)	SERVA, Heidelberg, Germany
Agar	European agar	Difco, BD sciences, Heidelberg, Germany
Amino acid standard solution	analytical standard	SIGMA-ALDRICH, Steinheim, Germany
Ammonium acetate; CH_3COONH_4	p.a.	Merck, Darmstadt, Germany
Ammonium chloride; NH_4Cl	≥99.5 %, p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ammonium hydroxide solution; NH_3, ≥ 25 %	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ammonium bicarbonate; $(NH_4)HCO_3$	≥99 %, p.a.	SIGMA-ALDRICH, Steinheim, Germany
Ammonium persulphate; $(NH_4)_2S_2O_8$	analytical grade	SERVA, Heidelberg, Germany
BICINE buffer solution (N, N-Bis(2-hydroxyethyl)glycine)	for molecular biology, 1 M in H_2O	SIGMA-ALDRICH, Steinheim, Germany
Bromophenol Blue	for electrophoresis	SIGMA-ALDRICH, Steinheim, Germany
Bruker Matrix HCCA (α-cyano-4-hydroxycinnamic acid solution)	-	Bruker Daltronics GmbH, Bremen, Germany
Calcein-AM	BioReagent; suitable for fluorescence	SIGMA-ALDRICH, Steinheim, Germany
CCCP	≥97 %	SIGMA-ALDRICH, Steinheim, Germany
Chloroform	≥99 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Cholesterol	≥95 %, ash free, powder	SIGMA-ALDRICH, Steinheim, Germany
D(-)-Fructose	-	OMNI Life Science GmbH & Co. KG, Bremen, Germany
D(+)-Glucose monohydrate	for microbiology	Merck, Darmstadt,

Chemical	Specification	Manufacturer
		Germany
Decane	≥99 %, ReagentPlus®	SIGMA-ALDRICH, Steinheim, Germany
Diethyl ether	≥99.8 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dimethyl sulphate	≥99.8 %	SIGMA-ALDRICH, Steinheim, Germany
DL-Lactic acid	90 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dodecane	analytical standard	SIGMA-ALDRICH, Steinheim, Germany
EDTA (ethylenediaminetetraacetic acid)	for molecular biology	SIGMA-ALDRICH, Steinheim, Germany
Egg phosphatidylcholine	E PC S	Lipoid GmbH, Ludwigshafen, Germany
Eicosane	analytical standard	SIGMA-ALDRICH, Steinheim, Germany
Ethanol, absolute	≥99.8 %	VWR, International, Foutenay-sous-Bois, France
Ethanol, denatured	99 %; with 1 % methylethylketone	Chemikalien und Laborbedarf Nierle, Freising, Germany
FMOC (9-fluorenylmethoxycarbonyl-chloride)	≥97 %	SIGMA-ALDRICH, Steinheim, Germany
Formaldehyde solution; CH_2O, 37 %	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Formic acid	98 - 100 %, p.a.	Merck, Darmstadt, Germany
Glycerol	anhydrous, ultra pure	J. T. Baker, Deventer, Netherlands
Glycine	for molecular biology and electrophoresis	GERBU Biotechnik GmbH, Heidelberg, Germany
Hexadecane	≥99 %	SIGMA-ALDRICH, Steinheim, Germany
Hydrochloric acid solution; HCl, 37 %	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$K_2HPO_4 \cdot 3\ H_2O$	p.a.	Merck, Darmstadt, Germany
KH_2PO_4	≥99 %, p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Chemical	Specification	Manufacturer
L-Cystein-HCl monohydrate	≥98.5 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
L-Glutamic acid	≥99 %	SIGMA-ALDRICH, Steinheim, Germany
MALDI-TOF MS bacterial test standard	-	Bruker Daltronics, Bremen, Germany
Maltose monohydrate	for microbiology	Merck, Darmstadt, Germany
Meat extract	for microbiology	Merck, Darmstadt, Germany
MES; 2-[N-morpholino]-ethanesulfonic acid	for molecular biology	GERBU Biotechnik GmbH, Heidelberg, Germany
Methanol	≥99.9 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$MgSO_4 \cdot 7H2O$	p.a.	Merck, Darmstadt, Germany
$MnSO_4 \cdot H_2O$	≥98 %, p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Molecular weight marker for peptides	for SDS-PAGE; Mw range 2.5 - 17 kDa	SIGMA-ALDRICH, Steinheim, Germany
Na_2HPO_4	p.a.	Merck, Darmstadt, Germany
NaH_2PO_4	p.a.	Merck, Darmstadt, Germany
Na-thiosulphate $\cdot 5H_2O$; $Na_2O_3S_2 \cdot 5H_2O$	p.a.	Merck, Darmstadt, Germany
n-Hexane	≥98 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Nigericin	approx. 98 % (TLC)	SIGMA-ALDRICH, Steinheim, Germany
n-Octacosane	analytical standard	SIGMA-ALDRICH, Steinheim, Germany
OPA (o-phtalaldehyde)	≥97 %	SIGMA-ALDRICH, Steinheim, Germany
Peptone from casein	for microbiology	Merck, Darmstadt, Germany
Perchloric acid solution; $HCLO_4$, 70 %	puriss., p.a.	SIGMA-ALDRICH, Steinheim, Germany
Phenol (redistilled, saturated in dH_2O)	Roti®-Aqua-Phenol for RNA extraction	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Piperazine (Diethylenediamine)	99 %	SIGMA-ALDRICH, Steinheim, Germany

Chemical	Specification	Manufacturer
Potassium chloride; KCl	≥99.5 %, p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Potassium hydroxide; KOH	p.a.	Merck, Darmstadt, Germany
Resazurin Na-salt	p.a.	SERVA, Heidelberg, Germany
Roti®-Aqua-Phenol	for RNA extraction, pH 4.5 - 5, redistilled in H_2O-saturated phenol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Roti®-Blue	5 x conc. solution for Colloidal Coomassie staining of protein gels	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
SDS (sodium n-dodecyl sulphate)	research grade	SERVA, Heidelberg, Germany
Silver nitrate; $AgNO_3$	≥99.9 %, p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium acetate; $C_2H_3O_2Na$	p.a.	Merck, Darmstadt, Germany
Sodium hydroxide solution; NaOH, 50 %	-	J. T. Baker, Deventer, Netherlands
Sodium hydroxide; NaOH	≥99 %, p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium sulphate; Na_2SO_4	p.a.	Merck, Darmstadt, Germany
Sodium carbonate; $NaCO_3$	≥99.8 %, p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sulphuric acid solution; H_2SO_4	95 - 97 %	Merck, Darmstadt, Germany
TEMED (N,N,N',N'-tetramethylethylen - diamine)	for electrophoresis	SIGMA-ALDRICH, Steinheim, Germany
Tetracosane	analytical standard	SIGMA-ALDRICH, Steinheim, Germany
Tetrahydrofuran	≥99.9 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Trichloroacetic acid	≥99.5 %, p.a.	Riedel-de Haën, Seelze, Germany
Tricine; N-[tris(hydroxymethyl)methyl] -glycine	≥99 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Trifluoroacetic acid	≥99.9 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Tris; tris(hydroxymethyl)-	ultra pure	MP Biomedicals, Solon,

Chemical	Specification	Manufacturer
aminomethane		Ohio, USA
Tris-HCl	99.89 %	GERBU Biotechnik GmbH, Heidelberg, Germany
Tween® 80 (Polyoxyethylenesorbitan monooleate)	for cell culture and bacteriology	GERBU Biotechnik GmbH, Heidelberg, Germany
Valinomycin	≥90 % (HPLC), ≥98 % (TLC)	SIGMA-ALDRICH, Steinheim, Germany
Water	Chromasolv®, for HPLC	SIGMA-ALDRICH, Steinheim, Germany
Yeast extract	for bacteriology	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
β-Cyclodextrin	≥97 %	SIGMA-ALDRICH, Steinheim, Germany

2.1.3 **Hop compounds**

Commercial hop compounds

Commercial hop compounds were kindly provided by Simon H. Steiner Hopfen GmbH (Mainburg, Germany). A list of commercial hop compounds is given in Tab. 3.

Tab. 3: Commercial hop compounds

Product name	Specification
Xantho-Flav™	65 - 85 % xanthohumol
Isoxantho-Flav™ 85	> 85 % isoxanthohumol
isoxanthohumol 95 %	> 95 % isoxanthohumol
Iso-α-acids 90 %	> 90 % iso-α-acids

Non-commercial hop compounds

Non commercial hop compounds were prepared and supplied by C. Vogt, Lehrstuhl für Lebensmittelchemie und Molekulare Sensorik, Technische Universität München (TUM).

In brief, a systematic, activity-orientated fractionation of hops and hop products was conducted. Green hop cones (gh), raw hops (rh; dried, ground

hop cones), hop pellets (pl) type 90, ethanol hop extract (et) and hop draff (df) were used as basic raw material originating from the following hop varieties: HALLERTAUER MAGNUM (M), HALLERTAUER TRADITION (Tr), HALLERTAUER TAURUS (Ts) and PERLE (Pe). According to MEBAK (Mitteleuropäische Brautechnische Analysenkommision) guidelines, MeOH/ether soluble total resin (tr), hexane soluble soft resin (sr) and hexane insoluble hard resin (hr) were prepared from the different raw materials listed above (Pfenninger 1997). Preparative high performance liquid chromatography (HPLC) was used to obtain α-acids (humulones) and β-acids (lupulones) from the sr fraction (Pfenninger 1996). The obtained hr was further fractionated to water soluble δ-resin (dr) and EtOH soluble ε-resin (er). Subsequently, subfractions (F01-11) and pure substances were obtained by flash chromatography and preparative chromatography. Structure elucidation of pure substances was achieved by liquid chromatography mass-spectrometry (LC-MS) and nuclear magnetic resonance spectroscopy (NMR) (Schurr et al. 2011). Further, various derivatives from isolated hop compounds were prepared and supplied by Christian Vogt, Lehrstuhl für Lebensmittelchemie und Molekulare Sensorik, TUM.

A list of thus obtained hop compounds and derivatives and assigned workup levels is given in Tab. 4.

Tab. 4: Non-commercial hop compounds. Key pattern: hop variety - raw material - fraction, e.g.: HALLERTAUER MAGNUM - ethanol extract - total resin (M-et-tr).
M, MAGNUM; Tr, TRADITION; Ts, TAURUS; Pe, PERLE; et, ethanol extract; pl, pellets; gh, green hops; rh, raw hops; df, draff; tr, total resin; sr, soft resin; hr, hard resin; er, ε-resin; dr, δ-resin; F01-11, subfraction 1-11.
Workup levels: 1, tr level; 2, sr/hr level; 3, α-/β-acids / δ-/ε-resin level; 4, F01-11; 5 pure substances, purified mixtures and derivatives.

Hop compound	Workup level
5-prenyl-xanthohumol	5
6-prenylnaringenine, 8-prenylnaringenine	5
isoxanthohumol	5
iso-α-acids	5

Hop compound	Workup level
M-et-er, M-et-dr	3
M-et-er-F01 - M-et-er-F11	4
M-et-sr, M-et-hr	2
M-et-tr	1
M-gh-sr, M-gh-hr	2
M-gh-tr	1
M-pl-sr, M-pl-hr	2
M-pl-tr	1
M-rh-sr, M-rh-hr	2
M-rh-tr	1
n-humulone, co-humulone	5
n-lupulone, co-lupulone	5
Pe-df-hr, Pe-df-sr	2
Pe-pl-hr, Pe-pl-sr	2
trans-iso-n-humulone	5
Tr-et-sr , Tr-et-hr	2
Tr-et-tr,	1
Ts-df-hr, Ts-df-sr	2
Ts-pl-hr, Ts-pl-sr	2
xanthohumol, xanthohumol H, xanthohumol L, xanthohumol C, xanthohumol I	5
α-acids, β-acids	5

Fresh hops

High alpha variety HALLERTAUER MAGNUM green hop cones were kindly provided by Simon H. Steiner Hopfen GmbH (Mainburg, Germany). Hops collected on the day of the harvest were promptly frozen and stored at -20 °C until further use

Outline of hop compounds

An overview over the fractionation pathway of supplied hop compounds and in-house hop compounds (cf. 2.2.6 - 2.2.9), as well as the assigned workup levels is given in Fig. 5.

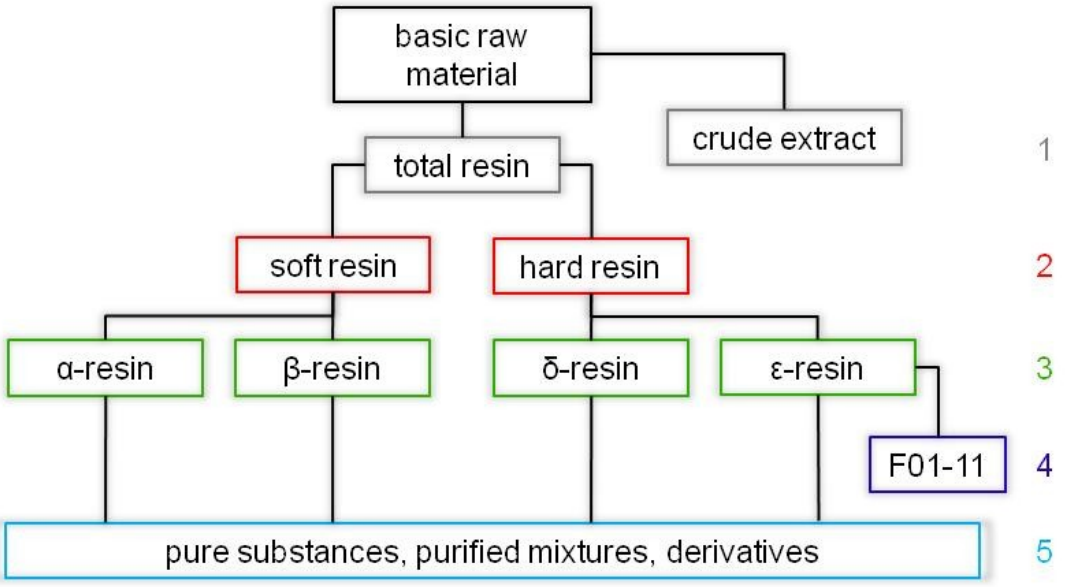


Fig. 5: Overview over hop compounds used in this work. Assigned workup levels are indicated.

2.1.4 **Consumables and other supplies**

Consumables and other supplies used in this work are listed in Tab. 5

Tab. 5: Consumables and other supplies used in this work

Item	**Specification**	**Manufacturer**
Acrylic cuvettes	10 × 10 × 45 mm, 10 × 4 × 45 mm	Sarstedt AG & Co., Nümbrecht, Germany
BLM cell electrodes	Sintered Ag/AgCl electrodes	In Vivo Metric, Healdsburg, CA, USA
Capillary GC column	Zebron ZB-1; 60 m, 0.25 mm i.d., 0.25 µm film thickness	Phenomenex, Torrance, CA, USA
Combitips	Combitips advanced®, sterile, 1 mL	Eppendorf AG, Hamburg, Germany

Item	Specification	Manufacturer
Cryo pure tubes	1.8 mL white, non-pyrogenic, non-mutagenic, non-cytotoxic	Sarstedt AG & Co., Nümbrecht, Germany
Folded filters	cellulose, 65 g/m^2	Munktell & Filtrak GmbH, Bärenstein, Germany
GC deactivated quartz wool liner	4.0 mm i.d. split/splitless tapered FocusLiner	SGE Analytical Science, Milton Keynes, UK
GC screw caps	Verex cap pre-assembled, 9 mm, PTFE/rubber septa	Phenomenex, Torrance, CA, USA
GC vials	Verex vial, 9 mm screw, 2 mL, clear 33, no patch	Phenomenex, Torrance, CA, USA
Hamilton syringe for GC autosampler	10 µl Hamilton® 701 N CTC	SIGMA-ALDRICH, Steinheim, Germany
HPLC column	Aeris-PEPTIDE 3.6 µm XB-C18 column, 150 x 4.6 mm	Phenomenex, Torrance, CA, USA
HPLC column	Gemini 5 µm C18 110 Å column, 150 x 3 mm	Phenomenex, Torrance, CA, USA
HPLC column	YMC Triart C18 RP, ID: 100 x 2.0 mm, 3 µm particles, 12 nm pores	YMC, Dinslaken, Germany
HPLC column	LiChrospher® 10 µm 100 RP-18 endcapped, 250 x 10 mm	Merck, Darmstadt, Germany
HPLC vial crimp caps	Verex seal, 11 mm Dia. Crimp, PTFE/rubber red	Phenomenex, Torrance, CA, USA
HPLC vials	Verex vial, crimp, 2 mL, clear 33, no patch	Phenomenex, Torrance, CA, USA
LightCycler® Capillaries	glass, 20 µL	Roche Diagnostics GmbH, Mannheim, Germany
MALDI-TOF MS stainless steel target plate	MSP 96	Bruker Daltronics, Bremen, Germany
Membrane filter units	Phenex™, RC-membrane, 0.2 µm, 4 mm non-sterile PP housing, lure/slip	Phenomenex, Torrance, CA, USA
Membrane filters	47 mm, cellulose, 0.2 µm	Sartorius AG, Göttingen, Germany
Microtitre plates	96 well, flat bottom with lid	Sarstedt AG & Co., Nümbrecht, Germany
Petri dishes	92 x 16 mm, without ventilation cams	Sarstedt AG & Co., Nümbrecht, Germany
Pipette tips	PIPETMAN TIPS Diamond; 0.1-20 µL	Gilson International B.V, Deutschland, Limburg-Offheim, Germany

Item	Specification	Manufacturer
Pipette tips	1-200 µL, 100-1000 µL	Peske GmbH, Karlsruhe, Germany
Quartz glass cuvettes	Quartz glass SUPRASIL® precision cuvettes	Hellma GmbH & Co. KG, Müllheim, Germany
Racked pipette tips for multichannel pipette	300 µL presterilised filter LTS® tips	Rainin Instrument LLC, Oakland CA, USA
Reaction tubes	200 µL, 1.5 mL, 2mL	Eppendorf AG, Hamburg, Germany
Sterile filters	Filtropur S 0.2 and S 0.45, sterile non-pyrogenic,	Sarstedt AG & Co., Nümbrecht, Germany
Sterile reagent and centrifuge tubes	5 mL, 15 mL, 50 mL	Sarstedt AG & Co., Nümbrecht, Germany
Syringes	single use, pyrogenfree, sterile; 2 mL, 10 mL, 20 mL	Dispomed Witt oHG, Gelnhausen, Germany

2.1.5 **Molecular biological kits and supplies**

Molecular biological kits used in this work are listed in Tab. 6

Tab. 6: Molecular biological kits used in this work

Kit	Specification	Manufacturer
RQ1 RNase-free DNase kit	Degradation of ds and ss DNA	Promega, Madison, WI, USA
M-MLV Reverse Transcriptase (RNase H-, point mutant) kit	cDNA synthesis	Promega, Madison, WI, USA
Random primers	cDNA synthesis	Promega, Madison, WI, USA
E.Z.N.A. Bacterial DNA Kit	DNA isolation	Omega Bio Tek Inc., Norcross, GA, USA
dNTP mix	10 mM each	MP Biomedicals, Solon, Ohio, USA
ABsolute™ QPCR SYBR® Green Capillary Mix kit	cDNA quantification	Thermo Scientific, Epsom, Surrey, UK

2.1.6 **Microorganisms**

Four beer spoiling *Lactobacillus (L.) brevis* strains isolated from different sources were used throughout this work (cf. Tab. 7).

Tab. 7: *Lactobacillus* strains used in this work

***Lactobacillus* species**	**TMW number**	**Isolation source**
L. brevis	1.6	faeces
L. brevis	1.1369	fermentation
L. brevis	1.465	spoiled soft drink
L. brevis	1.313	spoiled beer

2.2 Methods

2.2.1 **Determination of UV-Vis absorption profiles**

Absorption spectra of hop compounds were recorded with an UV/Vis- spectrophotometer between 200 nm and 600 nm excitation wavelength at room temperature. Absorption scans of 0.2 mM test compound in methanol were conducted with a scan speed of 1000 nm/min using quartz glass cuvettes. Subsequently, an aqueous $MnCl_2$ solution was added (final $MnCl_2$ concentration: 10 mM) and scans were repeated.

2.2.2 **Determination of Distribution ratios ($D_{org/aq}$)**

The distribution of hop derivatives in a liquid-liquid system was investigated using a classical shake-flask method with mutually saturated n-octanol serving as organic solvent and aqueous phosphate buffered solution (pH 4.3, according to $mMRS_2$) as it has widely been used (Sangster 1989). Concentrations of the respective analytes (A) were assessed by UV/Vis-spectroscopy. To this end individual absorption maxima and four point calibrations (linear fit, R^2 = 0.994 - 0.999) were determined in water-saturated n-octanol using a UV/Vis-spectrophotometer. Distribution ratios ($D_{org/aq} = [A]^{org} / [A]^{aq}$) were investigated after 1 h overhead shaking at

21 °C. Prior to mutual saturation of n-octanol and water and subsequent extraction, shake-flasks were flushed with nitrogen to prevent analyte oxidation. Experiments were conducted in duplicates.

2.2.3 **Isolation of single iso-co/n/ad-humulone analogues**

Cis- and trans-iso-α-acids were separated using β-cyclodextrin (β-CD) as a wet chemical method trans-iso-α-acids trap as demonstrated by KHATIB et al. with minor modifications (Khatib et al. 2010). Therefor, a 70 mM β-CD solution was prepared in EtOH:dH_2O (1:2, v/v) by heating the mixture to 50 °C under continuous stirring. 5 g iso-α-acids were dissolved in EtOH (final volume: 65 mL) and added gradually to the β-CD solution within 30 min. Subsequently, the solution was flushed with nitrogen, cooled to 4 °C and kept stirring over night (o.n.) in the dark. After vacuum filtration (cellulose filter, 65 g/m^2), cis-iso-α-acids were obtained from the supernatant by addition of 30 % dH_2O (pH 1 adjusted with 6 M HCl) and subsequent threefold liquid-liquid n-hexane extraction. The hexane phase was washed twice with acidified dH_2O to deplete potential β-CD carry over. In order to remove dH_2O from the organic phase, a spatula tip of anhydrous sodium sulphate was used. Following a final filtration, the organic solvent was evaporated with a rotary evaporator and the isolated cis-iso-α-acids were re-dissolved in EtOH. In a similar procedure, trans-iso-α-acids were recovered from the trans-iso-α-acids/β-CD complexes. The obtained filter cake was washed twice with 30 % EtOH. Thereafter, it was resuspended in methanol (MeOH) and shaken at 4 °C o.n. using an overhead shaker to release the trans-iso-α-acids from the complex. The procedure was repeated twice applying 2 h shaking. The collected MeOH was filtrated and mixed with 50 % dH_2O (pH 1) and hexane extraction and subsequent workup was conducted as described above. Separation of cis- and trans-iso-α-acids was verified by reversed phase high performance liquid chromatography (RP-HPLC) (cf. 2.2.4) and substances were obtained by preparative RP-HPLC (cf. 2.2.5).

2.2.4 **RP-HPLC analysis conditions of hop compounds**

RP-HPLC of hop compounds was conducted using an Aeris-PEPTIDE XB-C18 column. Mobile phases A (0.1 % formic acid (FA) in HPLC-grade water) and B (0.1 % FA in acetonitrile (AcN)) were used with a flow rate of 1.8 mL/min (-1 min 20 % B, 0.01 min 20 % B, 5 min 65 % B (curve 5, linear), 17.75 min 100 % B (curve 9, concave gradient), 19.75 min 100 % B, 21.75 min 20 % B (curve 5). Column temperature was set to 25 °C and controlled with a column heater. A Dionex UltiMate 3000 HPLC system served to conduct hop components' separation and UV mediated detection at 270 nm, 290 nm, 314 nm and 370 nm wavelength. Hop components were identified using an external standard and Chromeleon evaluation software version 6.80.

2.2.5 **Preparative RP-HPLC of hop compounds**

Preparative RP-HPLC was used to obtain single iso-co/n/ad-humulone analogues. Therefore, a LiChrospher 100 RP-18e column and the aforementioned equipment were used (cf. 2.2.4). Run conditions were changed regarding flow rate (2.5 mL/min) and mobile phase gradient (-10 min 30 % B, 0.01 min 30 % B, 16.7 min 52 % B (curve 5), 58.34 min 100 % B (curve 9), 75.8 min 100 % B, 77.8 min 20 % B (curve 5), 82.5 min 20 % B). Finally, the separated single iso-co/n/ad-humulone analogues were isolated from the HPLC eluents by liquid-liquid hexane extraction and taken to dryness using a rotary evaporator and subsequent lyophilisation in a freeze dry system.

2.2.6 **Methylation of an iso-α-acids mixture and cis-iso-cohumulone**

Dimethyl sulphate (DMS) which has widely been applied in synthetic and analytical chemistry was used as methylating agent (Lewis et al. 1930; Buchanan et al. 1944; Poonia & Yadav 1978; Kurán et al. 2008). DMS handling was conducted under particular safety measures by trained personnel in a team of two to ensure work safety. For the methylation of iso-α-acids and cis-iso-cohumulone, a 20 mg/mL solution of the respective

hop derivative was prepared using diethyl ether (DEE) as organic solvent. The solution was mixed with n-hexane and DMS (1:5:0.5) in a glass vial and sealed properly to avoid DMS discharge. A blank treatment was carried out replacing DMS with n-hexane. The mixtures were incubated for 24 h at 55 °C in a water bath. Excess DMS was exhausted in a two step reaction. To this end aliquots were taken, 50 % dH_2O was added and the two phase system was shaken for 24 h using an overhead shaker to completely deplete DMS. DMS depletion was verified by GC MS. Subsequently, H_2SO_4 was added to the blank treatment to acidify the reaction mixture as DMS hydrolyses to sulphuric acid and methanol in water (Robertson & Sugamori 1966). Reaction products were isolated by threefold liquid-liquid extraction with n-hexane and taken to dryness with a rotary evaporator at 55 °C.

2.2.7 **Preparation of imino-iso-α-acids**

Iso-α-acids were placed in an angled three-neck round-bottom flask which was continuously flushed with nitrogen via a reflux condenser. A thermometer and a pressure-equalising dropping funnel were inserted into the side necks of the flask. The iso-α-acids extract was dissolved in 10 mL KOH solution (70 mM) and 9 mL 1,4-dioxane and 7.5 mL ammonium acetate solution (2.87 M) were added under permanent stirring. The mixture's final iso-α-acids concentration was 25 mM (calculated for iso-n-humulone, MW = 362.46 g/mol). Finally, 50 mL n-hexane were added and the reaction mixture was stirred for 24 h at 50 °C in the dark. After the incubation, the aqueous phase was acidified (6 M HCl) and the reaction products were obtained by liquid-liquid hexane extraction.

2.2.8 **Preparation of humulinic acids**

Hydrolysis of iso-α-acids or α-acids leads to the formation of humulinic acids in alkaline aqueous solutions (Dierckens & Verzele 1969; Diffor et al. 1972; Molyneux & Wong 1973; Verzele & De Keukeleire 1991; Simpson 1993b). Accordingly, humulinic acids were prepared using a procedure similar to the

imination of iso-α-acids replacing ammonium acetate by a 2.87 M potassium hydroxide solution.

2.2.9 **Preparation of crude hop extracts**

Crude hop extracts were prepared from female HALLERTAUER MAGNUM hops (cf. 2.1.3). Hop cones were separated from leaves and bines and dried in accordance with BAYERISCHE LANDESANSTALT FÜR LANDWIRTSCHAFT (LfL) guidlines at 65 °C (Münsterer 2006) under evacuated nitrogen atmosphere for 5 h. Five dried hop cones (approx. 1 g dry weight) were pestled, transferred to a 100 mL conical flask using 30 mL ice cold EtOH to flush all laboratory materials. The hop/EtOH mixture was stirred for 3 h at 4 °C under nitrogen atmosphere. Subsequently, the mixture was filtered (65 g/cm^2 cellulose filter) and the filtrate was taken to dryness with a rotary evaporator. The obtained extract was redissolved in EtOH.

In addition to EtOH, tetrahydrofuran (THF) and hexane were used as extracting agent due to the organic solvents' different polarities (polarities: EtOH, 0.88; THF, 0.45; hexane, 0.00) (Küster & Thiel 2002).

2.2.10 **Identification of hop derivatives via LC-ESI MS/MS and GC MS**

A novel GC MS analysis method for hop derivatives without requisite analyte prederivatisation was developed for rapid and easy handling. An Agilent 7890 A gas chromatograph equipped with a CTC CombiPAL autosampler was coupled with a 5975 C model GC triple axis quadrupole mass selective detector. 1 µL samples were introduced into the preheated inlet compartment (230°C) equipped with a deactivated quartz wool liner (4.0 mm i.d. split/splitless tapered FocusLiner). Helium carrier gas flow rate and septum purge flow were set to 3 mL/min. Injection into a Zebron ZB-1 capillary GC column was conducted in the pulsed splitless mode (6min, 6.4918 bar; followed by a purge step via the split vent with 50 mL/min purge flow) and GC was performed using the following temperature program: 15 min at 100°C, 10°C/min ramp to 230°C, 65 min holding time. Detector temperature was 230 °C. Enhanced ChemStation software (Agilent

Technologies) was used for identification of analysed samples using the NIST MS search tool. Previously inexistent hop derivatives were introduced into the local NIST library before analysis.

For LC-ESI-MS/MS, an amaZon ETD ion trap mass spectrometer was coupled to an Agilent 1100 HPLC system. Analytes were separated on a YMC Triart C18 reversed-phase column using a 40 min gradient of mobile phases A (0.1 % FA in HPLC-grade water) and B (0.1 % FA in AcN) at 25 °C (gradient: 0 min 20 % B, 5 min 65 % B, 10 min 65 % B, 20 min 95 % B, 35 min 95 % B, 37 min 20 % B, 40 min 20 % B, flow rate 250 µL/min). Mass spectra were recorded in the range of 50 - 800 m/z in the positive ion mode. Intact and fragment masses of eluting analytes were determined in maximum resolution scan mode (4,650 amu/s) and the two most intense peaks were selected for further fragmentation by collision-induced dissociation (CID) with PAN mode enabled. Dynamic exclusion was enabled and dynamic exclusion duration was set to 10 s. Spectra were subsequently exported with the Bruker Daltonik DataAnalysis tool version 4.0 as mascot generic format (mgf) files. All measured fragment spectra of each precursor mass were merged into a consensus spectrum using a self tailored version of “elab.m” (settings: minimum peak detection rate PDR = 0.4, mass tolerance was set according to the resolution of the mass spectrometer to +/- 0.1 m/z) from the MASCAP platform (Mantini et al. 2010). Fragment consensus spectra were imported into the MS Interpreter application of the NIST MS search tool for evaluation of acquired MS data.

2.2.11 **Assessment of electrochemical membrane characteristics via bilayer lipid membrane (BLM) techniques**

Alterations of membrane potential and membrane conductivity of model egg phosphatidylcholine (PC) membranes were assessed to investigate the mode of action of hop derivatives on basis of the principles described by KALINOWSKI and FIGASZEWSKI (Kalinowski & Figaszewski 1995a; Kalinowski & Figaszewski 1995b) and according to procedures described by BEHR et al. with minor modifications (Behr 2008; Behr & Vogel 2009; Behr & Vogel 2010). The utilised custom made equipment consisted of a two chamber BLM

cell with sintered Ag/AgCl electrodes connected to a microchip controlled measurement apparatus (for detailed information consult: (Behr 2008)). Membranes were formed according to the MUELLER-RUDIN method (Mueller et al. 1963) using a 2 % egg PC, 1 % cholesterol membrane forming decane solution (Orlov et al. 1994). Membrane potential measurements were performed in a buffer solution of Tris (tris(hydroxymethyl)aminomethane), MES (2-[N-morpholino]ethanesulfonic acid) and citric acid (5 mM each) with potassium chloride (KCl, 0.2 M) serving as supporting electrolyte in the galvanostat mode. Membrane potential alterations were monitored subsequent to setting a pH gradient (ΔpH) of one pH unit by addition of 1 M HCl solution to the BLM cell's cis compartment or after a Mn^{2+}-gradient (ΔMn^{2+}) was formed by addition of 50 µM and 500 µM $MnCl_2$ to the cis and trans compartment, respectively. Hop derivatives' concentrations were 10 µM for ΔpH and 15 µM for ΔMn^{2+}, respectively. A control experiment for ΔpH was performed with 20 µM of the widely used protonophore carbonyl cyanide 3 chlorophenylhydrazone (CCCP) (Orlov et al. 1994). Two additional stainless steel current electrodes were mounted for membrane conductivity experiments in the potentiostat mode. Conductance was monitored after membrane formation in a 50 mM KCl buffer (pH 4) with 0.2 M KCl and application of a 50 mV potential in presence of 100 µM modified hop derivatives or purified cis-iso-cohumulone. A concentration of 30 µM was used for iso-α-acids. Accordingly, the measurements were conducted and 100 µM $MnCl_2$ was added after the potential was applied. All experiments were performed in independent triplicates under temperature controlled conditions at 23 °C.

2.2.12 **Media and culture conditions**

L. brevis strains used in this work (cf. Tab. 7) were kept at -80 °C as cryo stock cultures containing 20 % glycerol. Before further use, microorganisms were propagated on modified DE MAN, ROGOSA and SHARPE (MRS) medium 1 ($mMRS_1$) agar plates. Subsequently, microorganisms were precultured in $mMRS_1$ broth. Medium composition of $mMRS_1$ adjusted to pH 6.2 was (quantities per litre): 10 g peptone, 5 g yeast extract, 5 g meat extract, 4 g

K_2HPO_4, 2.6 g KH_2PO_4, 3 g NH_4Cl, 1 g Tween® 80, 0.5 g cysteine-HCl, 10 g maltose, 5 g glucose, 5 g fructose, 0.2 g $MgSO_4 \cdot 7H_20$, 0.038 g $MnSO_4 \cdot H_2O$. $mMRS_1$ plates contained 15 g/L agar. A variant of the medium ($mMRS_2$) adjusted to pH 4.3 was used throughout subsequent culturing or experiments. The divalent cation content of $mMRS_2$ was reduced to levels found in pilsner lager beer (98 mg/L magnesium, 0.16 mg/L manganese) (Behr & Vogel 2009). Further, addition of cysteine-HCl was omitted in $mMRS_2$, because beer contains comparatively low levels of cysteine (2.1 – 13.5 mg/L (Matsui et al. 1984)). 30 °C was used as culturing temperature throughout this work.

2.2.13 **Determination of minimum inhibitory concentration (MIC)**

Growth challenge tests were performed for *L. brevis* TMW 1.6, TMW 1.1369, TMW 1.465 and TMW 1.313 grown to stationary phase to determine individual MIC values. Therefore, microtitre plates containing $mMRS_2$, 1 mM resazurin and a respective test substance in concentrations increasing in 10 % steps from 0 % to 100 % of a predefined maximum were prepared. Liquid handling was conducted by an automated, computer controlled pipetting robot to ensure high accuracy and reduced throughput times. 8 % of 1:100 dilutions (*L. brevis* TMW 1.6, TMW 1.465 and TMW 1.313) or 1:40 dilutions (*L. brevis* TMW 1.1369) of stationary phase bacteria were used as inocula. After inoculation and 72 h incubation at 30°C, 80 mM Tris (pH 8.8) was added to reach the pH range at which the redox dye resazurin shows its characteristic blue colour (Behr & Vogel 2009) (resazurin pH-transition points according to supplier's product specification: pH 3.8 orange/red, pH 6.5 blue/purple). Subsequently, MIC values were assessed visually as shown before (Sarker et al. 2007). Metabolic activity was indicated by a colour shift from purple/blue (oxidised form resazurin) to pink (reduced form resorufin) (Twigg 1945).

2.2.14 **Cluster Analysis of MIC values**

A cluster analysis of determined MICs was conducted using the open source statistical R package ape (package for Analysis of Phylogenetic and Evolution). Therefore, means of the MICs of *L. brevis* TMW 1.6, 1.1369, 1.465 and 1.313 were calculated. MIC values above the experimental limit (e.l.) of 286.3 µg/mL were set to 314.9 µg/mL as a minimum MIC of MIC = e.l. * 1.1 can be assumed due to the experimental setup and evaluation method. Clustering results of overall MIC means were plotted as circular trees.

2.2.15 **DNA extraction**

Total cellular DNA was isolated according to supplier's instructions using the E.Z.N.A. Bacterial DNA Kit for DNA isolation.

2.2.16 **RNA extraction**

Total RNA was extracted using the hot phenol method as published by AIBA et al. with minor modifications (Aiba et al. 1981). In brief, cells grown to OD_{590} = 0.3 - 0.6 were harvested and shock frozen at -80°C. After o.n. storage, cells were resuspended in Aiba-buffer (20 mM Na-acetate, 0.5 % SDS, 1mM EDTA, pH 5.5) and aqueous phase was repeatedly shaken with a phenol solution at 60 °C. The extracted RNA was precipitated with EtOH, chilled to -80 °C and washed in Aiba-buffer/EtOH solution (1:3). The final RNA precipitate was collected and resuspended in RNAse-free dH_2O.

2.2.17 **SDS-PAGE**

One dimensional sodium n-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used for protein separation and size estimation. A protocol especially suitable for low molecular weight (lmw) proteins was selected and conducted with minor modifications (Schägger & von Jagow 1987).

8.3 x 7.3 cm gels of 0.75 mm thickness were prepared as described in the following. 17 % polyacrylamide separating gels consisted of 18.61 mL 30 % acrylamide, 11.1 mL Tris/SDS-buffer (3 M Tris, 0.3 % SDS, pH 8.45) and 4.46 g anhydrous glycerol. After ultrasonic treatment, 200 µL freshly prepared ammonium persulfate (APS) solution (10 %) and 20 µL N,N,N',N'-tetramethylethylendiamine (TEMED) were added to initiate cross-linking. Gels were poured immediately, overlaid with isopropanol and left to polymerise for 1 h. Subsequently, the isopropanol layer was discarded and a 4 % polyacrylamide stacking gel was prepared accordingly by mixing 1.35 mL acrylamide solution, 2.58 mL Tris/SDS-buffer, 6.42 mL dH2O, 70 µL APS and 7 µL TEMED. The stacking gel was filled onto the separating gel, a comb was inserted to form sample cavities and the gel was allowed to polymerise o.n.. For electrophoresis, the electrophoresis apparatus was assembled and running reagents were poured into the tank and gel chamber. The cathode buffer composition was 0.1 M Tris, 0.1 M N-[tris(hydroxymethyl)methyl]-glycine (Tricine) and 0.1 % SDS. Tris-buffer (0.2 M) adjusted to pH 8.9 was used as anode buffer. Electrophoresis commenced with a 20 min protein stacking step at 80 mA current, followed by approximately 6 h separation at 10 mA. Bromophenol Blue was used as tracking dye to monitor the protein separation progress.

Sample preparation was conducted according to the procedure described for MALDI-TOF MS analysis (cf. 2.2.24). Protein extracts from 5 mL *L. brevis* TMW 1.465 suspension (OD_{590} = 1) (sampling point: immediately after transfer to $mMRS_2$) were taken to dryness with a rotary evaporator to deplete FA, AcN and dH_2O and subsequently dissolved in SDS-PAGE sample buffer (4.33 mL dH_2O, 1 mL 0.5 M Tris-buffer (pH 6.8), 920 µL glycerol, 1.75 mL SDS solution (10 %) and 80 mg 1,4-dithio-D,L-threitol (DTT)). 10 µL samples were applied alongside a molecular weight marker (MW: 2,500 - 17,000) for protein size estimation.

2.2.18 **SDS-PAGE silver staining**

After protein separation by SDS-PAGE, proteins were visualised via silver staining according to BLUM et al. with modifications (Blum et al. 1987). Due

to its high sensitivity, the silver staining method was used for SDS-PAGE run conditions optimisation (Blum et al. 1987; Rabilloud 1992). The applied reagents and procedure are listed in Tab. 8 and Tab. 9.

Tab. 8: Composition of silver staining reagents according to BLUM et al. with modifications (Blum et al. 1987)

Reagent	**Composition**
Fixative	40 % EtOH, 10 % acetic acid
Washing solution	30 % EtOH
Thiosulfate reagent	0.02 % sodium thiosulfate
Silver nitrate reagent	0.2 % silver nitrate, 0.02 % formaldehyde solution (37 %)
Developer	3 % sodium carbonate, 0.05 % formaldehyde solution (37 %), 5 ppm sodium thiosulfate
Stop reagent	0.5 % glycine

Tab. 9: Silver staining procedure according to BLUM et al. with modifications (Blum et al. 1987)

Step	**Reagent**	**Application**
Fixation	Fixative	2 h
Wash	Washing solution	2 x 20 min
Wash	dH_2O	2 x 10 min
Sensitisation	Thiosulfate reagent	1 min
Wash	dH_2O	3 x 20 s
Silver impregnation	Silver nitrate reagent	20 min
Wash	dH_2O	3 x 20 s
Development	Developer	5 min
Wash	dH_2O	2 x 20 s
Stop	Stop reagent	5 min
Wash	dH_2O	3 x 10 min

2.2.19 **SDS-PAGE Coomassie Brilliant Blue staining**

For the quick and easy to apply Coomassie Brilliant Blue staining method, the gel was incubated in fixative (12 % trichloroacetic acid (TCA)) for 15 min. The gel was washed with dH_2O, covered with staining solution (200 mL Roti®-Blue solution, 92 mL acetic acid, 354 mL MeOH, 354 mL dH_2O), carefully heated in a microwave oven for 2 min and subsequently incubated at room temperature o.n., to achieve adequate staining results. After two washing steps (dH_2O), the gel was repeatedly destained in destaining solution I (50 % MeOH, 10 % acetic acid, 40 % dH_2O) and destaining solution II (10 % MeOH, 10 % acetic acid, 80 % dH_2O) until the background was appropriately destained. A final washing step (dH_2O) was performed and gels were stored in dH_2O at 4 °C until further use.

2.2.20 **Metabolic amino acid analysis**

Quantitative analysis of amino acids present in growth medium and fermented broths was conducted via RP-HPLC after o.n. protein precipitation with 3.5 % (v/v) perchloric acid. A Gemini C18 column and mobile phases A (20 mM Na_2HPO_4, 20 mM NaH_2PO_4, 0.8 % THF, pH 7.8) and B (30 % AcN, 50 % MeOH, 20 % HPLC-grade water) were used with a flow rate of 0.8 mL/min (linear gradients: 0 min 0 % B, 16 min 64 % B, 19 min 100 % B, 22 min 100 %B, 22.25 min 0 % B). Column temperature was set to 40 °C. A Dionex UltiMate 3000 HPLC system served to conduct amino acid separation and UV-mediated detection at 338 nm or 269 nm. Precolumn amino acid derivatisation using OPA, 3-MPA and FMOC was carried out according to principles described by BARTÓAK et al. (Bartóak et al. 1994). Amino acids were quantified using external standards and Chromeleon® evaluation software version 6.80 (Schurr et al. 2013).

2.2.21 **Determination of intracellular pH (pH_{in})**

The influence of iso-α-acids and derivatives of iso-α-acids on pH_{in} of energy depleted, resting cells was assessed according to the method described

previously by BEHR et al. (Behr et al. 2006) with minor modifications. *L. brevis* TMW 1.6 and TMW 1.465 were grown in $mMRS_2$ for 48 h, washed twice with phosphate buffer (50 mM, pH 6.2), resuspended to OD_{590} = 2.0 and incubated at 30 °C for 3 h with 2 µM Calcein-AM to allow dye internalisation and hydrolysis mediated by cellular esterases. Subsequently, the suspension was aliquoted to four parts and cooled to 4 °C. Cells were washed twice in lactate buffer (50 mM, pH 4.3) or in, respectively, lactate, piperazine and phosphate buffer (50 mM each, pH 4/5/6) to set up a calibration curve. Measurements for imino-iso-α-acids were performed in a buffer solution consisting of 0.2 M KCl and Tris, MES and citric acid (5 mM each), at pH 4.3. Linear correlation of Calcein-AM fluorescence and pH_{in} can be assumed due to the distribution of the fluorescence dye's six pK_a values (pK_{a1} 2.1, pK_{a2} 2.9, pK_{a3} 4.2, pK_{a4} 5.5, pK_{a5} 10.8, and pK_{a6} 11.7). Linear correlation of Calcein-AM fluorescence and pH_{in} could be verified in a six-point calibration pretest (linear fit, R^2 = 0.985) determining Calcein-AM fluorescence and pH_{in} in a pH 4.0 to pH 6.5 range (data not shown). Calcein-AM fluorescence (λ_{ex} = 495 nm, λ_{em} = 520 nm) was recorded for 60 min or until signal stabilised after addition of 80 µM iso-α-acids using a luminescence spectrometer. Measurements were performed every 2 s. 25 mM glucose and 20 mM glutamic acid, respectively, were used as energy source. The antibiotics valinomycin (Val) and nigericin (Nig) at a concentration of 1 µM served as reference ionophores and to equalise pH_{ex} and pH_{in} as required for the calibration (data not shown). Additionally, control experiments without addition of hop derivatives, Val/ Nig or energy source were performed.

2.2.22 **Transcriptional analysis of GAD-system associated genes**

Relative expression of GAD-system associated genes was investigated for reference acid stress conditions and two hop stress conditions using quantitative real-time polymerase chain reaction (qRT-PCR).

Total RNA was extracted from *L. brevis* TMW 1.6 and *L. brevis* TMW 1.465 grown in $mMRS_2$ and $mMRS_2$ containing 25 % or 50 % of the respective strains' MIC of iso-α-acids as described in 2.2.16. After purification of the extracted RNA with RQ1 RNase-free DNase according to the suppliers

guidelines, a 1:10 dilution in RNase-free dH_2O was prepared and cDNA was synthesised by reverse transcription using random primers and Moloney murine leukaemia virus reverse transcriptase following supplier's instructions of the molecular biological kit. Accordingly, a blank sample (without transcriptase) was prepared for subsequent control conditions of the transcriptional analysis. Quality and quantity of extracted RNA and synthesised cDNA (sample and control) were evaluated with a NanoDrop® spectrophotometer. The nucleotide sequences of the GAD system-associated genes *gadC*, *gadB*$_1$, *gadB*$_2$, *gad-tr*, a Na^+/H^+ antiporter (locus tag: LVIS_2211, found in the genetic neighbourhood of *gadB*$_2$) and the *tuf* nucleotide sequence encoding the elongation factor thermo unstable (EF-Tu) were determined using the National Center for Biotechnology Information (NCBI) nuccore online tool (http://www.ncbi.nlm.nih.gov/nuccore) on basis of the genome sequence of *L. brevis* ATCC 367. EF-Tu was used as housekeeping gene as shown previously (Arena et al. 2011; Duary et al. 2012). NCBI Primer-BLAST online tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast; default settings except: PCR product size: 230–270; Max T_m difference: 2) was used to design primers for genes to be amplified by qRT-PCR in accordance with producer guidelines for the LightCycler® apparatus. Primer sequences and respective annealing temperatures are shown in Tab. 10.

Tab. 10: Primers used in transcriptional analysis of GAD system–associated genes by LightCycler® qRT-PCR

Primer	Sequence (5' – 3')	Annealing temp. (°C)
gad-tr_F	AACGGCCGACAACTGGCTGG	59.90
gad-tr_R	TGCGAACAATCCCCTGTAAGCCG	59.51
$gadB_1$_F	TCGAGATGCGCCAAGTGCCA	59.07
$gadB_1$_R	ATCGACGAACGGCGCGAACA	59.78
$gadB_2$_F	CTCGCGGCTGAATCTCGCCA	59.57
$gadB_2$_R	ATCGCCAAACCGGCCAGCAT	59.69
gadC_F	GGCCGCTAAGTCGCGTTGGT	60.04

Primer	Sequence (5' – 3')	Annealing temp. (°C)
gadC_R	CCGGTCGGTGCTGCCACTTC	60.32
tuf_F	GCCGCTCAAATGGACGGTGC	59.23
tuf_R	AGCTGAACCGCGGATAACAGGA	60.50
LVIS_2211_F	TGGGGAATACAACACGAACGGCG	59.76
LVIS_2211_R	GGTGTCCGCGCAACGTTTCCT	60.50

qRT-PCR run conditions were as follows: initial denaturation (95 °C for 15 min), 45 repeated cycles of amplification and quantification (94 °C for 15 s, 60 °C for 20 s, 72 °C for 20 s in single fluorescence acquisition mode), melting curve determination confirming reaction specificity (95 °C for 0 s, 40 – 95 °C with temperature transition rate 0.1 °C/s and final cooling to 40 °C). All cDNA transcripts were quantified by fluorescence of ABsolute™ QPCR SYBR® Green Capillary Mix. Each LightCycler® reaction capillary was loaded with 5.25 µL ultrapure water, 7.5 µL SYBR Green Capillary Mix, 1.5 µL cDNA sample or blank (template RNA) and 0.75 µL primer mix (forward and reverse primer, 0.5 pM each).

Crossing points (CP; fluorescence response values appreciably higher than background fluorescence) were determined by the second derivative maximum method using the LightCycler® software version 3. Efficiency of the primer pairs was assessed with a dilution series of DNA extracted from *L. brevis* TMW 1.6 and *L. brevis* TMW 1.46 as described in 2.2.15.

In accordance with PFAFFL, primer efficiency was calculated according to the equation $E = 10^{(-1/\text{slope})}$ and the relative quantification (relative expression ratio, R) of the aforementioned target genes was calculated using equation (1) (Pfaffl 2001). The housekeeping gene EF-Tu was used as reference in the calculation to normalise the expression. The analysis was conducted in two independent experiments.

(1) $$R = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}(\text{control-sample})}}{(E_{\text{reference}})^{\Delta \text{CP}_{\text{reference}}(\text{control-sample})}}$$

2.2.23 MALDI-TOF MS based proteomic approach for hop constituents induced shock reactions

The influence of hop constituents on hop sensitive *L. brevis* TMW 1.6 and hop tolerant *L. brevis* TMW 1.465 was investigated on a proteomic level focusing on lmw proteins. In accordance with RUDD et al (Rudd et al. 1998), for this work, the term lmw proteins is arbitrarily defined as proteins of 150 amino acids or less (or 16.5 kDa, calculated on basis of an average amino acid molecular weight of 110 Da; (Berg et al. 2003)). For the assay, *L. brevis* TMW 1.6 and *L. brevis* TMW 1.465 were grown in $mMRS_1$ at 30 °C o.n.. Cells were washed in $mMRS_1$ and transferred to fresh broth to allow readjustment pH_{in} and to minimise environmental stressors. After a 3 h resting period, cells were harvested and aliquots were resuspended in one of three different shock broths. $mMRS_2$ served to mimic reference acid stress conditions. Hop shocks were performed in $mMRS_2$ containing iso-α-acids (50 % of the respective strains' MIC) or xanthohumol. Xanthohumol was used at respective equivalent molarity, since no MIC could be determined within limitations of solubility.

Samples for MALDI-TOF MS analysis were taken in order to determine characteristic mass spectra. Sampling was conducted prior to the resting period, every 30 min during the 3 h resting period, immediately after the transfer to shock broths and at various points over a 48 h incubation period (30 min, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h). All experiments were conducted in triplicates.

Additionally a control experiment was set up to mimic the transition from $mMRS_1$ to $mMRS_2$ in order to ensure that changes in MALDI-TOF mass spectra could clearly be attributed to the influence of hop compounds instead of media specific features. Therefore, media corresponding to either one of the two broths' pH-value, cysteine, manganese and magnesium

concentration were prepared in all possible combinations and sampling was conducted prior to the medium transition, as well as immediately afterwards and at the 1 h incubation point

2.2.24 **MALDI-TOF MS analysis**

An automatically operating Microflex LT MALDI-TOF mass spectrometer in linear positive ion detection mode was used under Biotyper Automation Control 2.0. Ions were generated with a nitrogen laser (λ = 337 nm) and mass spectra ranging from 2 to 20 kDa were acquired accumulating 240 shots per sample. External mass calibration was done using a bacterial test standard. Samples of 1 mL fermentation broth were taken and sample preparation was conducted according to KERN et al. with minor modifications (Kern et al. 2013). Therefore, cells were harvested, washed in dH_2O and inactivated with EtOH (75 %). Cells were collected again by centrifugation and all supernatant was removed thoroughly. The protein extract was obtained by application of formic acid (FA), dH_2O and AcN (35 : 15 : 50, v/v). Triplicates of 1 µL suspension were transferred to the stainless steel target plate, overlaid with 1 µL matrix solution (10 mg/mL α-cyano-4-hydroxycinnamic acid in AcN, dH_2O and trifluoroacetic acid (TFA); (50 : 47.5 : 2.5, v/v)) and taken to dryness before measurements.

2.2.25 **Bioinformatic evaluation MALDI-TOF MS raw data**

Data processing for the identification of stress induced peaks was carried out according to KERN et al. as summarised in the following (Kern et al. 2014). An open sharedroot computer cluster (ATIX; http://opensharedroot.org), running a self tailored MASCAP (Mantini et al. 2010) software application, implemented in octave (Eaton & Rawlings 2003) was used to analyse spectra exported using FlexAnalysis 3.3. Job control was conducted via a message passing interface (MPI) and BASH (http://www.gnu.org/software/bash) scripts were used to create software pipelines. Processing, detection and alignment of peaks was performed as described in detail by MANTINI et al. with 600 ppm

defined as limit of distance tolerance for alignment and clustering of peaks (Mantini et al. 2010).

In order to detect shock broth induced peaks, test spectra were checked for differences with a minimum accepted peak detection rate of 0.4, excluding all peaks absent in 60 % of the analysed spectra. Lowest accepted peak intensity was set to 50 % of a respective peak's average and a limit of 20 % standard deviation was defined for signal intensity.

2.2.26 **Protein identification strategy for shock induced peaks**

SDS-PAGE was used for protein separation and size estimation (cf. 2.2.17 and 2.2.19). Following the staining procedure, areas of interest were excised from the polyacrylamide gel and sent to the analysis laboratory ZFP (Ludwig-Maximilians-Universität München, Germany). All working materials were wiped with acetone to remove protein stains potentially interfering with LC-ESI MS/MS analysis. Samples were alkylated and reduced prior to shipping. Therefore, samples were shrunk and destained with AcN. Then, the gel particles were incubated in 0.1 M ammonium bicarbonate (AmBic) buffer containing 10 mM DTT at 56 °C for 45 min. After two washing steps in AcN, an equal amount of 0.1 M AmBic buffer containing 55 mM iodoacetamide was added and samples were kept in the dark for 30 min. In order to render the gel particles completely free of staining residues, the samples were repeatedly washed in 0.1 M AmBic buffer and AcN, respectively (Imhof 2004).

Finally, data obtained via LC-ESI MS/MS were evaluated bioinformatically. The identification of proteins from *L. brevis* TMW 1.465 was principally done according to BEHR et al. (Behr et al. 2007). A protein database was created according to JOHNSON et al. (Johnson et al. 2005). All available protein sequences from *L. brevis* were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/). Three hundred contaminant proteins including keratins and proteases were added into the database (Zhu et al. 2004). For protein identification, the created database was searched by the MS-BLAST version (Shevchenko et al. 2001) of the WU-BLAST2 program

(http://blast.wustl.edu/). For the latter, mgf files (Perkins et al. 1999) derived from LC-ESI MS/MS analysis were processed to peptide sequences with PepNovo (Frank & Pevzner 2005; Frank et al. 2005; Frank et al. 2007; Frank 2009). Therefore, MGF files were split in parts and processed in parallel by an open sharedroot computer cluster (ATIX; http://opensharedroot.org/) using a MPI for job control (Gabriel et al. 2004). BASH scripts and programs written in C generated and submitted catenations of peptide sequences to MS-BLAST for protein identification.

3 Results

3.1 Chemical and physical characterisation of hop compounds and hop derivatives

3.1.1 Analysis of modified hop derivatives via GC MS and LC-ESI MS/MS

Hop iso-α-acids and chemically modified iso-α-acids derivatives were analysed and identified by a novel GC MS method without prederivatisation and by LC-ESI MS/MS. In the following, the analysed compounds are listed according to IUPAC nomenclature and corresponding trivial names, which, for this work, were assigned to previously inexistent compounds (Tab. 11). Analytical experiments were performed in duplicates. Molecular weight, retention time (RT) and most intense peaks of electron impact (EI) spectra of the GC MS analysis of the analytes are listed in Tab. 12. Standard deviations of determined RTs were ≤ 0.008 min. An exemplary total ion chromatogram of an iso-α-acids mixture (A) and the total ion count of iso-n-humulone (B) are depicted in Fig. 6.

Tab. 11: IUPAC names of iso-α-acids and modified iso-α-acids analysed by GC MS and LC-ESI MS/MS

Compound	IUPAC name
iso-n-humulone	3,4-dihydroxy-5-(3-methylbut-2-en-1-yl)-2-(3-methylbutanoyl)-4-(4-methylpent-3-enoyl)cyclopent-2-en-1-one
iso-adhumulone	3,4-dihydroxy-5-(3-methylbut-2-en-1-yl)-2-(2-methylbutanoyl)-4-(4-methylpent-3-enoyl)cyclopent-2-en-1-one
iso-cohumulone	3,4-dihydroxy-5-(3-methylbut-2-en-1-yl)-2-(2-methylpropanoyl)-4-(4-methylpent-3-enoyl)cyclopent-2-en-1-one
methyl-iso-n-humulone	4-hydroxy-3methoxy-5-(3-methylbut-2-en-1-yl)-2-(3-methylbutanoyl)-4-(4-methylpent-3-enoyl)cyclopent-2-en-1-one
methyl-iso-adhumulone	4-hydroxy-3methoxy -5-(3-methylbut-2-en-1-yl)-2-(2-methylbutanoyl)-4-(4-methylpent-3-enoyl)cyclopent-2-en-1-one

Compound	IUPAC name
methyl-iso-cohumulone	4-hydroxy-3methoxy -5-(3-methylbut-2-en-1-yl)-2-(2-methylpropanoyl)-4-(4-methylpent-3-enoyl)cyclopent-2-en-1-one
imino-iso-n-humulone	3,4-dihydroxy-5-(3-methylbut-2-en-1-yl)-2-(3-methylbutanoyl)-4-(4-methylpent-3-enoyl)cyclopent-2-en-1-imine
imino-adhumulone	3,4-dihydroxy-5-(3-methylbut-2-en-1-yl)-2-(2-methylbutanoyl)-4-(4-methylpent-3-enoyl)cyclopent-2-en-1- imine
imino-cohumulone	3,4-dihydroxy-5-(3-methylbut-2-en-1-yl)-2-(2-methylpropanoyl)-4-(4-methylpent-3-enoyl)cyclopent-2-en-1- imine
n-humulinic acid	3,4-dihydroxy-5-(3-methylbut-2-en-1-yl)-2-(3-methylbutanoyl)cyclopent-2-en-1-one
adhumulinic acid	3,4-dihydroxy-5-(3-methylbut-2-en-1-yl)-2-(2-methylbutanoyl)cyclopent-2-en-1-one
cohumulinic acid	3,4-dihydroxy-5-(3-methylbut-2-en-1-yl)-2-(2-methylpropanoyl)cyclopent-2-en-1-one

Tab. 12: Hop derived compounds, retention times (RT) (min), molecular weights (Mw) and largest peaks EI spectrum determined by GC MS analysis. Standard deviations of RT were ≤ 0.008 min.

compound	Mw	RT	largest peaks EI spectrum
iso-n-humulone	362.47	35.412	69; 197; 41; 43; 57; 97; 293; 266; 55
iso-adhumulone	362.47	35.166	69; 41; 197; 57; 43; 55; 39; 97; 293
iso-cohumulone	348.44	33.401	69; 183; 41; 43; 252; 279; 97; 55; 71
methyl-iso-n-humulone	376.49	37.126	209; 41; 69; 43; 289; 55; 57; 347; 180; 249
methyl-iso-adhumulone	376.49	36.813	289; 41; 69; 57; 55; 249; 43; 233; 209; 304
methyl-iso-cohumulone	362.47	35.008	43; 275; 41; 69; 235; 55; 290; 219; 277; 209
imino-iso-n-humulone	361.48	52.809	196; 41; 69; 208; 197; 292; 232; 126; 55; 247
imino-adhumulone	361.48	51.482	196; 41; 69; 28; 18; 208; 247; 197; 55
imino-cohumulone	347.45	47.993	182; 41; 69; 233; 112; 194; 183; 96; 165
n-humulinic acid	266.34	28.340	41; 69; 197; 57; 43; 124; 71; 39; 55; 85

compound	Mw	RT	largest peaks EI spectrum
adhumulinic acid	266.34	28.235	69; 41; 57; 197; 43; 55; 71; 29; 124; 39
cohumulinic acid	252.31	27.275	69; 41; 43; 183; 71; 166; 39; 165; 55; 113

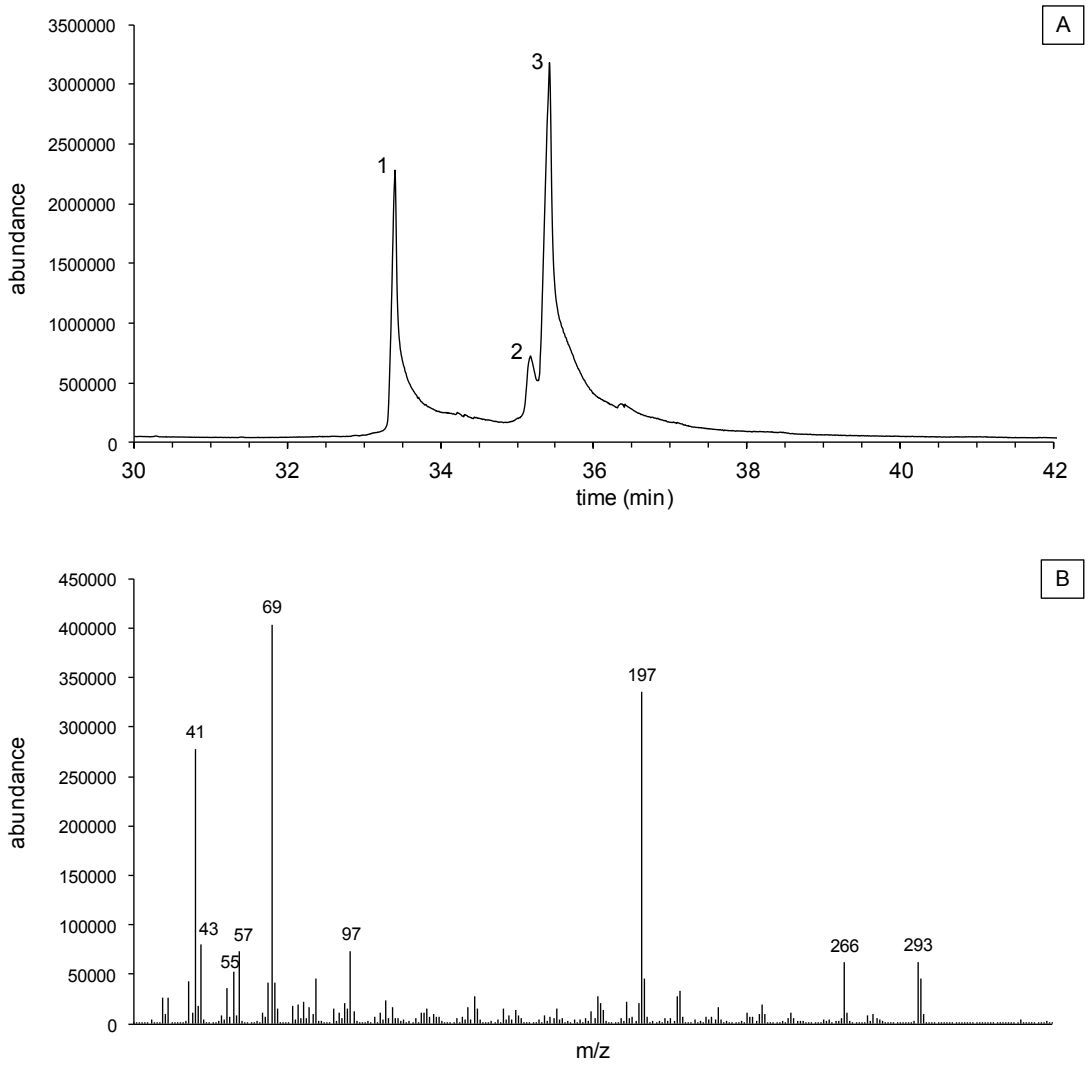


Fig. 6: Total ion chromatogram of iso-α-acids (A) (1, iso-cohumulone; 2, iso-adhumulone; 3, iso-n-humulone) and EI mass spectrum of iso-n-humulone (B) determined by GC MS analysis.

The fragmentation behaviour of iso-α-acids and modified iso-α-acids acquired by LC-ESI MS/MS was as follows:

Iso-cohumulone; MW = 348. m/z 349 $[M+H]^+$; m/z 348 $[M+H-H]^+$; m/z 331 $[M+H-H_2O]^+$; m/z 302 $[M+H-CH_3O_2]^+$; m/z 294 $[M+H-C_4H_7]^+$; m/z 280 $[M+H-C_5H_9]^+$; m/z 266 $[M+H-C_6H_{11}]^+$; m/z 238 $[M+H-C_7H_{11}]^+$; m/z 212 $[M+H-C_{10}H_{17}]^+$; m/z 206 $[M+H-C_8H_{15}O_2]^+$; m/z 204 $[M+H-C_7H_{13}O_3]^+$.

Iso-n/ad-humulone; MW = 362. m/z 363 $[M+H]^+$; m/z 345 $[M+H-H_2O]^+$; m/z 294 $[M+H-C_5H_9]^+$; m/z 252 $[M+H-C_7H_{11}O]^+$.

Methyl-iso-cohumulone; MW = 362. m/z 363 $[M+H]^+$; m/z 345 $[M+H-H_2O]^+$; m/z 294 $[M+H-C_5H_9]^+$; m/z 260 $[M+H-C_5H_{11}O_2]^+$.

Methyl-iso-n/ad-humulone; MW = 376. m/z 377 $[M+H]^+$; m/z 359 $[M+H-H_2O]^+$; m/z 320 $[M+H-C_4H_9]^+$; m/z 308 $[M+H-C_5H_9]^+$; m/z 288 $[M+H-C_5H_{13}O]^+$; m/z 278 $[M+H-C_6H_{11}O]^+$; m/z 234 $[M+H-C_8H_{15}O_2]^+$; m/z 166 $[M+H-C_{13}H_{23}O_2]^+$.

Imino-iso-cohumulone; MW = 347. m/z 348 $[M+H]^+$; m/z 330 $[M+H-H_2O]^+$; m/z 291 $[M+H-C_4H_9]^+$; m/z 279 $[M+H-C_5H_9]^+$; m/z 278 $[M+H-C_4H_6O]^+$; m/z 252 $[M+H-C_6H_8O]^+$; m/z 238 $[M+H-C_7H_{12}N]^+$.

Imino-iso-n/ad-humulone; MW = 361. m/z 362 $[M+H]^+$; m/z 344 $[M+H-H_2O]^+$; m/z 305 $[M+H-C_4H_9]^+$; m/z 293 $[M+H-C_5H_9]^+$; m/z 277 $[M+H-C_5H_9O]^+$; m/z 207 $[M+H-C_8H_{13}NO_2]^+$.

LC-ESI MS/MS spectra and characteristic fragments of iso-α-acids and modified iso-α-acids obtained by data processing via the MS Interpreter application are depicted in Fig. 7 - Fig. 9 and in Appendix Fig. 1 - Appendix Fig. 3. It could be concluded from these results that methylation of iso-α-acids occurred in the C-3 position bound acidic hydroxyl group. Further, substitution of the C-1 carboxylic group yielded imination products. The characteristic parent compounds of methyl-iso-n-humulone and imino-iso-n-humulone (m/z 377 and m/z 362, respectively) could not be detected in the measurements of iso-α-acids. This clearly indicates the formation of the desired target compounds.

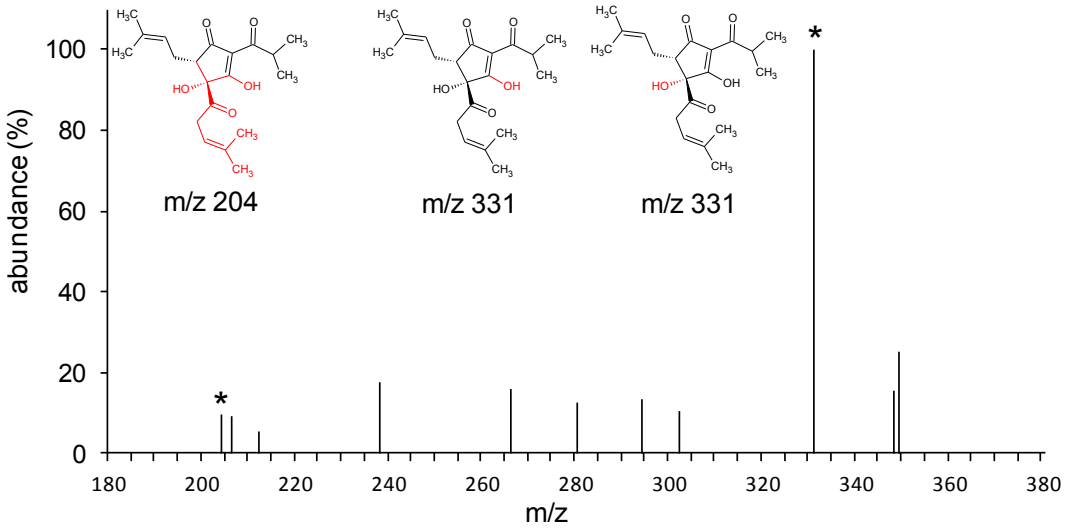


Fig. 7: Mass spectrum and characteristic fragmentation behaviour of iso-cohumulone obtained by LC-ESI MS/MS and subsequent data processing. Arbitrary abundance units were normalised using the peak of highest intensity as reference. Molecule parts marked in red indicate loss. Asterisks indicate depicted fragments.

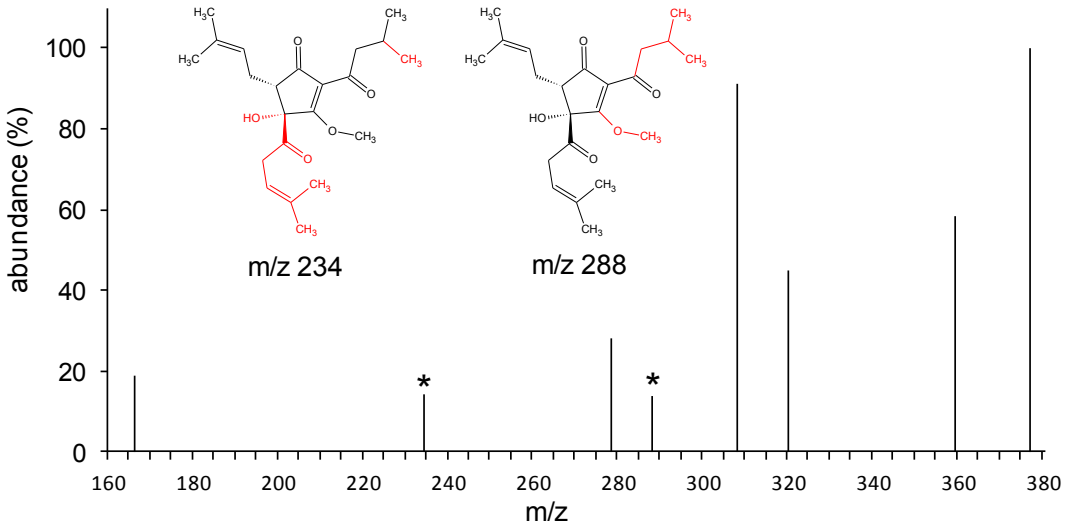


Fig. 8: Mass spectrum and characteristic fragmentation behaviour of methyl-iso-n-humulone obtained by LC-ESI MS/MS and subsequent data processing. Arbitrary abundance units were normalised using the peak of highest intensity as reference. Molecule parts marked in red indicate loss. Asterisks indicate depicted fragments.

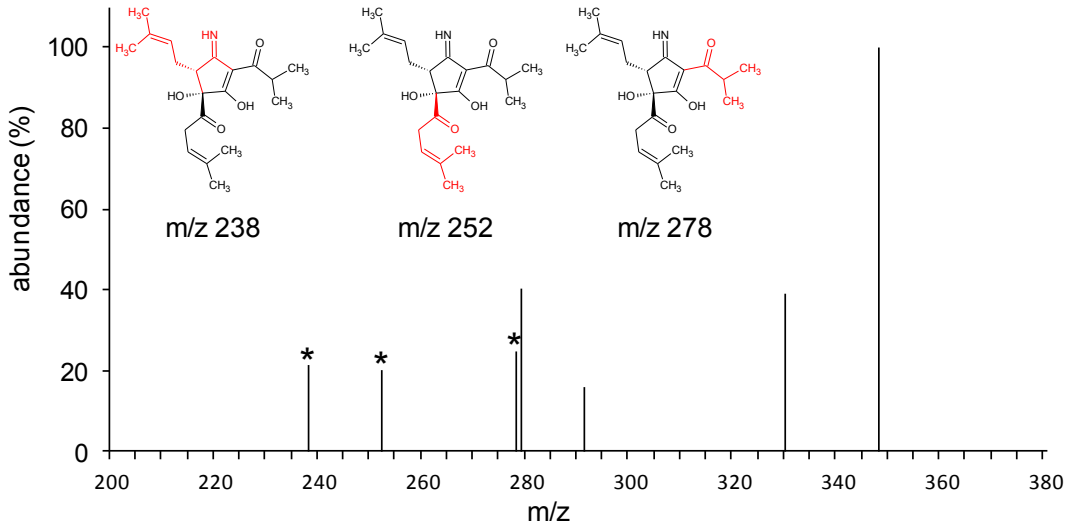


Fig. 9: Mass spectrum and characteristic fragmentation behaviour of imino-iso-cohumulone obtained by LC-ESI MS/MS and subsequent data processing. Arbitrary abundance units were normalised using the peak of highest intensity as reference. Molecule parts marked in red indicate loss. Asterisks indicate depicted fragments.

3.1.2 **Manganese binding ability of hop compounds**

UV-Vis spectra were recorded to investigate the manganese binding ability of iso-α-acids and modified iso-α-acids. The scans showed a shift of test substance-characteristic absorption maxima (approx. 10 nm to 20 nm) towards the upper end of the spectrum for all compounds except for imino-iso-α-acids. Exemplary UV-Vis absorption spectra of iso-α-acids and imino-iso-α-acids are shown in Fig. 10 and Fig. 11. Further scans are depicted in Appendix Fig. 8 - Appendix Fig. 10.

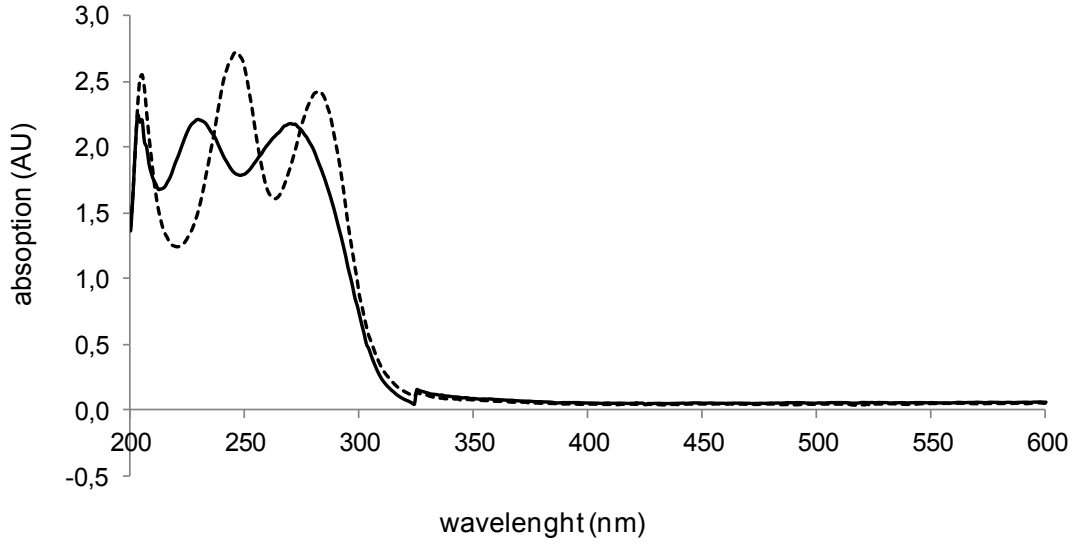


Fig. 10: UV-Vis absorption spectra of iso-α-acids (solid line) and iso-α-acids in presence of 10 mM manganese (dotted line) determined in methanol.

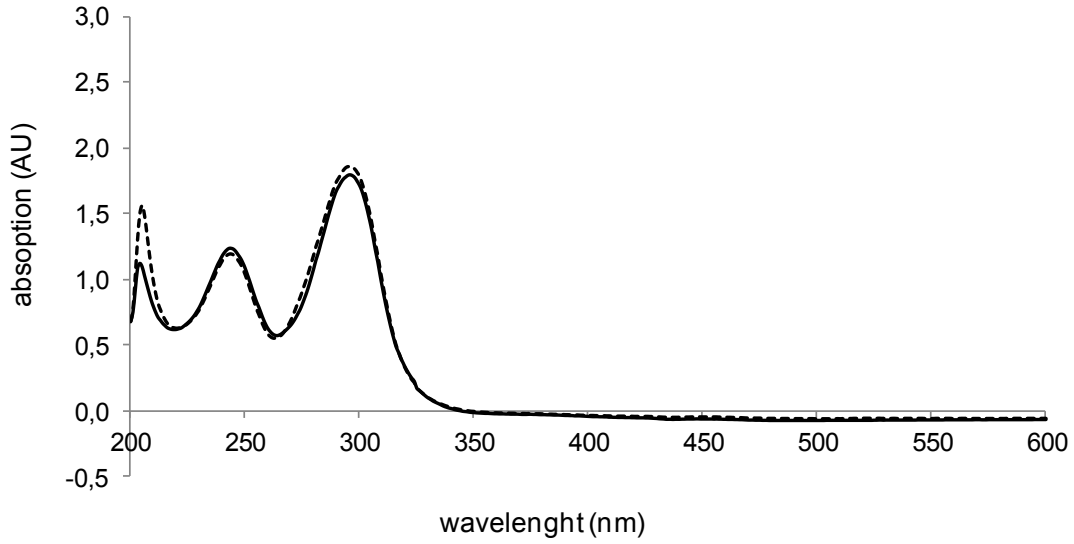


Fig. 11: UV-Vis absorption spectra of imino-iso-α-acids (solid line) and imino-iso-α-acids in presence of 10 mM manganese (dotted line) determined in methanol.

3.1.3 **Membrane solubility of hop compounds**

The partitioning of hop derivatives and modified hop derivatives in a liquid two phase system was evaluated under conditions reflecting the compounds' ionisation in physiological and electrochemical experiments to evaluate the influence of the respective modification on membrane solubility and hence guarantee sound interpretability of the findings. Distribution ratios were determined by the shake flask method using n-octanol as organic solvent and

an aqueous phosphate buffered solution (pH 4.3). By definition, high membrane solubility is reflected by high values of the dimensionless measure $D_{org/aq}$, and vice versa. $D_{org/aq}$ values were assessed for iso-α-acids, methyl-iso-α-acids, methyl-cis-iso-cohumlone, imino-iso-α-acids and humulinic acids. Methylation of iso-α-acids at the C-3 position did not change the molecule's membrane solubility, whereas hydrolysis of the isohexenoyl side chain at C-4 and imination at the C-1 position led to a differently pronounced decrease of membrane solubility (cf. Fig. 12).

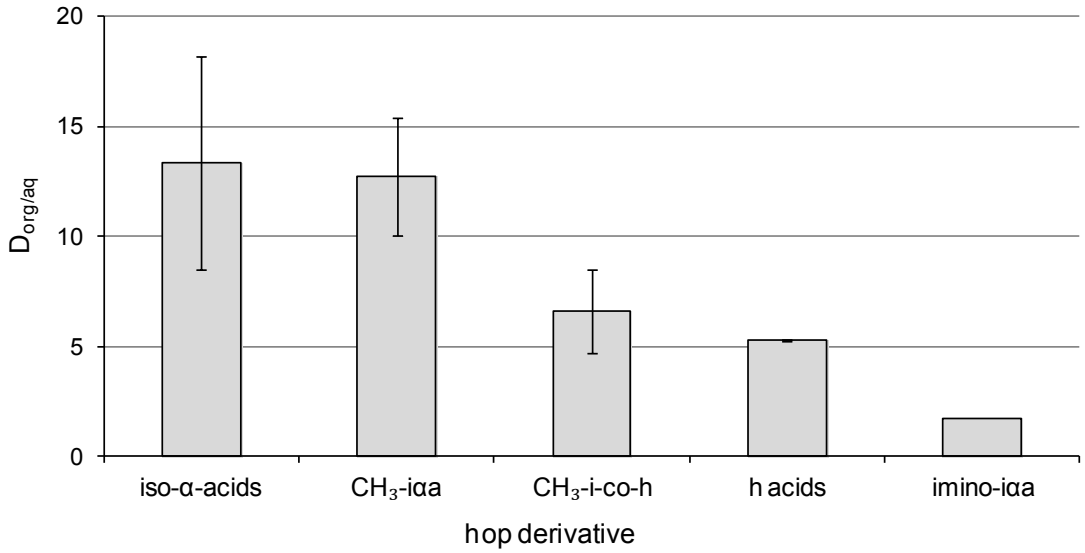


Fig. 12: Distribution ratios ($D_{org/aq}$) of iso-α-acids, methyl-iso-α-acids (CH_3-iαa), methyl-cis-iso-cohumulone (CH_3-i-co-h), humulinic acids (h acids) and imino-iso-α-acids (imino-iαa) determined by shake-flask method using n-octanol and phosphate buffered solution (pH 4.3) in a two phase liquid system. High $D_{org/aq}$-values reflect high membrane solubility and vice versa.

3.2 Inhibitory potential of hop compounds and hop derivatives

3.2.1 **Antimicrobial activity-orientated screening of hop compounds**

An antimicrobial activity-orientated screening of various hop compounds (crude hop extracts, commercial hop products, non-commercial hop extracts, pure substances and hop derivatives) was achieved by determination of individual MIC values for four different *L. brevis* strains. No less than three

biological replicates were conducted to determine MICs. A detailed list of MICs for all assessed compounds is shown in Appendix Tab. 1. An exemplary depiction of a MIC determination microtitre plate is shown in Fig. 13.

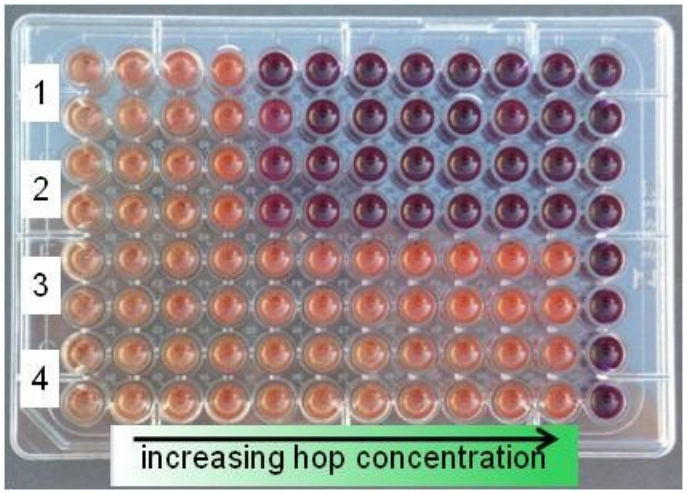


Fig. 13: Exemplary MIC determination microtitre plate. MIC determined in $mMRS_2$ after 72 h incubation at 30 °C with 1 mM resazurin. Test strains 1 - 4: *L. brevis* TMW 1.6, 1.1369, 1.465 and 1.313, respectively were investigated in technical duplicates for hop compounds increasing in 10 % steps of a predefined maximum concentration. Metabolic activity indicated by a colour shift from purple/blue (oxidised form resazurin) to pink (reduced form resorufin).

MICs of crude hop extracts from green HALLERTAUER MAGNUM hop cones were determined to examine the influence of the extraction agent's polarity on the antimicrobial potential of the extract. A tentative decrease of antimicrobial activity, corresponding with higher polarity of the extractant (polarities: hexane, 0.0; THF, 0.45; EtOH, 0.88. (Küster & Thiel 2002)) could be observed. However, except for *L. brevis* TMW 1.465, no significant differences could be found (Fig. 14).

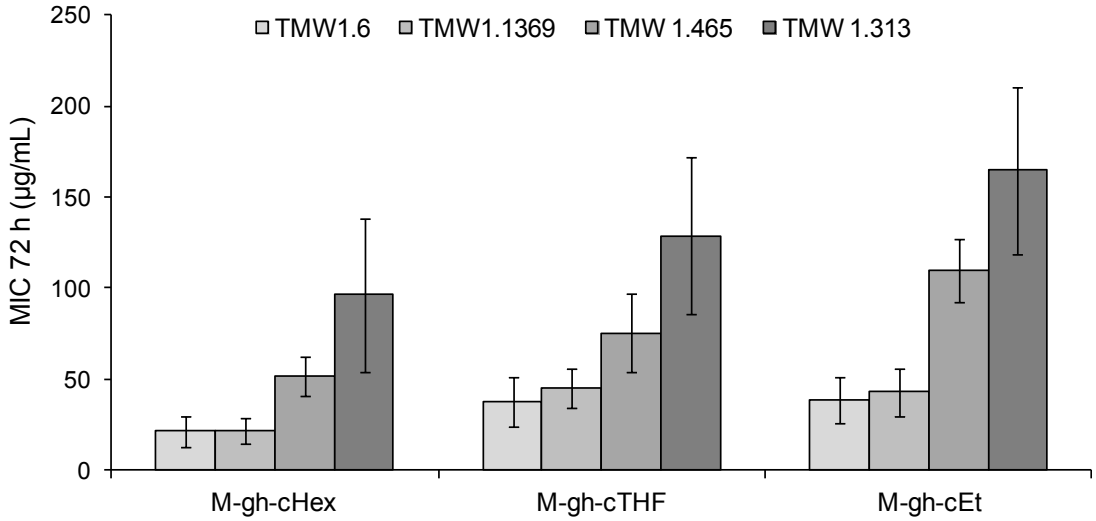


Fig. 14: MIC of crude hop extracts from green HALLERTAUER MAGNUM hop cones. MIC determined after 72 h incubation at 30 °C for *L. brevis* TMW 1.6, 1.1369, 1.465 and 1.313.

Accordingly, total resins prepared from different HALLERTAUER MAGNUM raw materials (EtOH extract (et), green hops (gh), raw hops (rh), pellets type 90 (pl)) following MEBAK guidelines (cf. 2.1.3) were investigated. No differences between extracts from different raw materials could be found for the two more hop sensitive *L. brevis* strains TMW 1.6 and TMW 1.1369 for all extracts and for the two more hop tolerant strains TMW 1.465 and TMW 1.313, except for the total resin prepared from pellets which had lower inhibitory potential (Fig. 15). A similar pattern could be found for the soft resin fraction (Appendix Fig. 4).

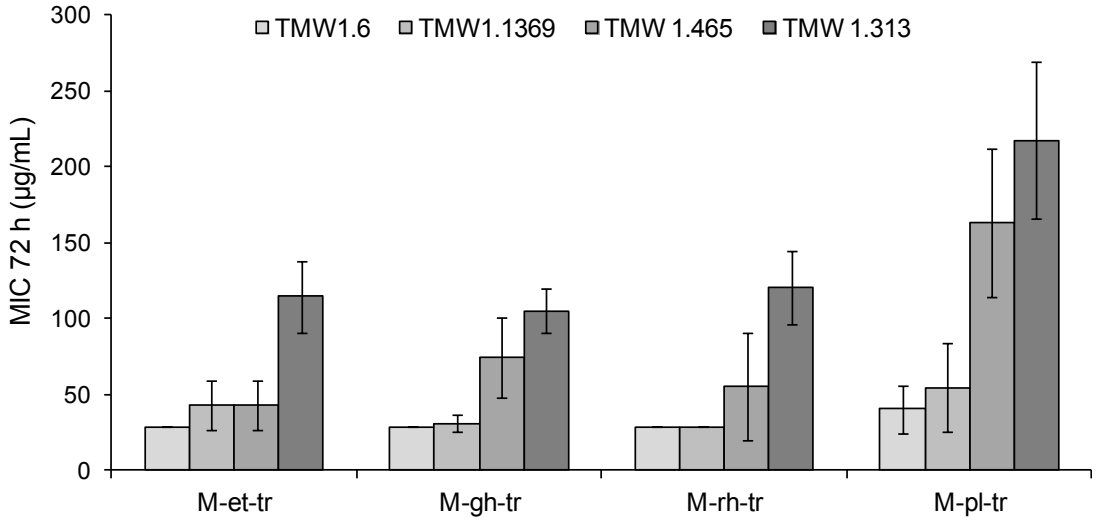


Fig. 15: MIC of total resins prepared from different HALLERTAUER MAGNUM hop raw materials. MIC determined after 72 h incubation at 30 °C for *L. brevis* TMW 1.6, 1.1369, 1.465 and 1.313.

Fig. 16 shows the MIC of tr, sr and hr prepared from HALLERTAUER MAGNUM ethanol extract. The tr and sr extracts exhibited similar MICs. The hr extract appeared to be less antimicrobially active. However, due to partially high standard deviations, merely a trend could be identified. Extracts from gh, rh and pl led to the same result regarding tr and sr. The hr fraction of these raw materials was found to be clearly less antimicrobially active with MIC values above the experimental limit of 286.3 µg/mL (cf. Appendix Fig. 5, Appendix Fig. 6, Appendix Fig. 7).

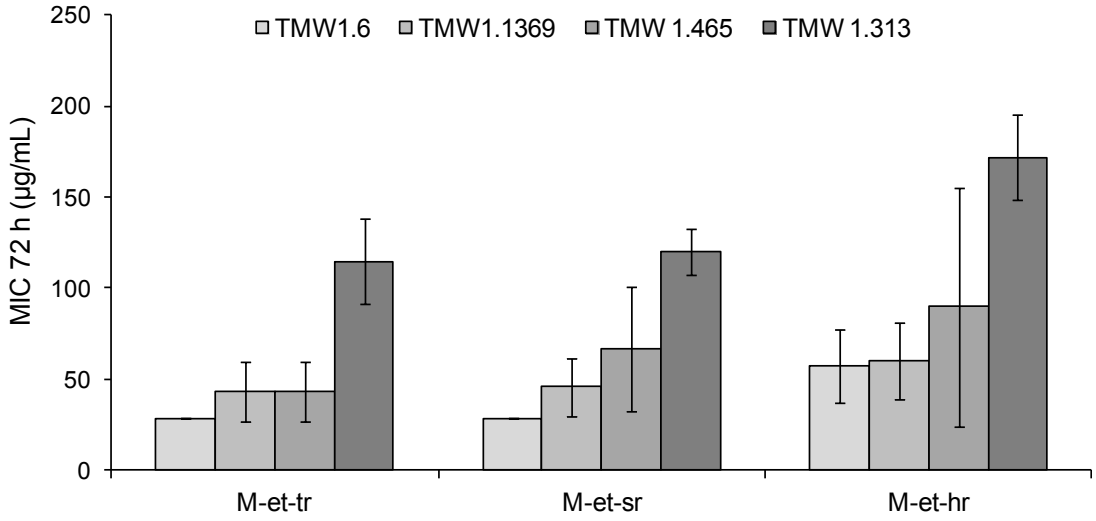


Fig. 16: MIC of total, soft and hard resin prepared from HALLERTAUER MAGNUM ethanol extract. MIC determined after 72 h incubation at 30 °C for *L. brevis* TMW 1.6, 1.1369, 1.465 and 1.313.

Further, the hard resin subractions (δ-resin, ε-resin) and the ε-resin subractions F01-11 were investigated. The inhibitory effect of δ-resin and ε-resin did not differ. Regarding the ε-resin, subfractions F06 - 09 were found to be the most inhibitory for all four *L. brevis* strains (Fig. 17).

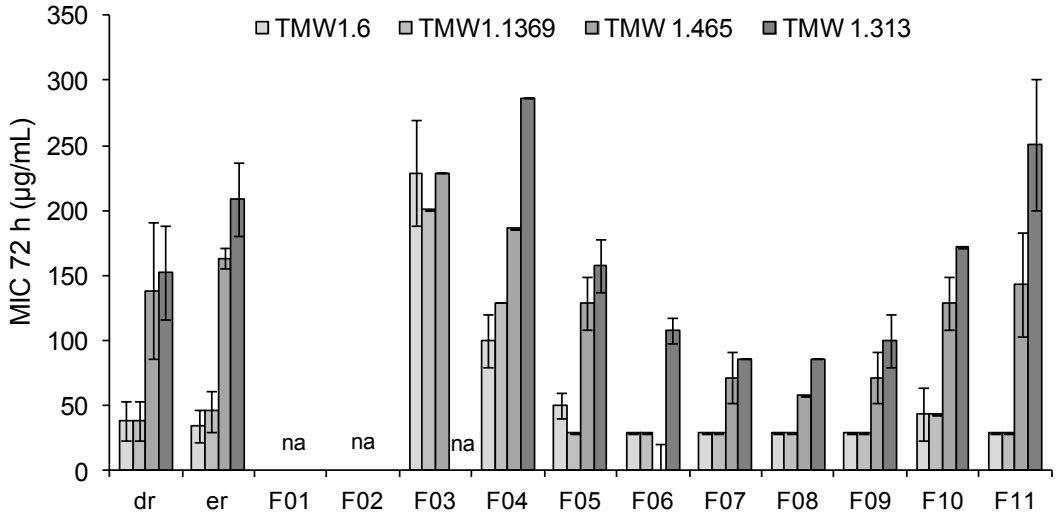


Fig. 17: MIC of δ-resin, ε-resin and ε-resin subfractions F01-11, prepared from HALLERTAUER MAGNUM ethanol extract. MIC determined after 72 h incubation at 30 °C for *L. brevis* TMW 1.6, 1.1369, 1.465 and 1.313. na, MIC above experimental limit (286.3 mg/mL).

Additionally, pure substances obtained from a fractionation process and commercial hop compounds (cf. 2.1.3) were assessed. The antimicrobial activity of these hop compounds partially varied within a wide range. A selection of exemplary results is depicted in Fig. 18. Further MIC values are listed in Appendix Tab. 1.

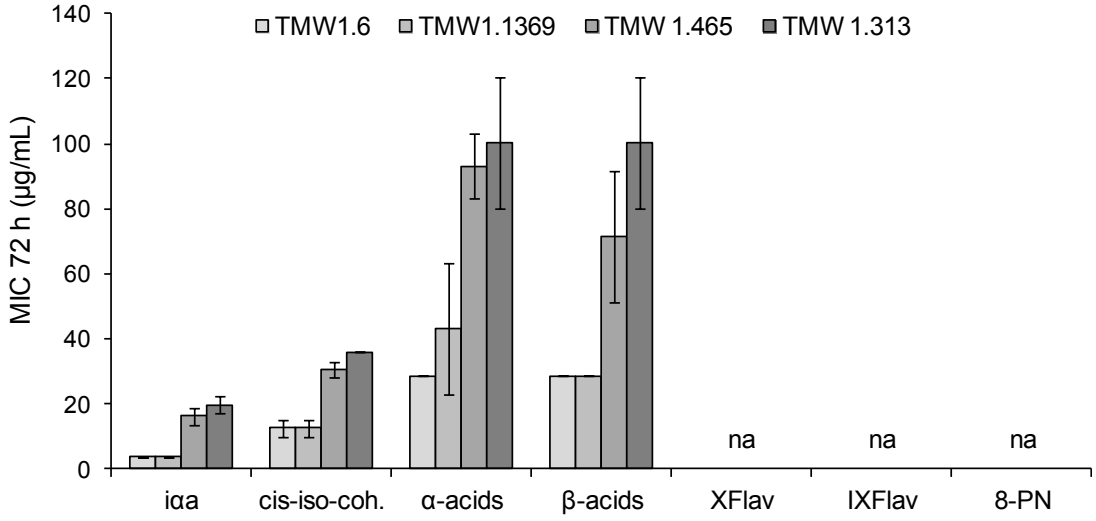


Fig. 18: MIC of selected commercial and non-commercial hop compounds. MIC determined after 72 h incubation at 30 °C for *L. brevis* TMW 1.6, 1.1369, 1.465 and 1.313. na, MIC above experimental limit (286.3 µg/mL).

In order to achieve a better understanding of MIC-determining factors regarding hop varieties, basic raw materials, individual fractions and workup levels and to allow for a comprehensible presentation, a cluster analysis of overall MIC means was conducted. In a first approach, MICs of different hop compounds comprising crude extracts (prepared with different extracting agents), total-, soft- and hard-resins from different hop varieties and different raw materials were clustered (analysis 1). In a second approach, MICs of hop compounds comprising different workup levels from a HALLERTAUER MAGNUM ethanol extract, as well as pure substances, purified mixtures and hop derivatives were clustered (analysis 2).

Circular trees of obtained clustering results are presented in the following. As depicted in Fig. 19 and Fig. 20, analysis 1 showed that the applied extracting agent and the hop variety had rather subordinate influence on the antimicrobial potential of the tested compound. Also, the workup level of these compounds (level 1 and 2) was not a determining factor. The biggest differences were found between the two groups total-/soft-resin and hard-resin, with the latter being less effective.

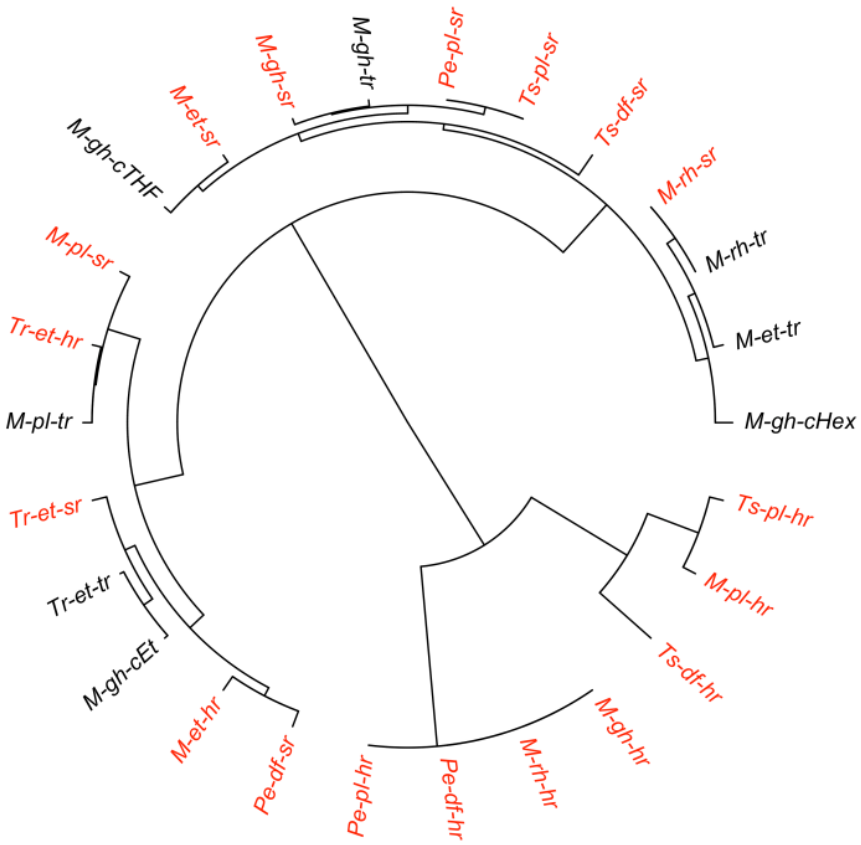


Fig. 19: Circular tree of MIC cluster analysis 1. Colour code indicates workup level of hop fraction according to Fig. 5 and Tab. 4. Key pattern: hop variety - raw material - fraction, e.g.: HALLERTAUER MAGNUM – ethanol extract - total resin (M-et-tr). M, MAGNUM; Tr, TRADITION; Ts, TAURUS; Pe, PERLE; et, ethanol extract; pl, pellets; gh, green hops; rh, raw hops; df, draff; tr, total resin; sr, soft resin; hr, hard resin; cHex, crude hexane extract; cTHF, crude THF extract; cEt, crude EtOH extract.

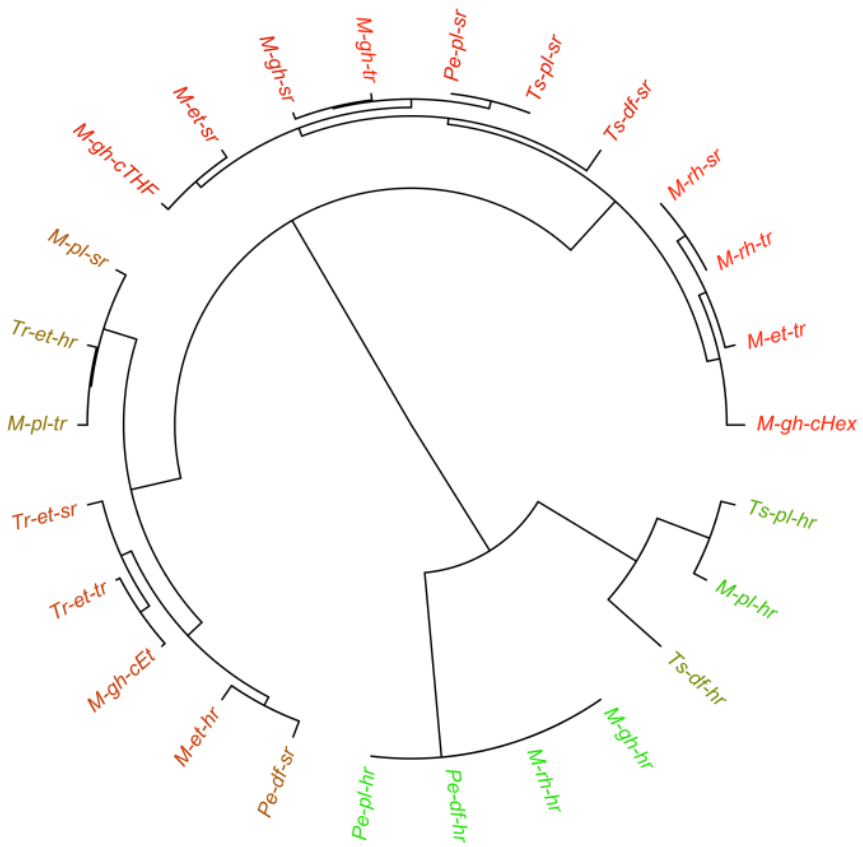


Fig. 20: Circular tree of MIC cluster analysis 1. Colour code indicates MICs ascending from low (bright red) to high (light green). Key pattern: hop variety - raw material - fraction, e.g.: HALLERTAUER MAGNUM – ethanol extract - total resin (M-et-tr). M, MAGNUM; Tr, TRADITION; Ts, TAURUS; Pe, PERLE; et, ethanol extract; pl, pellets; gh, green hops; rh, raw hops; df, draff; tr, total resin; sr, soft resin; hr, hard resin; cHex, crude hexane extract; cTHF, crude THF extract; cEt, crude EtOH extract.

In Fig. 21 and Fig. 22, results for analysis 2 focusing on different workup levels of compounds prepared from a HALLERTAUER MAGNUM ethanol extract, as well as pure substances, purified mixtures and hop derivatives are presented. The clustering showed that the workup level itself is not a determining factor regarding the antimicrobial activity of the test compound as pure substances could be found to be highly toxic (iso-α-acids) or completely harmless (xanthohumol) for the investigated *L. brevis* strains. However, all fractions of levels 1-3 exerted antimicrobial effects to some extent. Also, some hard resin subfractions (F06-F09) were found to be similarly effective as the hard resin's counterpart soft resin or the superordinate total resin.

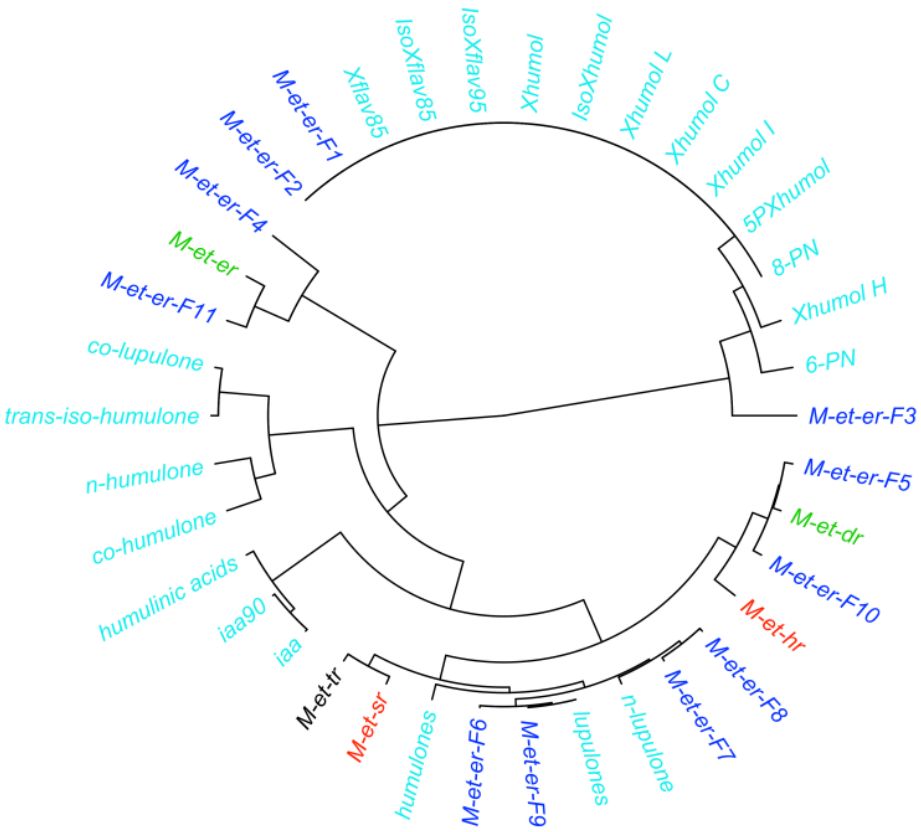


Fig. 21: Circular tree of MIC cluster analysis 2. Colour code indicates workup level of hop fraction according to Fig. 5 and Tab. 4. Key pattern: hop variety - raw material - fraction, e.g.: HALLERTAUER MAGNUM – ethanol extract - total resin (M-et-tr). M, MAGNUM; et, ethanol extract; tr, total resin; sr, soft resin; hr, hard resin; er, ε-resin; dr, δ-resin; F01-11, subfraction 1-11; iaa, iso-α-acids; iaa90, commercial iso-α-acids mixture; Xflav85, commercial xanthohumol product; Isoflav85/95, commercial isoxanthohumol products; Xhumol, xanthohumol; IsoXhumol, isoxanthohumol, Xhumol L/C/I/H, xanthohumol derivatives; 5PXhumol, 5-prenyl-xanthohumol; 8-PN, 8-prenylnaringenine, 6-PN, 6-prenylnaringenine.

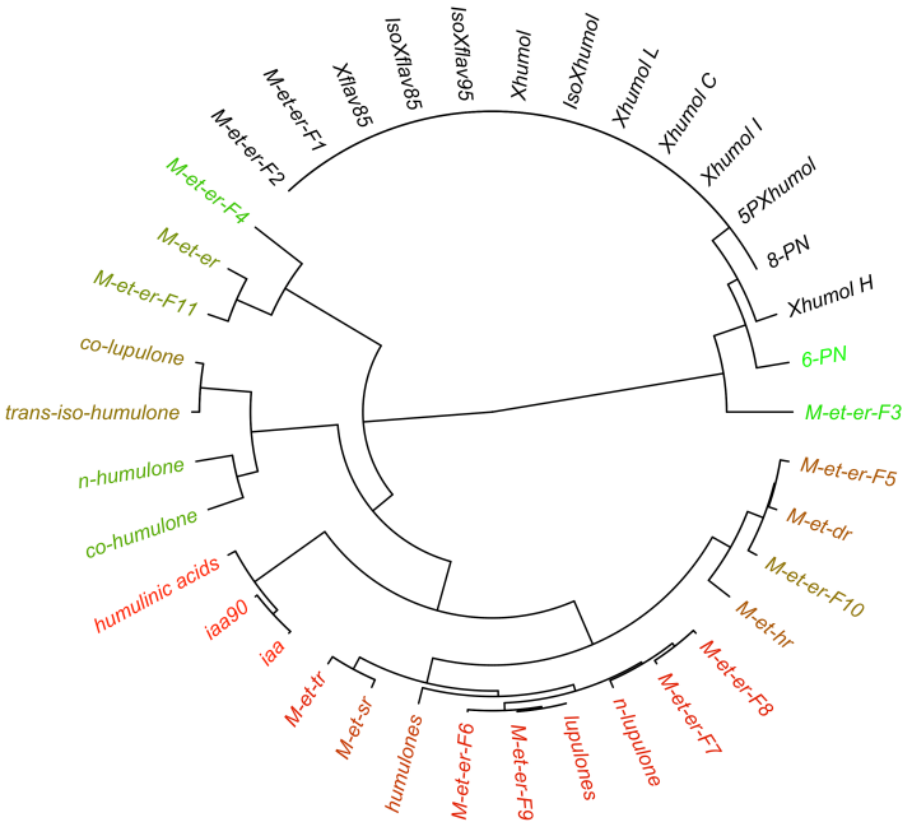


Fig. 22: Circular tree of MIC cluster analysis 2. Colour code indicates MICs ascending from low (bright red) to high (dark grey). Key pattern: hop variety - raw material - fraction, e.g.: HALLERTAUER MAGNUM – ethanol extract - total resin (M-et-tr). M, MAGNUM; et, ethanol extract; tr, total resin; sr, soft resin; hr, hard resin; er, ε-resin; dr, δ-resin; F01-11, subfraction 1-11; iaa, iso-α-acids; iaa90, commercial iso-α-acids mixture; Xflav85, commercial xanthohumol product; Isoflav85/95, commercial isoxanthohumol products; Xhumol, xanthohumol; IsoXhumol, isoxanthohumol, Xhumol L/C/I/H, xanthohumol derivatives; 5PXhumol, 5-prenyl-xanthohumol; 8-PN, 8-prenylnaringenine, 6-PN, 6-prenylnaringenine.

3.2.2 **Antimicrobial activity of modified iso-α-acids**

To assess the influence of molecular modifications on the antimicrobial potential of iso-α-acids, growth challenge tests were conducted and MICs were determined. Loss of the acidic hydroxyl group in the C-3 position, which was brought about by methylation, resulted in increased MICs, i.e. in lowered antimicrobial activity of the test substances. Similarly, but to a much greater extent, imination at the C-1 position and the resulting loss of Mn^{2+}-binding ability also affected the test substances, as their antimicrobial activity was entirely abolished within the experimental limit (c_{max} = 791.9 µM). Humulinic

acids exhibited the greatest antimicrobial potential with MICs below the range found for iso-α-acids. A typical pattern of susceptibility towards the test substances could be found for hop sensitive *L. brevis* TMW 1.6 and 1.1369 and *L. brevis* TMW 1.465 and 1.313. The observed pattern remained consistent for all tested substances Fig. 23.

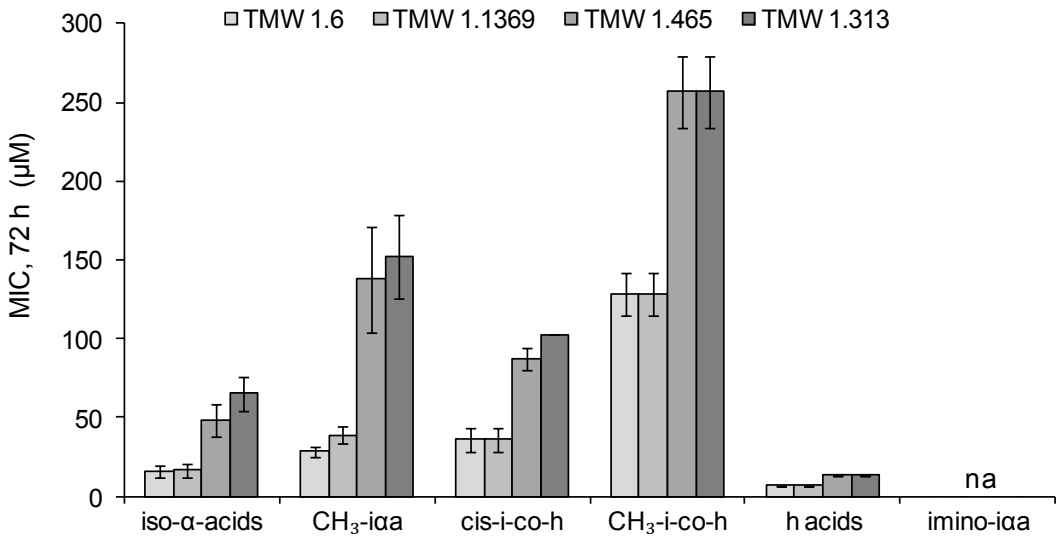


Fig. 23: Minimum inhibitory concentration (MIC) of iso-α-acids, methyl-iso-α-acids (CH_3-iαa), cis-iso-cohumulone (cis-i-co-h), methyl-cis-iso-cohumulone (CH_3-cis-i-co-h), humulinic acids (h acids) and imino-iso-α-acids (imino-iαa), determined after 72 h incubation at 30 °C for *L. brevis* TMW 1.6, 1.1369, 1.465 and 1.313. Imino-iαa: MICs > experimental limit; (c_{max} = 791.9 µM).

3.3 Metabolic and physiologic characteristics of *L. brevis* under hop stress

3.3.1 **Viability, growth and broth acidification**

To investigate the living conditions of *L. brevis* TMW 1.6 and TMW 1.465 under hop stress and reference acid stress conditions, broth acidification, viable cell counts and OD_{590} were assessed.

Therefore, cells were cultivated in $mMRS_1$ medium at pH 6.2 to allow stress-free preculturing. Subsequently, cells were grown in $mMRS_2$ until OD_{590} = 0.3 - 0.6 was reached and broth acidification and OD were measured. Viable cell counts were determined by plate count method using

$mMRS_1$ agar plates. Effects of hop stress (25 % and 50 % of $MIC_{iso\text{-}\alpha\text{-}acids}$) were examined in comparison to reference acid stress conditions in $mMRS_2$ at pH 4.3.

As shown in Fig. 24, the ability to further acidify $mMRS_2$ of both *L. brevis* strains remained unaltered in the presence of iso-α-acids. While *L. brevis* TMW 1.465 lowered the medium's pH-value (pH 4.11), the more hop-sensitive strain *L. brevis* TMW 1.6 propagated at unchanged pH conditions. The determination of live cell numbers revealed a more pronounced ability of *L. brevis* TMW 1.465 to survive the given adverse living conditions than it was the case for *L. brevis* TMW 1.6, when grown to a similar OD. There could, however, not be determined any differences in live cell numbers comparing hop stress and reference acid stress conditions for either of the strains (Fig. 25).

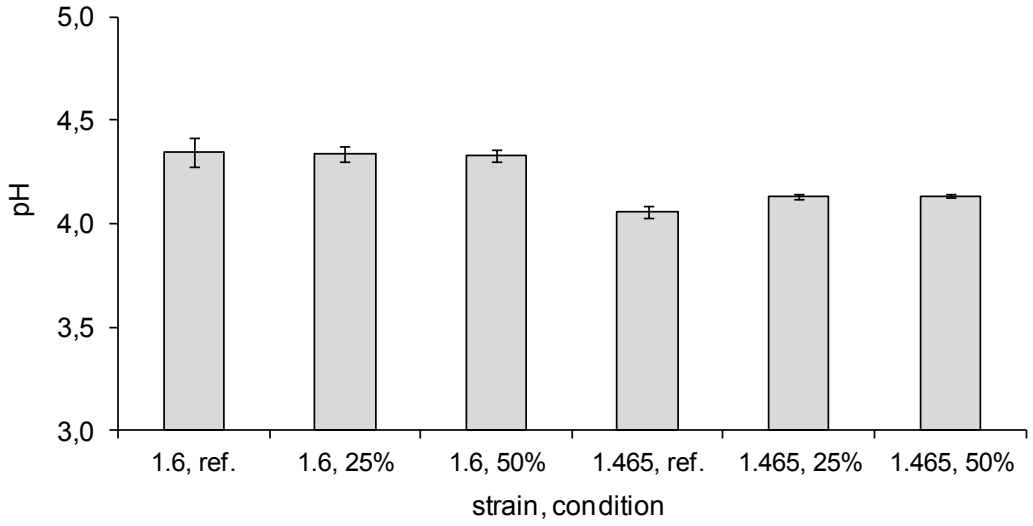


Fig. 24: Acidification of $mMRS_2$ by *L. brevis* TMW 1.6 and TMW 1.465 grown to OD_{590} = 0.3 - 0.6 in presence of 25 % or 50 % $MIC_{iso\text{-}\alpha\text{-}acids}$ and reference acid stress conditions, respectively. $MIC_{iso\text{-}\alpha\text{-}acids}$: *L. brevis* TMW 1.6 = 10 µM, *L. brevis* TMW 1.465 = 44 µM.

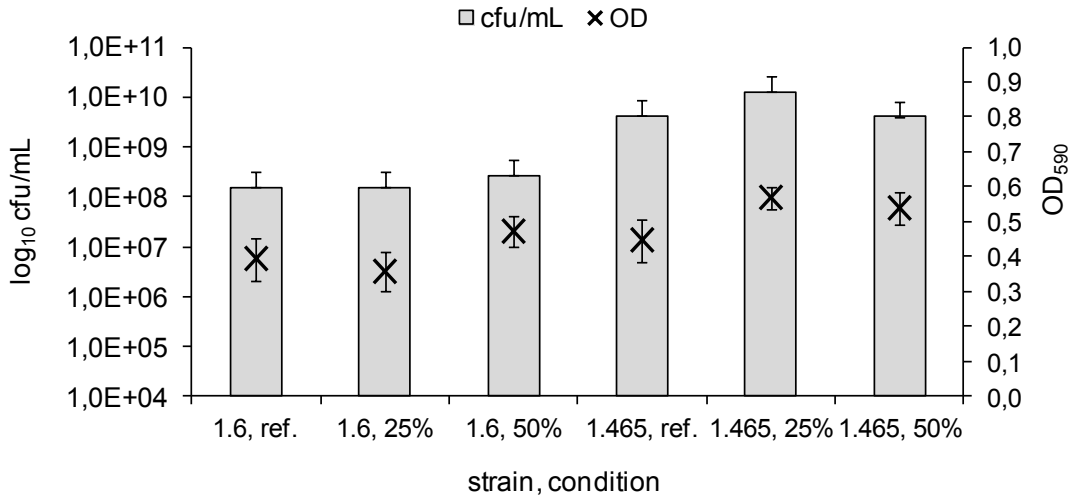


Fig. 25: Live cell numbers (bars) determined at given OD_{590} (X) of *L. brevis* TMW 1.6 and TMW 1.465 grown in $mMRS_2$ in presence of 25 % or 50 % $MIC_{iso\text{-}\alpha\text{-}acids}$ and reference acid stress conditions, respectively. $MIC_{iso\text{-}\alpha\text{-}acids}$: *L. brevis* TMW 1.6 = 10 µM, *L. brevis* TMW 1.465 = 44 µM.

3.3.2 **GAD-system associated amino acid metabolism**

GAD-system associated amino acid metabolism was examined by RP-HPLC analysis, monitoring glutamic acid (Glu) decarboxylation and γ-aminobutyric acid (GABA) yield. In accordance with 3.3.1, *L. brevis* TMW 1.6 and TMW 1.465 were grown in $mMRS_2$ and $mMRS_2$ containing 25 % or 50 % $MIC_{iso\text{-}\alpha\text{-}acids}$ to OD_{590} = 0.3 - 0.6 for the determination.

Subset results for GABA yield are depicted in Fig. 26. The analysis showed that the GABA production of hop-sensitive *L. brevis* TMW 1.6 was not affected by hops. The amount of detected GABA under reference or hop stress conditions did not vary significantly from the mean GABA yield of 4.45 mM. In contrast, hop-tolerant *L. brevis* TMW 1.465 GABA yield was greatly influenced by hops resulting in about the fourfold production (4.36 mM) as compared to control conditions (1.10 mM). Consequently, the GABA production of *L. brevis* TMW 1.465 was within the range of yield of *L. brevis* TMW 1.6 under hop stress. However, *L. brevis* TMW 1.465 produced significantly lower amounts of GABA in the absence of iso-α-acids.

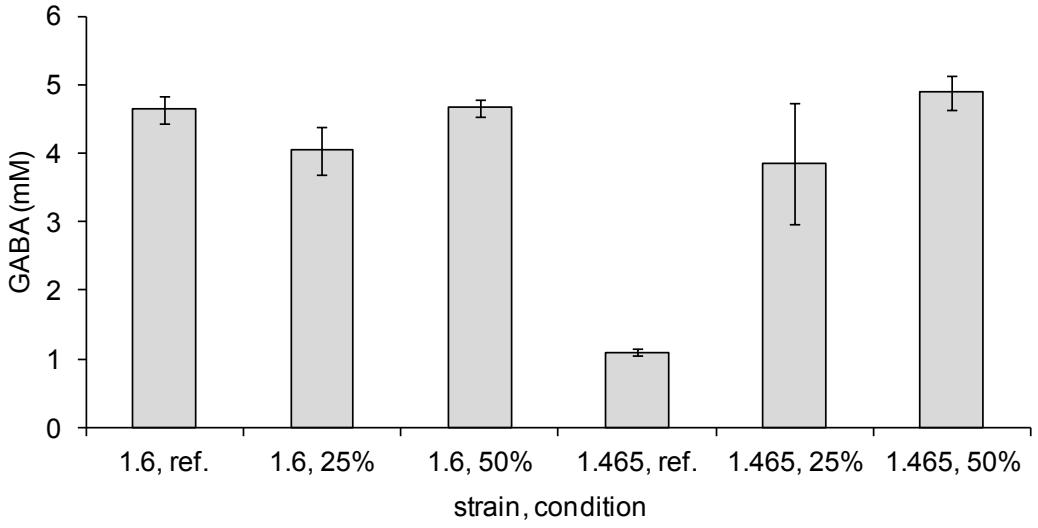


Fig. 26: GABA production of *L. brevis* TMW 1.6 and TMW 1.465 grown to OD_{590} = 0.3 - 0.6 in $mMRS_2$ under reference conditions and in presence of 25 % or 50 % $MIC_{iso\text{-}\alpha\text{-}acids}$. $MIC_{iso\text{-}\alpha\text{-}acids}$: *L. brevis* TMW 1.6 = 10 µM, *L. brevis* TMW 1.465 = 44 µM.

3.3.3 **Intracellular pH**

The influence of hop derivatives on pH_{in} maintenance of *L. brevis* was investigated with a Calcein-AM fluorescence-based assay. Therefore, a calibration was prepared, and (relative fluorescence units) RFU measured at excitation wavelength λ_{ex} = 495 nm and emission wavelength λ_{em} = 520 nm were converted to corresponding pH-values (data not shown; $R^2 > 0.982$).

Firstly, energy source dependent maintenance of pH_{in} of *L. brevis* TMW 1.6 and TMW 1.46 was examined. For this purpose, the course of pH_{in} after addition of 1 µM Val/Nig and 80 µM iso-α-acids, respectively, was monitored. Concomitant addition of 25 mM glucose (Glc) or 20 mM Glu served as potential energy source.

For hop-sensitive *L. brevis* TMW 1.6, Fig. 27 shows the almost immediate decrease of pH_{in} after addition of the ionophore antibiotics mix, also when combined with potential energy sources. After 60 min incubation, the final pH_{in} values were pH 4.3 (Val/Nig and Val/Nig + Glc) and pH 4.5 (Val/Nig and Val/Nig + Glu) hinting at a slightly improved maintenance of pH_{in} of *L. brevis* TMW 1.6 when supplied with Glu. The cells' pH_{in} decreased noticeably slower in the presence of hops or hops together with Glc and Glu,

respectively. Nonetheless, after 60 min, the pH_{in} dropped to equally low values as in the presence of Val/Nig and no difference could be determined between the runs with solely hops and those with concomitant addition of energy sources.

In the presence of Val/Nig, *L. brevis* TMW 1.465 exhibited pH_{in} courses similar to those described above for *L. brevis* TMW 1.6. After a rapid drop, the final pH_{in} values were reached almost immediately as pH_{in} and pH_{ex} were equilibrated Fig. 28. In contrast to *L. brevis* TMW 1.6, a rapid and short-term increase of pH_{in} could be determined, when iso-α-acids were added to *L. brevis* TMW 1.465. In consistence with the observations illustrated above, the pH_{in} of *L. brevis* TMW 1.465 decreased slower in the measurements for hops and pH equilibrium was reached after 60 min in the experiment with hops and Glc addition. In contrast, the measurements with hops and hops together with Glu, in particular, revealed that *L. brevis* TMW 1.465 succeeded in maintaining pH_{in} to a certain extent (pH 4.5 and pH 4.8).

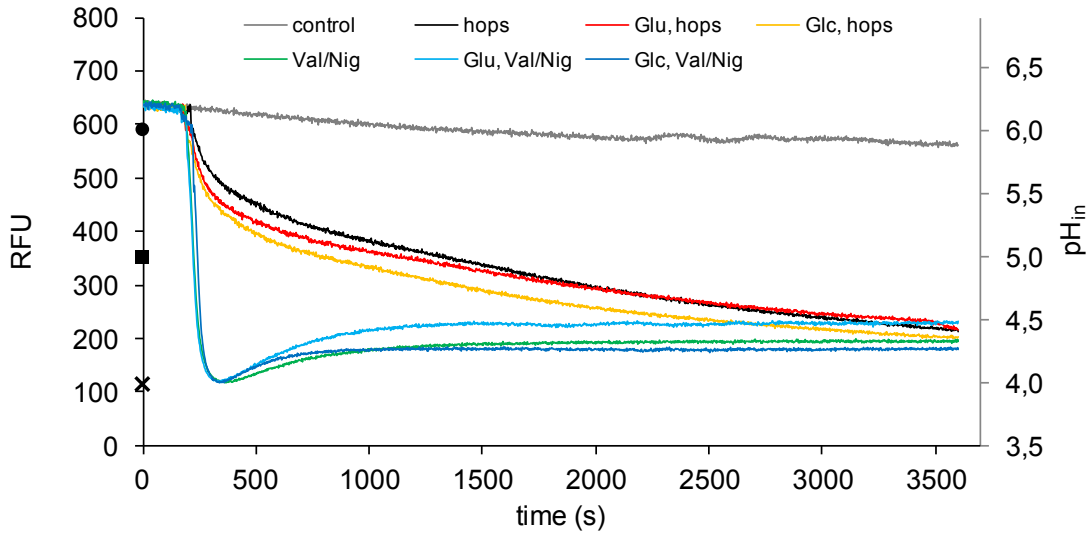


Fig. 27: *Coloured graphs*: Course of pH_{in} of energy depleted, resting cells of *L. brevis* TMW 1.6 (control) and after addition of 1 µM Val/Nig, 80 µM iso-α-acids (hops). Concomitant addition of 25 mM Glc or 20 mM Glu serving as energy sources. *Symbols*: RFU of calibration points at pH 4 (*cross*), pH 5 (*square*) and pH 6 (*circle*). λ_{ex} = 495 nm, λ_{em} = 520 nm.

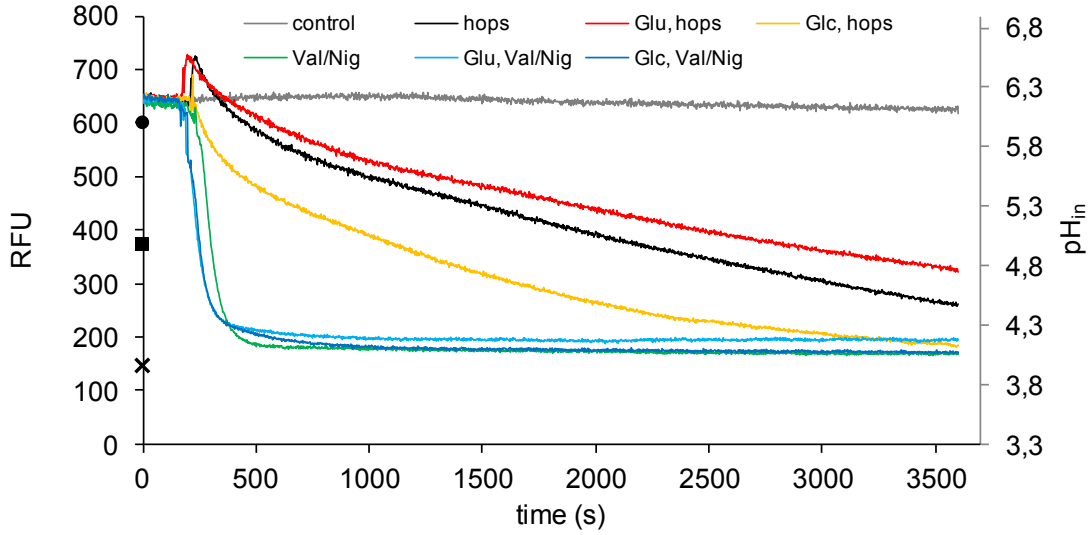


Fig. 28: *Coloured graphs*: Course of pH_{in} of energy depleted, resting cells of *L. brevis* TMW 1.465 (control) and after addition of 1 µM Val/Nig, 80 µM iso-α-acids (hops). Concomitant addition of 25 mM Glc or 20 mM Glu serving as energy sources. *Symbols*: RFU of calibration points at pH 4 (*cross*), pH 5 (*square*) and pH 6 (*circle*). λ_{ex} = 495 nm, λ_{em} = 520 nm.

Secondly, the influence of differently modified iso-α-acids on pH_{in} of *L. brevis* TMW 1.6 was assessed to investigate the ionophore activity of iso-α-acids and its derivatives on a physiological level to evaluate the role of different functional groups in the disruption of ΔpH. Therefore, three point calibrations were prepared for two independent measurements (linear fit, R^2 = 0.978 and 0.988). Exemplary measurements for a control experiment without a test substance, for iso-α-acids and for Val/Nig are presented in Fig. 29.

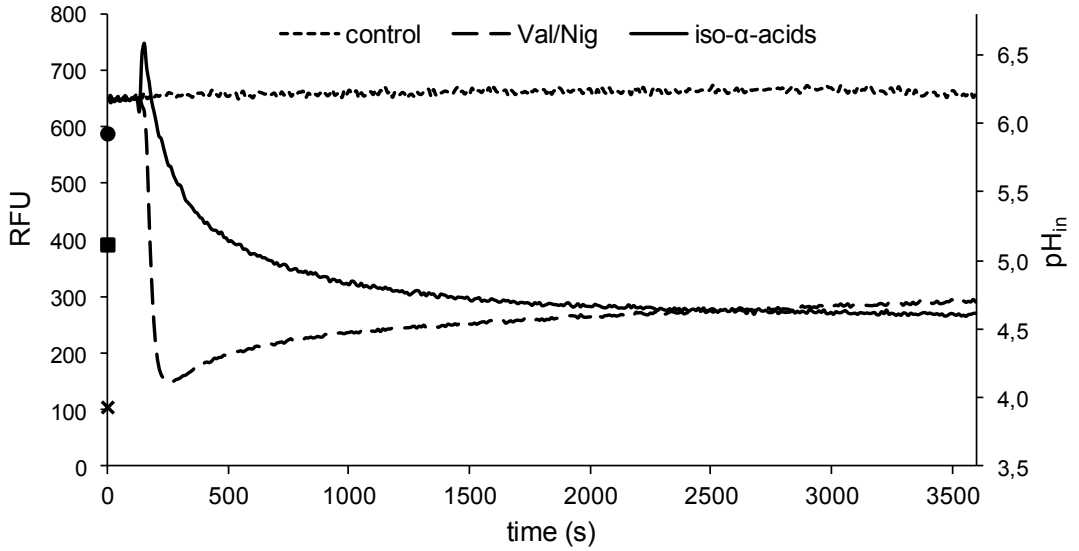


Fig. 29: Intracellular pH value (pH_{in}) of energy depleted, resting cells of *L. brevis* TMW 1.6 (control), and after addition of 1 µM valinomycin and nigericin (Val/Nig) or 80 µM iso-α-acids. Values are depicted as relative fluorescence units (RFU) and corresponding pH. Symbols: RFU of calibration points at pH 4 (cross), pH 5 (square) and pH 6 (circle).

The overall decrease of pH_{in} ($pH_{in,\ initial}$ - $pH_{in,\ final}$) is depicted as ΔpH_{in} for all test substances in Fig. 30. The application of the reference ionophore combination Val/Nig, as well as the iso-α-acids mixture and cis-iso-cohumulone alone led to a complete disruption of the transmembrane pH gradient. Methylation of iso-α-acids' acidic hydroxyl group in the C-3 position decreased the test substances influence on pH_{in}. Imination at the C-1 position, on the other hand, had no influence on the molecule's activity. The decrease of pH_{in} caused by humulinic acid was similar the methylation products Fig. 30.

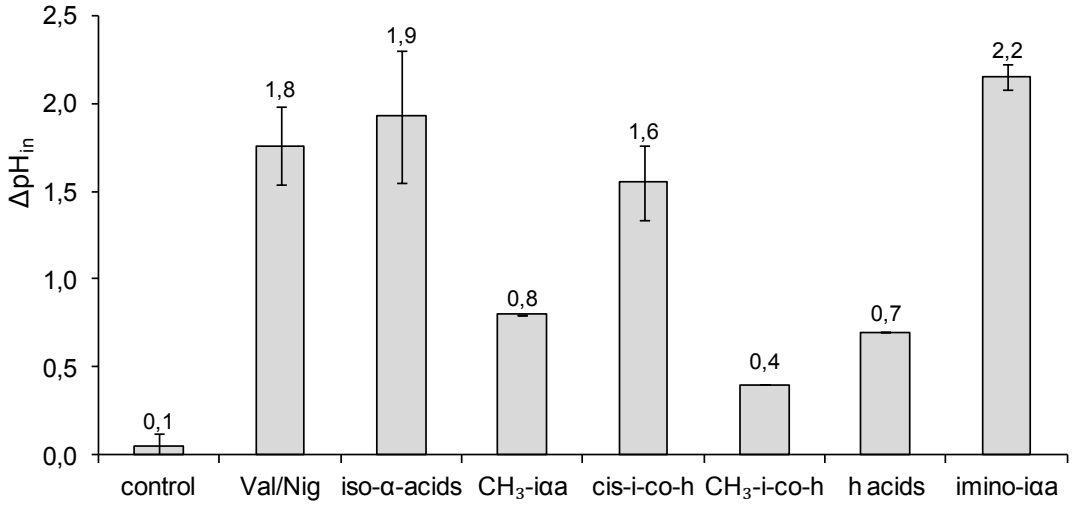


Fig. 30: Decrease of intracellular pH ($\Delta pH_{in} = pH_{in, initial} - pH_{in, final}$) of energy depleted, resting cells of *L. brevis* TMW 1.6 (control), and after incubation in presence of 1 µM valinomycin, nigericin (Val/Nig) or 80 µM iso-α-acids, methyl-iso-α-acids (CH_3-iαa), cis-iso-cohumulone (cis-i-co-h), methyl-cis-iso-cohumulone (CH_3-cis-i-co-h), humulinic acids (h acids) or imino-iso-α-acids (imino-iαa).

3.4 Transcriptional analysis of GAD-system associated genes

The regulation of GAD system-associated genes was investigated by means of qRT-PCR to elucidate the potential role of the acid stress alleviating GAD system in hop tolerance.

The elongation factor Tu (EF-Tu) was used in the LightCycler® PCR as housekeeping gene to normalise the relative gene expression of *gadC*, *gadB*$_1$, *gadB*$_2$, *gad-tr* and the investigated Na^+/H^+ antiporter. For each gene, the expression level under reference conditions was defined as 1.0. Fig. 31 depicts the results of the transcriptional analysis for *L. brevis* TMW 1.6. The analysis revealed that all investigated genes were expressed during growth in $mMRS_2$ under reference conditions. A general repression of transcription could be observed for the genes *gad-tr*, *gadB*$_1$, *gadB*$_2$ and *gadC* when the microorganism was exposed to hop stress (iso-α-acids). However, under 50 % MIC conditions, the transcription of *gad-tr* and *gadB*$_2$ appeared to be rather unaltered. Also, the expression of Lvis_2211 was found to be identical under all three conditions.

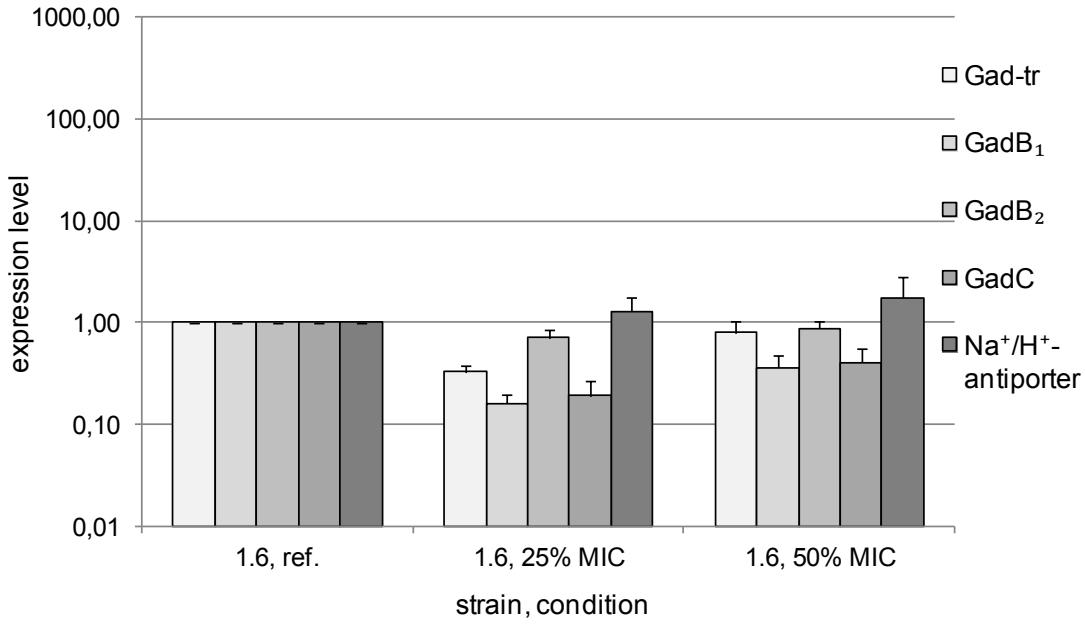


Fig. 31: Relative gene expression levels of *L. brevis* TMW 1.6 grown under reference conditions and hop stress conditions (25 % and 50 % $MIC_{iso\text{-}\alpha\text{-}acids}$) in $mMRS_2$. $MIC_{iso\text{-}\alpha\text{-}acids}$: *L. brevis* TMW 1.6 = 10 µM. The GAD system-associated genes *gad-tr*, *gadB*$_1$, *gadB*$_2$, *gadC* and Lvis_2211 (a Na^+/H^+-antiporter) were analysed by qRT-PCR using the *tuf* gene as housekeeping gene to normalise gene expression. Data represent mean and sd from two independent experiments.

In accordance with the findings described above, *gad-tr*, *gadB*$_2$ and *gadC* were expressed by *L. brevis* TMW 1.465 under reference conditions (cf. Fig. 32). On the contrary, the gene *gadB*$_1$ was found not to be transcribed. The level of the determined expression under reference conditions is therefore designated as not detectable, and the expression level under hop stress is depicted as 1,000 to clearly illustrate the induction of *gadB*$_1$ expression in the presence of iso-α-acids. Likewise, *gad-tr*, *gadC* and Lvis_2211 were overexpressed compared to reference conditions. *gadB*$_2$ expression, in turn, was repressed at the same time.

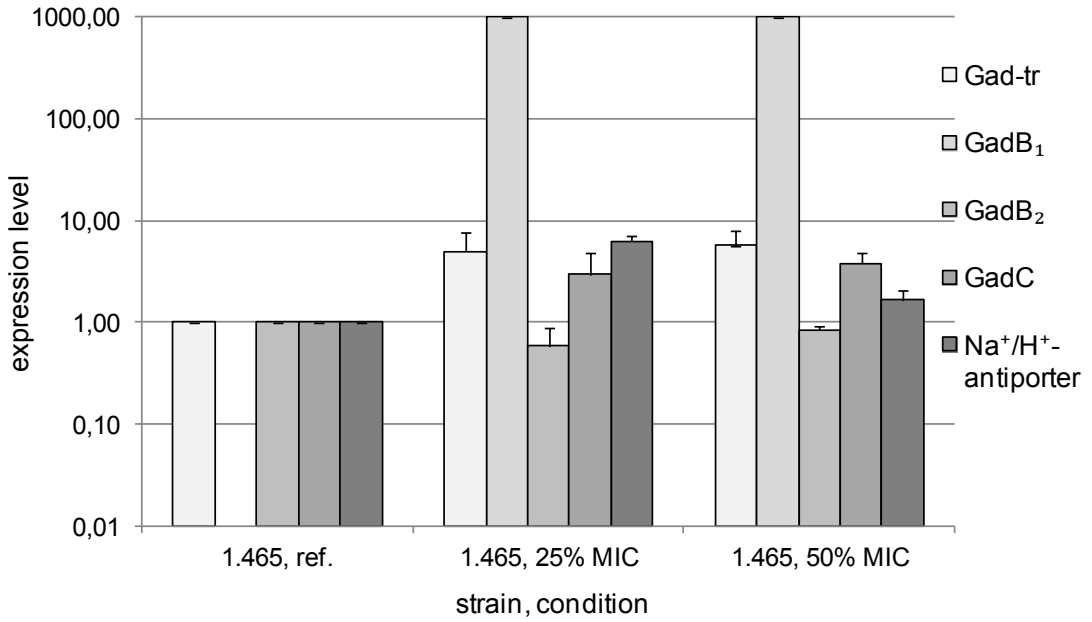


Fig. 32: Relative gene expression levels of *L. brevis* TMW 1.465 grown under reference conditions and hop stress conditions (25 % and 50 % $MIC_{iso\text{-}\alpha\text{-}acids}$) in $mMRS_2$. $MIC_{iso\text{-}\alpha\text{-}acids}$: *L. brevis* TMW 1.465 = 44 µM. The GAD system-associated genes *gad-tr*, *gadB*$_1$, *gadB*$_2$, *gadC* and Lvis_2211 (a Na^+/H^+-antiporter) were analysed by qRT-PCR using the *tuf* gene as housekeeping gene to normalise gene expression. Data represent mean and sd from two independent experiments. ND, not detected.

3.5 MALDI-TOF MS based proteomic approach for acid and hop shock induced responses

3.5.1 Evaluation of stress induced characteristic MALDI-TOF mass spectra

MALDI-TOF mass spectra were recorded for hop sensitive *L. brevis* TMW 1.6 and hop tolerant *L. brevis* TMW 1.465 under reference, acid stress and two different hop shock qualities mediated by iso-α-acids or xanthohumol. All samples taken immediately after the transfer into shock broths exhibited a characteristic peak at approximately m/z = 3146. Control experiments with a variety of different media (all possible combinations of pH-value, cysteine, manganese and magnesium concentration of either $mMRS_1$ or $mMRS_2$ were assessed) showed that this characteristic peak resulted from the difference of pH-values and the cysteine concentration (reducing conditions) present in the

two different broths. Manganese or magnesium levels did not have an influence.

In *L. brevis* TMW 1.6, the peak could not be detected under reference conditions or in presence of xanthohumol at any other assessed point of the 48 h incubation period. In presence of iso-α-acids, however, it could be found at any sampling point. In contrast, in *L. brevis* TMW 1.465, the peak could be detected under all conditions at any sampling point. Exemplary mass spectra obtained for *L. brevis* TMW 1.6 and *L. brevis* TMW 1.465 illustrating characteristic differences between the two strains are depicted in Fig. 33. In total, 2475 spectra including shock experiments, control experiments and culture modifications were included in the analysis.

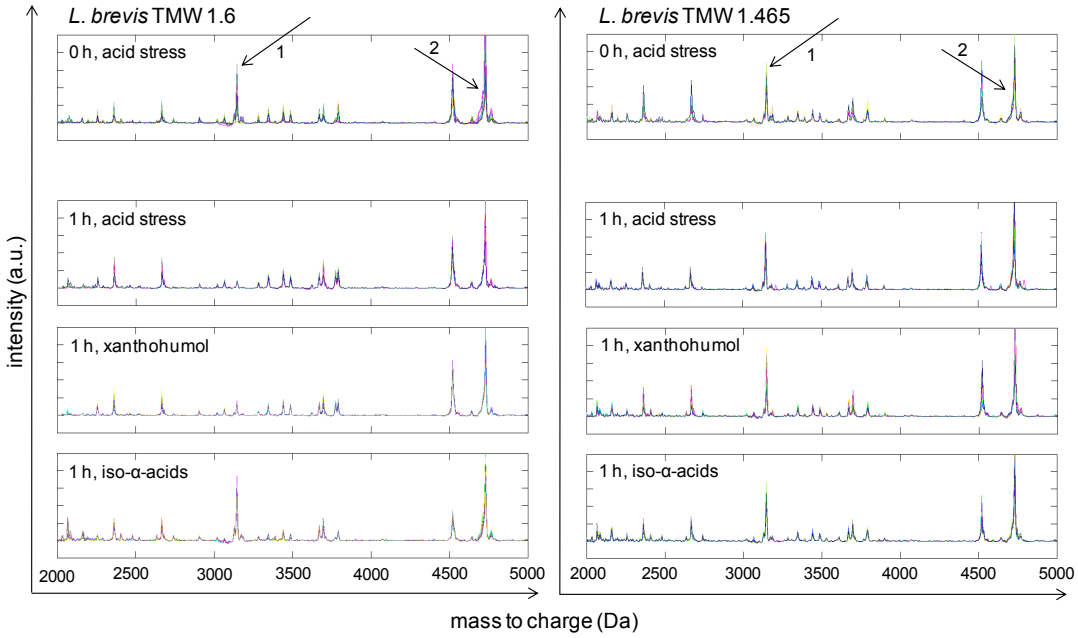


Fig. 33: Mass spectra obtained from *L. brevis* TMW 1.6 and *L. brevis* TMW 1.465 in the mass range from 2,500 Da to 5,000 Da. Respective stress conditions and sampling points are indicated. Acid stress: $mMRS_2$. Hop stress: 10 µM (TMW 1.6) and 44 µM (TMW 1.465) hop compound. Arrows 1 and 2 mark additional peaks at 3146 m/z and 4719 m/z as a shoulder of the 4729 m/z peak, respectively.

3.5.2 Protein identification via LC-ESI MS/MS and bioinformatic data processing

In order to identify the protein accounting for the approx. 3146 m/z peak detected via MALDI-TOF MS spectra via SDS PAGE combined with LC-ESI MS/MS analysis, the probable molecular weight of the protein accounting for the latter peak had to be predicted. In general MALDI-TOF-MS is assumed to mainly generate peptides containing only one charge. However, experiments with lysozyme spiked samples from *L. brevis* protein extracts exhibited intense additional peaks with m/z values of 14303, 7153 and 4769 (Kern et al. 2013). This indicates that under the conditions used for MALDI-TOF MS spectra acquisition, the generation of double, as well as triple charged peptides was possible. As the mass resolution of the mass spectrometer used, did not allow the charge determination directly from the mass differences of the monoisotopic peaks, the m/z peak lists were searched for with 3146 m/z peak concomitant occurring additional/higher intensity peaks in the mass spectra. A set of peaks was found with m/z values of 3146, 4719 and 9436, suggesting a probable charge of 3 for the 3146 m/z peak and a calculated molecular weight of approx. 9435 Da for the corresponding protein. The generated protein database containing all available protein sequences from *L. brevis* (NCBI; http://www.ncbi.nlm.nih.gov/) was converted to a mass list by a perl script using “get_mw_wt” function (Bio::Tools::SeqStats; http://search.cpan.org/). Possible protein candidates were identified and are displayed in Tab. 13 (all other possible charge states, as well as common posttranslational modifications were considered as well, but did not show any hit in the created mass list, data not shown)

Tab. 13: Possible protein candidates with molecular weight of 9435 Da +/- 600 ppm

Accession number	Function	MW (Da)
gi|545616974|ref|WP_021740885.1|	hypothetical protein	9436.5
gi|544229969|gb|ERK43181.1|	hypothetical protein HMPREF0495_01650	9436.5
gi|227191629|gb|EEI71696.1|	phosphocarrier, HPr family	9438.6

Accession number	Function	MW (Da)
gi|489647992|ref|WP_003552424.1|	phosphocarrier protein HPr	9438.6
gi|116098938|gb|ABJ64087.1|	acyl carrier protein	9439.2
gi|544228539|gb|ERK41830.1|	acyl carrier protein	9439.2
gi|472406752|ref|YP_007653857.1|	acyl carrier protein	9439.2
gi|116333591|ref|YP_795118.1|	acyl carrier protein	9439.2
gi|499986959|ref|WP_011667677.1|	acyl carrier protein	9439.2
gi|472233687|dbj|BAN06652.1|	acyl carrier protein	9439.2
gi|227190931|gb|EEI70998.1|	hypothetical protein HMPREF0496_1732	9439.3
gi|490601207|ref|WP_004466227.1|	hypothetical protein	9439.3

Subsequently, a protein extract of *L. brevis* TMW 1.465 was prepared (sampling point: immediately after transfer to $mMRS_2$). SDS-PAGE was used for protein size separation. The area of interest was excised from the gel and proteins were analysed via LC-ESI MS/MS. Bioinformatic evaluation of the obtained data as described above identified the acyl carrier protein as only overlap with the proteins listed in Tab. 13. This suggests the identification of the protein of interest as the 83 amino acid acyl carrier protein (ACP) with a molecular weight of 9,438.0 Da. The alignment of the ACP amino acid sequence and protein fragments generated from LC-ESI MS/MS raw data by de novo peptide sequencing using the PepNovo tool is depicted in Fig. 34.

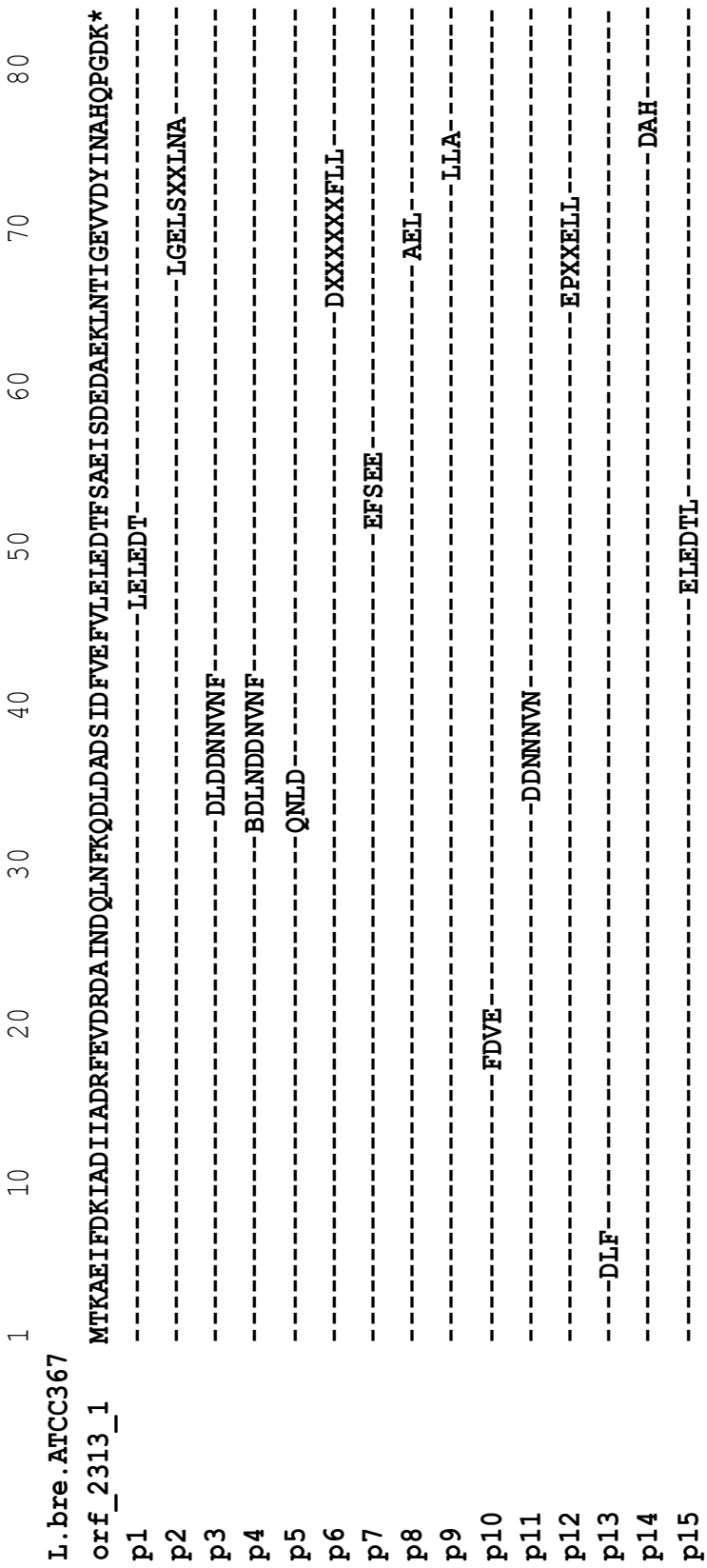


Fig. 34: Alignment of the acyl carrier protein (ACP) sequence of *L. brevis* ATCC 367 ACP (ref|YP_795118.1|) and protein fragments (p1 - p15) identified by LC-ESI MS/MS and de novo protein sequencing using PepNovo.

3.6 Electrochemical membrane characteristics

Membrane potential generation and membrane conductivity changes of egg PC/cholesterol BLMs were investigated to assess the impact of modifications of target functional groups and the C-4 side chain of iso-α-acids. All tested substances led to membrane potential generation in presence of a transmembrane pH gradient (ΔpH) of one pH unit. Exemplary measurements are depicted in Fig. 35. When a Mn^{2+}-gradient was set between the cis- and trans-chamber of the BLM measurement cell, the membrane potential formation was completely abolished, when imino-iso-α-acids were assessed. This finding indicates that the carbonyl group in the C-1 position is prerequisite for Mn^{2+} binding. Exemplary measurements are depicted in Fig. 36. Accordingly, membrane conductance experiments showed that imino-iso-α-acids were incapable of increasing membrane conductivity after addition of 100 µM $MnCl_2$ (Fig. 37). The results of all independently conducted experiments are summarised in Tab. 1.

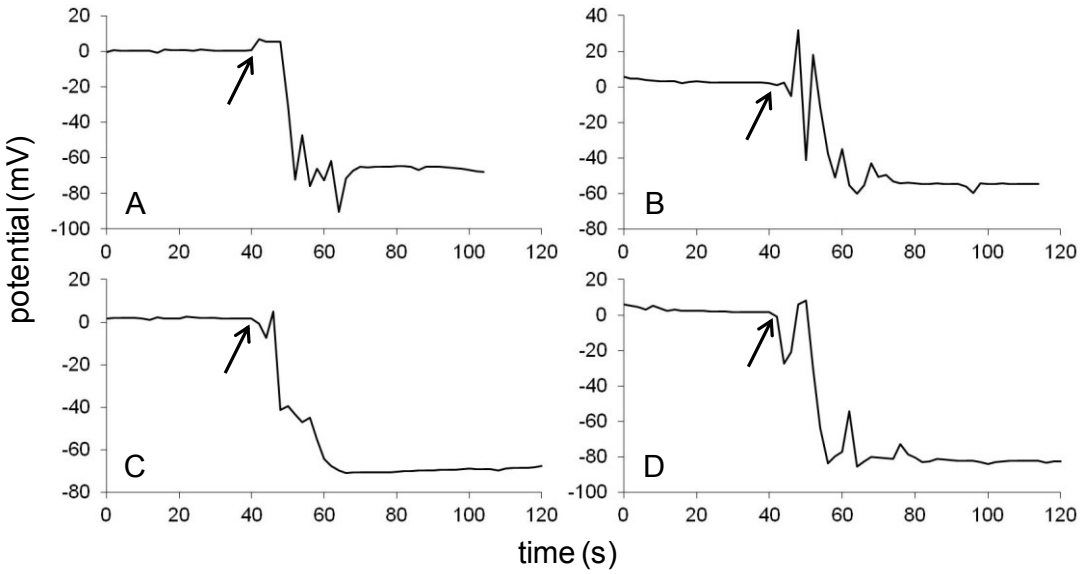


Fig. 35: Membrane potential of egg PC/cholesterol BLMs after generation of ΔpH = 1 in presence of: A, 10 µM iso-α-acids; B, 10 µM CH_3-iso-cohumulone; C, 10 µM humulinic acids; D, 10 µM imino-iso-α-acids. Arrows indicate ΔpH generation. Aqueous buffer solution: Tris, MES, citric acid (5 mM each), KCl (0.2 M), pH 5.

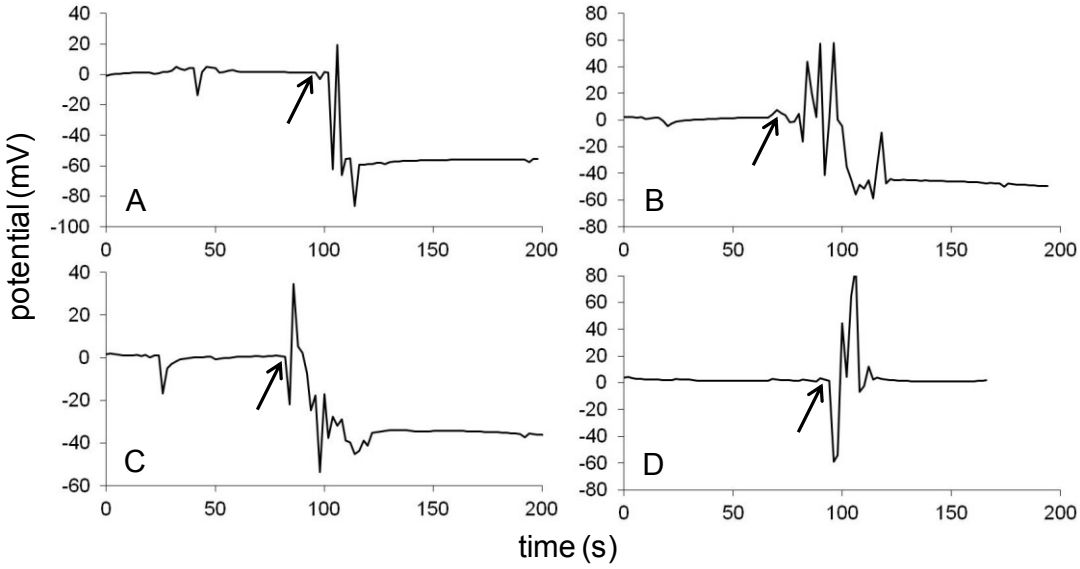


Fig. 36: Membrane potential of egg PC/cholesterol BLMs after generation of ΔMn^{2+} (50 µM / 500 µM $MnCl_2$) in presence of: A, 15 µM iso-α-acids; B, 15 µM CH_3-iso-cohumulone; C, 15 µM humulinic acids; D, 15 µM imino-iso-α-acids. Arrows indicate ΔMn^{2+} generation. Aqueous buffer solution: Tris, MES, citric acid (5 mM each), KCl (0.2 M), pH 5.

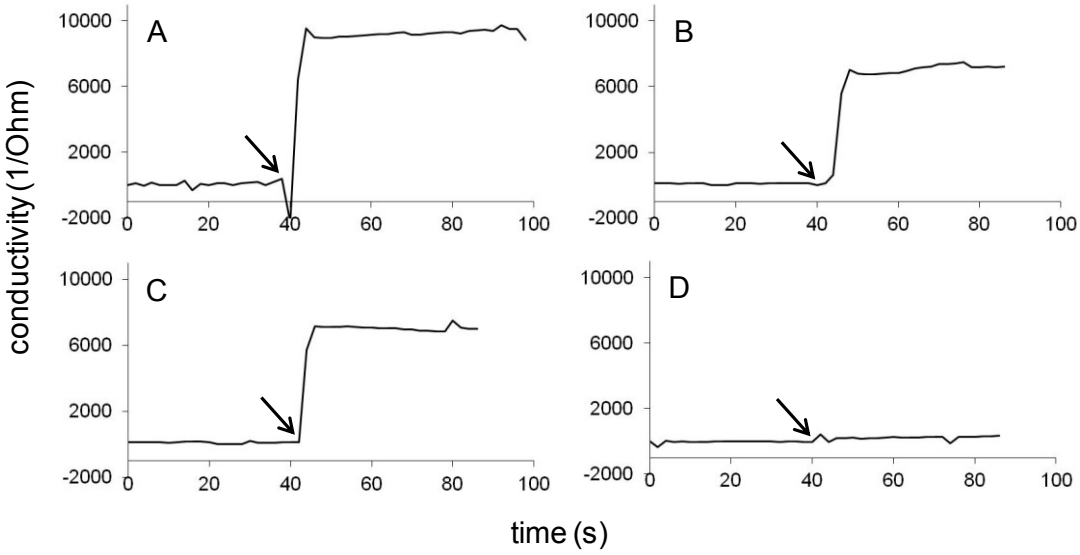


Fig. 37: Membrane conductivity of egg PC/cholesterol BLMs in presence of: A, 30 µM iso-α-acids; B, 100 µM CH_3-iso-cohumulone; C, 100 µM humulinic acids; D, 100 µM imino-iso-α-acids. A 50 mV potential was applied. Arrows indicate addition of 100 µM $MnCl_2$. Aqueous buffer solution: 50 mM potassium phosphate, 0.2 M KCl, pH 4.

Tab. 14: Influence of iso-α-acids and modified iso-α-acids on electrochemical properties of BLMs. Increased membrane conductivity and membrane potential generation indicated by (+). Unchanged electrochemical characteristic indicated by (-). ΔpH = 1, ΔMn^{2+} = 50 µM / 500 µM $MnCl_2$. Test substances: iso-α-acids; methyl-iso-α-acids (CH_3-iαa), cis-iso-cohumulone (cis-i-co-h), methyl-cis-iso-cohumulone (CH_3-i-co-h), humulinic acids and imino-iso-α-acids (imino-iαa).

Hop derivative	increased membrane conductivity	membrane potential generation	
	100 µM Mn^{2+}	ΔpH	ΔMn^{2+}
iso-α-acids	+	+	+
CH_3-iαa	+	+	+
cis-i-co-h	+	+	+
CH_3-i-co-h	+	+	+
humulinic acids	+	+	+
imino-iαa	-	+	-

4 Discussion

Over the last millennia, the alcoholic beverage nowadays known as beer has evolved from such different brews like ancient Egyptian *Nekhen-Hoffmann beer* (Maksoud et al. 1994; Hornsey 2003) to medieval *gruit beer* (Verzele & De Keukeleire 1991; Behre 1998; Hornsey 2003) to the variety of beers available today. This last big step of the evolution was probably mainly driven by the enactment of the German purity law in 1516 by the Bavarian duke Wilhelm IV, exclusively allowing the use of water, barley malt and hops for the production of beer (Verzele & De Keukeleire 1991; Behre 1998). The restriction of licit ingredients and the concomitantly increased use of hops did however not only coin the flavour and taste of beer, but serendipitously also improved its microbial stability since several antimicrobial hop components and hop derivatives are known to protect beer from spoilage (Simpson & Fernandez 1992; Garetz 1994; Sakamoto 2002; Sakamoto & Konings 2003; Haseleu et al. 2009; Intelmann & Hofmann 2010). Among the hop compounds and hop derivatives which can be found in beer, iso-α-acids play a prominent role as they are considered the main bittering component (De Keukeleire & Verzele 1971; Palamand & Aldanhoff 1973; Jaskula et al. 2008; Intelmann & Hofmann 2010; Intelmann et al. 2011) and highly antimicrobial active (Teuber & Schmalreck 1973; Simpson 1993a; Behr et al. 2006; García-Villalba et al. 2006; Schurr et al. 2013). In addition to hops, the composition of beer (pH value, redox state, nutrients, ethanol concentration etc.) represents another obstacle for microbial viability (Back 1994; Sakamoto & Konings 2003). However, a fairly small group of ingenious microbial specialists nonetheless is capable to grow in beer and cause beer-spoilage. *Lactobacillus* (*L.*) *brevis* is the microorganism responsible for most detected beer-spoilage incidents (Back 1994; Sakamoto & Konings 2003; Thelen et al. 2006; Suzuki 2011).

Hop tolerance of beer spoiling bacteria and the antimicrobial mode of action of hop compounds have been in the focus of scientists since the beginning of the last century (Shimwell 1937a). Although since that time many researchers have worked on the topics, still neither remained fully elucidated.

This work has been designed to achieve a better understanding of the molecular mechanism of hop inhibition in *L. brevis* by a dissective approach spotlighting microbial viability, physiology, metabolism and electrochemical membrane characteristics.

This work demonstrates that hop inhibition is determined by various factors and that there are distinct levels of hop tolerance towards different antimicrobial effects for different *L. brevis* strains. It is shown for different substances that the mechanism behind hop inhibition can clearly be attributed to molecular functions and the antimicrobial mode of action is elucidated. Other substances are demonstrated to act as synergistic partners in mixtures of hop components. The overall inhibitory effect of hops can therefore not be attributed to individual effects of hop constituents. Also, hop tolerance is multifaceted and varies for different strains as their susceptibility towards different inhibitory effects also differs.

The obtained results are discussed in the following.

4.1 Antimicrobial activity of hop compounds

A screening of a broad spectrum of hop compounds was conducted to obtain an overview over general principals influencing the antimicrobial activity of hop compounds. Therefore, MICs of crude hop extracts, hop fractions obtained by an activity orientated extraction procedure (performed and supplied by C. Vogt, Lehrstuhl für Lebensmittelchemie und Molekulare Sensorik, TUM, cf. 2.1.3), commercial hop products and pure hop constituents were determined for four *L. brevis* strains (TMW 1.6, TMW 1.1369, TMW 1.465, TMW 1.313). The microorganisms were precultured under optimal conditions ($mMRS_1$). For the assessment of the hop compounds' antimicrobial potential, a growth broth was used which exhibited beer-like characteristics ($mMRS_2$) regarding acidity (pH 4.3), reducing components (cysteine-free) and divalent cations concentration (Mn^{2+} and Mg^{2+} concentration according to levels found in pilsner lager beer).

The antimicrobial activity of crude hop extracts from HALLERTAUER MAGNUM green hop cones was investigated using hexane, THF or EtOH as extracting

agents. The inhibitory potential tentatively showed a decrease with increasing polarity of the extractant (polarities: hexane, 0.0; THF, 0.45; EtOH, 0.88 (Küster & Thiel 2002)). As the hard resin fraction (hr), by definition, is hexane insoluble (Pfenninger 1997), a crude hop extract containing only the soft resin fraction (sr) (and potentially other hexane soluble substances extracted from the hop cone bracts and bracteoles) by trend showed the highest antimicrobial effect. However, a total resin extract (tr) prepared according to MEBAK guidelines (cf. 2.1.3) using the same raw material, also showed similar MICs. Hence, it can be concluded that the extracting agent used did not have significant influence on the crude and total extracts' inhibitory effect.

HALLERTAUER MAGNUM tr and sr extracts prepared from different raw materials also showed consistent MICs, except for the tr obtained from pelleted hops (pellets type 90, pl), which was less effective against the two more hop tolerant strains *L brevis* TMW 1.465 and TMW 1.313. Since all supplied raw materials (cf. 2.1.3, Non-commercial hop compounds) were handled equally, regarding the influence of light, temperature or oxygen, it can only be assumed that the absolute composition of the tr and sr prepared from pellets varied from the other raw materials' and consequently they were less effective against *L. brevis* TMW 1.465 and TMW 1.313. Considering the nowadays common cold storage of harvested hops (1 - 5 °C), packaging under inert gas and the use of diffusion-resistant composite material packs (Biendl et al. 2012), the underlying reason for differences between e.g. ethanol extracts and hop pellets remains unclear. Comparing green hops and hop pellets though, it must be kept in mind that the antimicrobially active α-acids are degraded three times as fast in pelleted hops, as in green hops, since the protecting lupulin glands are damaged during the pelletising process (Biendl et al. 2012).

A comparison of tr, sr and hr extracts showed that the tr and sr fraction bear similar inhibitory potential, whereas the hr fraction showed lower or no activity, depending on the HALLERTAUER MAGNUM raw material. A subfractionation of the hr obtained from the EtOH-extract raw material revealed that the resulting ε-resin (EtOH soluble) and δ-resin (water soluble) were equally inhibitory as the hr fraction. Further subfractionation of the

ε-resin yielded 11 subfractions (F01 - 11) with varying MICs, the most inhibitory ones being F06 - F09. Since F01-11 were obtained by preparative RP-HPLC, it can be deduced from the expectable elution order, that F06 - 09 represent amphiphilic or rather non-polar fractions of a fairly limited range since all ε-resin subfractions are ethanol soluble. This clearly points towards a dependence of membrane solubility and antimicrobial activity of these ε-resin subfractions. The effect of hop compounds on cell membranes could be demonstrated in several scientific papers before (Teuber & Schmalreck 1973; Behr & Vogel 2009; Behr & Vogel 2010). Further subfractionation of the ε-resin yielded several pure substances, such as xanthohumol, isoxanthohumol, 6-prenylnaringenin or 8-prenylnaringenin. These constituents, however, did not cause inhibition of any of the investigated microorganisms.

A cluster analysis using the open source statistical R package ape was conducted to achieve an enhanced understanding of the causal factors for differences and similarities between the investigated hop compounds.

A clustering approach focusing on different extractants, raw materials and hop varieties showed, that the biggest differences of MICs were found between the two groups tr/sr and hr, resulting from the content of humulones and lupulones in the sr fraction. Consequently, this finding also showed that the choice of bitter hops or aroma hop varieties did not contribute drastically to the inhibitory potential of the extracts. This conclusion might seem surprising at first sight as the α-acid content of hops plays a major role in the hop industry. It is widely agreed that a classification of hops as aroma and bitter hop varieties along their constituents is not feasible. However, hop varieties with mild and pleasant aroma and increased polyphenol content and α-acids contents below 10 % are generally referred to as aroma hops (Nance & Setzer 2011; Biendl et al. 2012). As the α-acids content of the four hop varieties used in this work is considerably different (PERLE (7.4 %), HALLERTAUER TRADITION (6.2 %), HALLERTAUER MAGNUM (13.9 %), HALLERTAUER TAURUS (15.9 %); determined according to EBC method 7.4 (Pfenninger 1996; Biendl et al. 2012)), it can be assumed that there could not be found striking differences between the varieties due the low solubility of

α-acids (Spetsig 1955; Stevens 1966; Jaskula-Goiris et al. 2010). The solubility and antimicrobial effect of α-acids is increased significantly upon isomerisation during the wort boiling process (Simpson & Smith 1992; García-Villalba et al. 2006).

A second clustering approach focusing on different degrees of complexities of the hop compounds showed that the workup level of the test substance alone did not correlate with the antimicrobial potential as e.g. pure substances could be found to be either comparably effective (co-lupulone) or completely ineffective (xanthohumol). Further, subfractions could be shown to be both, more (M-et-er-F06) or less (M-et-hr) antimicrobially active as their respective superordinate fraction. Taken together, these findings indicate that some hop compounds are solely antimicrobially active in presence of synergistic partners, whereas other compounds can exert full antimicrobial activity also in absence of other hop constituents.

The prenylated chalcone xanthohumol, a substance which besides hops so far has solely been found in a Chinese medicinal plant (*Sophora flavescens*), has received increasing attention in recent years because more and more biological effects of xanthohumol have been discovered (Biendl et al. 2012). Therefore, a closer look at substance's properties will be taken in the following passage.

In addition to its antioxidative capacity (Miranda et al. 2000; Yamaguchi et al. 2009), anti-inflammatory properties (Cho et al. 2008; Lee et al. 2011), anti-atherosclerotic effects (Hirata et al. 2012), anticancerous and chemopreventive potential (Gerhäuser et al. 2002; Stevens & Page 2004; Pan et al. 2005; Deeb et al. 2010) and other biological effects, xanthohumol has been described to be antimicrobial active against a variety of microorganisms. Antimicrobial activity has been shown for bacteria (Mizobuchi & Sato 1984; Bhattacharya et al. 2003), fungi (Mizobuchi & Sato 1984), viruses (Buckwold et al. 2004; Wang et al. 2004) and different strains of the protozoan malaria parasite *Plasmodium falciparum* (Herath et al. 2003).

For this study, xanthohumol was purified from one of the most antimicrobially active ε-resin subfractions. Considering the above-mentioned biologic effects

and the fact that this ε-resin subfraction consisted mainly of xanthohumol, it was rather unexpected that xanthohumol alone did not have any antimicrobial effect on the *L. brevis* test stains. It can hence be concluded that xanthohumol can only exert antimicrobial activity against *L. brevis* together with (a) synergistic partner(s). The application of antibiotic combinations is widely used in medicine and various synergistic effects of combinations of two or more antibiotics have been under investigation since the early 1950s (Acar 2000). The mechanism behind the synergisms of the ε-resin subfraction's single constituents could not be investigated due to lack of test material from further subfractionation of the compound. However, NATARAJAN et al. were able to show a synergistic effect of xanthohumol or lupulone and a variety of antibiotics (with different target sites) against a wide range microbes (both Gram-negative and Gram-positive) in vitro and hypothesised that the hop compounds facilitated the antibiotics' penetration of the cell membranes (Natarajan et al. 2008). A similar effect was first demonstrated for streptomycin and penicillin G in *Enterococcus faecalis* (Moellering & Weinberg 1971) and has since been shown for numerous other substances in several other species (Acar 2000).

The discussed investigation of the inhibitory effect of a broad spectrum of hop compounds and hop derivatives consequently revealed that the inhibitory potential of a compound cannot be deduced and hence must be assessed separately for every new identified pure substance or for combinations of different hop compounds.

As iso-α-acids proved to be amongst the most active tested substances, the compound was used for most subsequent investigations. Additionally, iso-α-acids also play a crucial role in the hop industry due to their sensory characteristics and they do not have to be prepared in a tedious fractionation process, as they are commercially available.

4.2 Detection and evaluation of acid and hop shock-induced responses in beer spoiling *L. brevis* by MALDI-TOF MS

Within the fairly limited group of beer-spoiling bacteria, *L. brevis* plays a role of outstanding importance because it is responsible for most beer-spoilage incidents (Back 1994; Sakamoto & Konings 2003; Thelen et al. 2006). It is therefore axiomatic that this microorganism must be able to adapt to or be prepared for the detrimental conditions present in beer, including the presence of hop derived compounds, which have been shown to be antimicrobial active by a multitude of scientific studies (Shimwell 1937a; Mizobuchi & Sato 1984; Mizobuchi & Sato 1985; Simpson & Fernandez 1992; Simpson & Smith 1992; Fernandez & Simpson 1993; Simpson 1993b; Simpson 1993a; Back 1994; Sakamoto & Konings 2003; Schurr et al. 2013). Accordingly, in order to survive and grow in beer, a set of protective mechanisms of short term stress responses and middle- to long-term adaptation processes is an indispensable requisite of *L. brevis* and may be regarded a beer-spoilage potential delineating factor.

In a previous study of this working group, proteomic approaches using 2D gel electrophoresis were taken to assess hop stress responses, adaptation processes and differences between various *L. brevis* strains (Behr et al. 2007; Preissler 2011). However, immanent limitations of 2D gel electrophoresis did not allow to investigate proteins below 10 kDa molecular weight.

Furthermore, 2D gel electrophoresis can be a time consuming, laborious and therefore tedious process. Accordingly, a comparison of several different stress conditions with multiple sampling points throughout a prolonged incubation period appears to be a mammoth task. Additionally, the control experiment, which was set up for this work to mimic the transition to shock broths in all possible combinations of the differing growth medium ingredients would be another multiplier for the amount of necessary 2D gels.

Mass spectrometry has recently become the gold standard for biomarker detection (Mantini et al. 2010). Due to the high sensitivity (low fM to aM levels) (Caprioli et al. 1997) and short throughput times, tools like

MALDI-TOF MS are very well suited to discover differences between protein patterns of several sample collections. Accordingly, these analytical technologies offer supreme properties for the examination of certain biological circumstances or stress responses (Mantini et al. 2010).

Therefore, the use of MALDI-TOF MS analysis as a tool for stress reaction analysis was chosen in the present work, to compare different stress qualities in a long term screening and to facilitate the assessment of low molecular weight proteins ranging from 2 kDa to 16 kDa for the first time.

For this purpose, a hop shocks experiment was designed, to examine microbial responses to hop derived xanthohumol and iso-α-acids on a proteomic level. An acidic medium ($mMRS_2$) was used as a reference condition, enabling the discrimination of acid and hop induced stress responses. Two *L. brevis* strains exhibiting different levels of hop sensitivity were used (hop sensitive *L. brevis* TMW 1.6, hop tolerant *L. brevis* TMW 1.465).

Under reference and hop stress conditions, a characteristic peak could be found in the MALDI-TOF mass spectra at 3146 m/z immediately after the cells were suspended in the stress broths. At any later sampling point of the 48 h incubation time, *L. brevis* TMW 1.6 formed this peak only in presence of iso-α-acids, whereas for *L. brevis* TMW 1.465, the peak could be detected in all stress samples. Sampling points prior to the transfer to shock broths, control experiments with different media and samples from the reference acid stress treatment furthermore rule out the sheer dependence of peak formation on bacterial growth phases, one might assume. Consequently, peak formation could clearly be attributed to stress reactions.

The protein accounting for the detected peak could be identified with high probability as the 83 amino acid *L. brevis* acyl carrier protein (ACP) via LC-ESI MS/MS and subsequent bioinformatic data processing.

The universal and highly conserved ACP forms part of the multi enzyme complex fatty acid synthase (FAS) and functions as donor for acyl intermediates in the FA biosynthesis (Byers & Gong 2007). In bacterial type II FAS, in which the enzymatic subunits are dissociated (Campbell & Cronan

2001; Heath et al. 2002), ACP acts like a shuttle for the acyl intermediates between the subunits during the FA elongation process. The acyl intermediates are covalently bound to the ACP's 4'-phosphopantetheine prosthetic group with a thioester linkage (Sabaitis & Powell 1976; Heath et al. 2002; Berg et al. 2003). FA degradation is independent of ACP, as β-oxidation uses CoA-bound acyl moieties instead of acyl-ACP (Heath et al. 2002; Berg et al. 2003).

Thus, the ACP is inextricably tied to FA and glycerol phospholipid biosynthesis and consequently to cell membrane de novo synthesis and structural cell membrane alterations, too.

As a reaction to acidic conditions, bacterial membranes contain FA of increased chain length and higher degree of saturation, leading to reduced membrane fluidity (Quivey et al. 2000; Fozo & Quivey 2004; Behr et al. 2006). Unsaturated, short and branched chain FAs (low melting point FAs) are considered to increase membrane fluidity and permeability. In contrast, saturated, long, cyclic and straight FAs (high melting point FAs) render membranes more rigid and impermeable (Beales 2004). Similarly, changes in the membrane composition of *L. brevis* TMW 1.465 were demonstrated by BEHR et al. for acid stress and iso-α-acids induced stress. Membrane fluidity was reduced, when cells were grown at pH 4 instead of pH 6 and hop stress led to an even more pronounced reduction of membrane fluidity (Behr et al. 2006). Accordingly, microorganisms make use of lowered membrane permeability as a tool to prevent penetration of undesirable substances like hop compounds.

The pattern of detected ACP under acidity, xanthohumol and iso-α-acids induced stress, respectively, can consequently be regarded an indicator for ongoing membrane changes in the two *L. brevis* strains. In the presence of iso-α-acids, ACP could be found in all samples, indicating an enduring reaction to the inhibitory substance. In contrast, xanthohumol led to the same result as the reference acid treatment. Since xanthohumol was found incapable to inhibit growth of the test strains, this finding seemed little surprising. However, for the hop tolerant strain *L. brevis* TMW 1.465, ACP could as well be detected under these conditions throughout the entire

incubation time, hinting to a more pronounced stress reaction or adaptation process, which in consequence enables long-term survival. It may hence be deduced that tolerance mechanisms in *L. brevis* TMW 1.465 are provoked by comparatively weaker stressors, which could be one of the factors determining its (lower) susceptibility to iso-α-acids. These findings coincide with examinations of PREISSLER, showing that cell membranes of *L. brevis* strains with higher beer-spoilage potential were of a FA composition which accounts for lower fluidity (Preissler 2011).

Beyond that, the studies showed that MALDI-TOF MS can be used as an excellent tool for the characterisation of hop stress responses of beer spoiling *L. brevis*.

4.3 Role of the GAD system in hop tolerance of *L. brevis*

In the following section, the contribution of the microbial acid stress tolerance mechanism glutamic acid decarboxylase (GAD) system to hop tolerance and concomitant maintenance of intracellular pH (pH_{in}) in *L. brevis* is discussed.

In *L. brevis*, the GAD system comprises a transcriptional regulator (Gad-tr), a glutamate/γ-aminobutyrate antiporter (GadC) and two glutamate decarboxylases ($GadB_1$, $GadB_2$) (Li et al. 2013). Hop iso-α-acids act as ionophores, which impair cells' proton motive force (Behr & Vogel 2009). Hop-tolerant bacteria must therefore be able to express effective mechanisms of pH maintenance such as the GAD system. To elucidate the specific roles of the two Gad isoenzymes, the influence of iso-α-acids on the GAD system was investigated on a metabolic and transcriptional level for two *L. brevis* strains. Highly hop-tolerant *L. brevis* TMW 1.465 and hop-sensitive *L. brevis* TMW 1.6 were selected for the experiments. Microorganisms were cultured in $mMRS_2$ at pH 4.3 under reference acid stress conditions and together with iso-α-acids at concentrations of 25 % and 50 % of the respective microorganism's MIC. Several metabolic characteristics and the expression of GAD system-associated genes on a transcriptional level were investigated.

4.3.1 **Metabolic characteristics**

Heterofermentative LAB like *L. brevis* are incapable of glycolysis due to the lack of aldolase and triosephosphate isomerase (Krämer 2002; Berg et al. 2003). Hence, LAB use the pentose phosphate pathway (PPP) to funnel hexoses like glucose (Glc) and fructose (Frc) into the phosphoketolase pathway (PKP) to generate ATP (DeMoss et al. 1951; Kandler 1983; Krämer 2002; Berg et al. 2003). The pathway generates 1 and 2/3 mol ATP from 1 mol Glc or Frc, respectively, and 2 mol NADH + H^+. The formation of ethanol from acetyl phosphate (acetyl-P) allows the recovery of NAD^+ and thus a balanced redox state (Kandler 1983; Krämer 2002). The availability of an additional electron acceptor (e.g. Frc) renders the generation of an additional mol ATP possible as Frc is reduced to mannitol (mannitol dehydrogenase) and acetic acid is concomitantly formed from acetyl-P (acetate kinase). Consequently, the end products of the metabolic pathway are CO_2 and lactic acid at equimolar amounts and ethanol and acetic acid depending on the oxidation-reduction potential of the fermentation (Kandler 1983; Krämer 2002; Rodríguez et al. 2012). Pentoses entering the PKP at the xylulose-5-phosphate level are metabolised without production of CO_2 (Kandler 1983; Berg et al. 2003). This type of carbohydrate catabolism and the associated production of end products lead to inevitable acidification of the bacterial environment. Even though lactobacilli are considered intrinsically resistant to acid stress within limitations, it was found that pH values below strain-specific thresholds unavoidably lead to a disrupted gradient between the pH of extracellular and intracellular space and consequently cause cell death (Kashket 1987; Charalampopoulos et al. 2003). It can therefore be assumed that ionophoric hop components such as iso-α-acids render the living conditions of LAB in acidic environments even more difficult. The analysis of metabolic characteristics showed that final broth acidification and survival of *L. brevis* TMW 1.6 and TMW 1.465 was not influenced by addition of iso-α-acids, when cells were grown to similar optical density (OD_{590} = 0.3-0.6). Yet, hop dosage caused severalfold prolonged lag phases, representing extensive microbial adaptation processes connected to hop stress (data not shown). In comparison, the higher beer-spoilage potential of *L. brevis* TMW 1.465 was reflected by higher survival rates and

the lowered pH of the growth medium. Although the GAD system was found in a relatively limited number of bacteria, it is widely spread amongst lactobacilli and plays a major role in acid resistance of various *Lactobacillus* species, amongst which *L. brevis* was found to be one of the most potent GABA producers (Cotter et al. 2001; Li & Cao 2010). It could be shown that GABA production of *L. brevis* TMW 1.6 was independent of the hop concentration. *L. brevis* TMW 1.465 reached equally high levels of GABA in the presence of iso-α-acids but only produced 25 % of the amount under reference acid stress conditions. These findings are in accordance with studies concluding that GAD system activity and GABA production are strain-specific characteristics and beyond that underlie variation depending on environmental conditions (Li & Cao 2010; Li et al. 2010). It could hence be demonstrated that the strain bearing higher hop tolerance exhibited higher survival rates and was able to increase GABA production by the factor four in presence of additional hop stress. Consequently, a resting cells assay to determine the strains' pH_{in} under various conditions was designed. The investigations showed that *L. brevis* TMW 1.6 could not maintain a transmembrane pH gradient independent of the presence of Glc and Glu as energy source, since no difference between pH_{in} and pH_{ex} could be detected, when iso-α-acids were present. *L. brevis* TMW 1.465 on the other hand exhibited complete disruption of the gradient only in the presence of Glc, possibly as a consequence from acidic components (lactic acid) formed via PKP metabolism as hops without an energy source lead to a more moderate decreased of pH_{in}. In contrast to GABA, lactic acid can lead to acidification of the cytoplasm as it can passively enter the cell and dissociate into lactate and protons at equimolar amounts (van de Guchte et al. 2002; Krämer 2002). The application of the ionophoric antibiotics valinomycin and nigericin was used as control experiment to induce disruption of the transmembrane pH gradient. In comparison with iso-α-acids, the antibiotics exhibited a higher activity and rapidly equilibrated pH_{ex} and pH_{in}. It must be taken into account, however, that although the iso-α-acids' concentration (80 µM) was higher than the concentration of valinomycin and nigericin (1 µM each), only the smallest part of the added iso-α-acids can act as ionophores under the given conditions. SIMPSON and SMITH showed that iso-α-acids can act as

ionophores only in their undissociated form as they cannot permeate the cell membrane otherwise (Simpson & Smith 1992). At pH 4.3, only 5.9 % of the added iso-α-acids are present in the undissociated form (calculated for trans-iso-n-humulone (pK_a 3.1), according to ALBERT (Albert 1985)). On the contrary, the efficiency of nigericin increases exponentially under acidic conditions due to the formation of oligomers (Toro et al. 1987). In the experiments with hop-tolerant *L. brevis* TMW 1.465, a rapid and short-term increase of pH_{in} could be observed directly after the addition of iso-α-acids. This increase could not be detected when valinomycin and nigericin (with or without energy source) were added. Further, it did not appear during the measurements for hop-sensitive *L. brevis* TMW 1.6. Therefore, it was concluded that this phenomenon seems to reflect a hop-mediated reaction which specifically occurred in hop-tolerant *L. brevis* TMW 1.465. It can hence be speculated that membrane ion gradient-dependent H^+-transport could cause this increase of pH_{in} in *L. brevis* TMW 1.465 as the effect of iso-α-acids on the membrane potential is weaker than the effect on the trans-membrane proton gradient (Simpson 1993b). Marginal residues of the intracellular ATP-pool of *L. brevis* TMW 1.465 could however also be a possible explanation for the findings, as in comparison with hop-sensitive strains, hop-tolerant strains showed higher intracellular ATP levels (Simpson 1993b; Simpson & Fernandez 1994). Interestingly, *L. brevis* TMW 1.465 mastered to control pH_{in} best when supplied with Glu, resulting in the highest transmembrane pH gradient. In a similar assay, BEHR et al. showed that a hop adapted variant of the strain (*L. brevis* TMW 1.465A) also failed in maintaining a trans-membrane pH gradient when Glc was supplied as only energy source. When arginine was used to energise the cells, *L. brevis* TMW 1.465A could preserve pmf to some extend by metabolising the energy source via the arginine deiminase pathway (Behr et al. 2006). These findings are in line with the elevated survival and stronger broth acidification compared to *L. brevis* TMW 1.6. Considering the aforementioned strain specificity of the GAD system, these findings demonstrate that this tolerance mechanism can play a role in the alleviation of hop stress of some strains and may delineate beer-spoiling strains.

4.3.2 **Transcriptional analysis**

To further investigate the mutual relation of hop stress and the GAD system and to gain insight into the molecular biological background of the system's activity under hop stress, a transcriptional analysis was conducted. In comparison with reference acid stress conditions, the expression of the assessed genes (*gad-tr*, *gadB$_1$*, *gadB$_2$*, *gadC*, LVIS_2211) of *L. brevis* TMW 1.6 remained rather unchanged or was merely slightly decreased. This pattern of expression clearly reflects the results of the metabolic analysis including unaltered broth acidification and GABA production throughout reference and stress conditions. The additional iso-α-acids-mediated stress could thus not be compensated for by improved maintenance of pH_{in}. For *L. brevis* TMW 1.465, the transcriptional analysis revealed a considerably different picture. Unlike *L. brevis* TMW 1.6, the hop-tolerant strain did not express the isoenzyme $GadB_1$ under reference conditions and accordingly produced less GABA, indicating an overall lower stress level. Nevertheless, the expression of $GadB_2$ under all conditions was equal to the level found in the hop-sensitive strain. All other investigated genes were found to be upregulated under hop stress, including $GadB_1$ which was only induced in the presence of iso-α-acids and can therefore be held responsible for the detected fourfold higher GABA production. The upregulation of *gadC* and LVIS_2211 encoding a Na^+/H^+-antiporter furthermore accounts for improved maintenance of the transmembrane pH gradient. The revealed expression patterns determined under reference and hop stress conditions of the two *L. brevis* strains are in line with the investigated metabolic characteristics. It could be shown that differences between *L. brevis* TMW 1.6 and *L. brevis* TMW 1.465 regarding the GAD system are not only delineated by the sheer quantity of produced GABA but also by strain-specific expression of GAD system-associated genes and the respective strains' ability to respond to given living conditions. It can further be concluded that, under reference acid stress conditions, the highly hop-tolerant strain *L. brevis* TMW 1.465 is not forced to exploit all defence mechanisms at disposal and can therefore react to additional stressors.

The results demonstrate that the application of iso-α-acids can further increase the yield of GABA production in biotechnological fermentation processes as shown before for various other parameters (Li et al. 2010; Tung et al. 2011).

4.4 Molecular mechanism behind the antimicrobial activity of hop iso-α-acids

In the beer brewing process, iso-α-acids are formed during the kettle boiling step (De Keukeleire & Verzele 1971; Molyneux & Wong 1973; Palamand & Aldanhoff 1973; Schulze et al. 1981). These hop derivatives exhibit elevated water solubility and increased bitterness as compared to their mother compounds, the α-acids (García-Villalba et al. 2006). Therefore, iso-α-acids are regarded the main bittering component in beer (De Keukeleire & Verzele 1971; Palamand & Aldanhoff 1973; Jaskula et al. 2008; Intelmann & Hofmann 2010; Intelmann et al. 2011). Beyond imparting a distinct comfortable bitter aroma to beer, iso-α-acids act as a potent antimicrobial agents (Teuber & Schmalreck 1973; Simpson 1993a; Behr et al. 2006; García-Villalba et al. 2006; Schurr et al. 2013) against a variety of beer spoiling microorganisms (Back 1994; Sakamoto & Konings 2003). Hence, iso-α-acids are to be considered to be of prime importance to beer quality. For that reason, a new approach to further elucidate the mode of action of these hop derived compounds on a molecular level was sought for. Therefor, modified iso-α-acids were designed and synthesised and the obtained derivates' properties and their physiological and electrochemical effects on membranes were investigated in BLM model experiments and in live cells.

Three differently modified types of iso-α-acids were synthesised to get a better understanding of the hop derivative's antibacterial mode of action. As iso-α-acids were described as ionophores, which transport protons across cytoplasmic membranes similarly to weak acids (Simpson & Smith 1992; Sakamoto et al. 2002; Blanco et al. 2006), the molecule was methylated in the C-3 position (cf. Fig. 2). This modification facilitated the evaluation of the share of the C-3 bound acidic hydroxyl group in the substance's ionophore

activity. Secondly, an imino form of iso-α-acids was prepared by substitution of the carbonyl group in the C-1 position (cf. Fig. 2) to reassess the role of the postulated manganese binding site (Simpson & Hughes 1993). Hydrolysis of the C-4 bound isohexenoyl side chain (cf. Fig. 2) results in the formation of humulinic acids, which can be found in commercially available isomerised hop extracts and aged beers (Drewett & Laws 1970; Verzele et al. 1973; Intelmann et al. 2011). Hence, furthermore a humulinic acids mixture was prepared in order to evaluate the C-4 bound side chain's influence on iso-α-acids' characteristics. The synthesised modified iso-α-acids are depicte in Fig. 38.

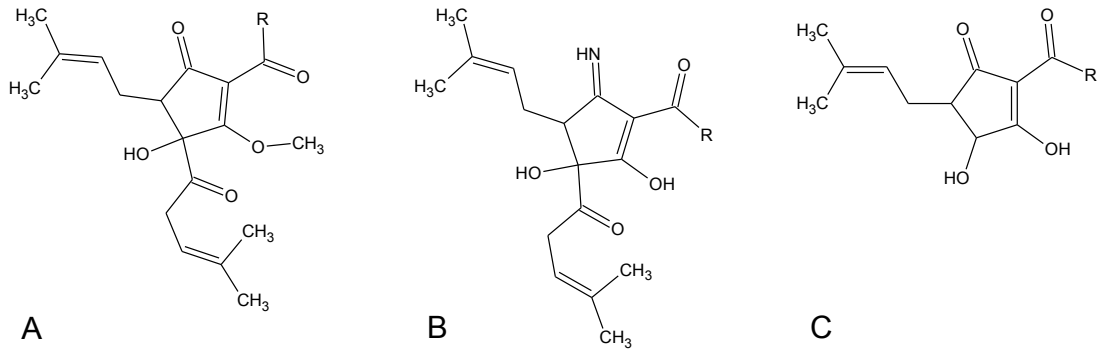


Fig. 38: Modified iso-α-acids. A: methyl-iso-α-acids, B: imino-iso-α-acids, C: humulinic acids. co-, n-, ad-congeners: R_{co} = $CH(CH_3)_2$, R_n = $CH_2CH(CH_3)_2$, R_{ad} = $CH(CH_3)C_2H_5$.

An analysis of UV-Vis absorption profiles of the hop derivatives under investigation revealed that the C-1 carbonyl group is involved in the interaction of iso-α-acids and manganese, as spectral changes indicate binding of manganese to the hop derivatives' chromophore β-triketone group (Simpson & Hughes 1993). This conclusion could be drawn from the finding that imino-iso-α-acids did not cause a shift of compound characteristic absorption maxima. It can therefore be deduced that neither the iso-α-acids' C-4 side chain, nor the C-3 hydroxyl group are involved in manganese complexation and the resulting effects on cell membranes and microorganisms.

This phenotypically observed loss of manganese binding capability of imino-iso-α-acids could be demonstrated by two types of model BLM experiments based on previously described principles and procedures (Kalinowski & Figaszewski 1995a; Kalinowski & Figaszewski 1995b; Behr 2008; Behr & Vogel 2009; Behr & Vogel 2010). Firstly, potentiostat mode measurements showed that imino-iso-α-acids failed in enhancing BLM conductivity in presence of manganese. Test substances without modifications in the C-1 position uniformly caused higher membrane conductance. These findings are consistent with those of BEHR et al. who demonstrated a manganese dependent increase of transmembrane charge permeation caused by iso-α-acids (Behr & Vogel 2009). The detected changes of membrane conductivity, which could not be attributed to an electroneutral H^+/Mn^{2+} exchange mechanism (as proposed for trans iso-n-humulone (Simpson 1993a)) were reported to arise from a transmembrane redox reaction (Behr & Vogel 2010). BEHR et al. illustrated that the transmembrane electron transfer was mediated by Mn^{2+} acting as electron donor and electron accepting iso-α-acids (Behr & Vogel 2010). In biological systems, manganese most commonly has valences of +2 and +3 (Archibald 1986) and the ion couple Mn^{2+}/Mn^{3+} can undergo redox reactions (Wariishi et al. 1989). Thus, BEHR et al. postulated the following transmembrane charge transfer model, elucidating oxidative stress caused by iso-α-acids: uncomplexed iso-α-acids can permeate the cytoplasmic membrane and enter the cytosol, where they interact with Mn^{2+} due to the reducing conditions present (higher pH and Mn^{2+} conc.). In the following, the formed electron donor at the inner membrane/solution interface can accept and subsequently transfer an electron through the membrane to the extracellular membrane/solution interface, where the prevailing more oxidising conditions (lower pH and Mn^{2+} conc.) cause electron release. Intracellularly, the restored Mn^{2+}/iso-α-acids complex can now again accept an electron and thus cause oxidative stress (Behr & Vogel 2010). The transmembrane charge transfer furthermore influences the membrane potential ($\Delta\Psi$) and consequently also the cell's pmf (Berg et al. 2003; Foster 2004). Consequently, the effect of imino-iso-α-acids and differently modified iso-α-acids on membrane conductivity can clearly be attributed to the

respective compound's manganese binding capability. It can further be expected that no oxidative stress is exerted on live cells by the imino derivative.

A second type of experiment was conducted to assess the formation of membrane potentials in presence of pH or manganese gradients across a BLM. In accordance with the delineated lack of manganese complexation ability, imino-iso-α-acids were shown to generate membrane potentials solely when a pH gradient was built in the BLM cell. This finding reflects the unimpaired function of the acidic hydroxyl group and the loss of manganese binding ability, resulting in cancelled transmembrane charge transfer mediated by the redox reaction described above. As expected, the membrane potential generation power of humulinic acids did not differ from the iso-α-acids', since neither of the two target groups (C-1 and C-3 moiety) had been modified. These findings rule out a possible role of the C-4 bound side chain in model BLM experiments regarding membrane conductivity alterations and membrane potential generation. Interestingly, that was also the case for methyl-iso-α-acids for both tested gradients (ΔpH, ΔMn^{2+}). While manganese binding and the involved redox reaction could be anticipated, the ionophore action as weak acid function was rather unexpected. This led me to question the mechanism behind the membrane potential formation in this experimental setting caused by methyl-iso-α-acids in presence of a pH gradient.

β-Diketones can undergo keto-enol tautomerisation and β-diketones and polyketones with β-diketone groups (e.g. cyclic β-triketones) have therefore widely been studied (Burdett & Rogers 1964; Otway & Rees 2000; Wu et al. 2002; Sojka 2006; Vigato et al. 2009). In various biologically active compounds, such as iso-α-acids, part of the molecular skeleton is formed by the three keto-group 2 acylcycloalkane-1,3-dione (Rubinov et al. 1996; Blanco et al. 2003). Consequently, iso-α-acids can well likely be subject to keto-enol tautomerisation, similar to many different other plant derived substances, which for convenience have been termed β-triketones (Sojka 2006; Blanco et al. 2007). Iso-α-acids are therefore presumed herein to undergo a tautomerisation reaction in aqueous solutions as depicted in Fig.

39. In the enol form, iso-α-acids exhibit an additional acidic hydroxyl group in the C-1 position.

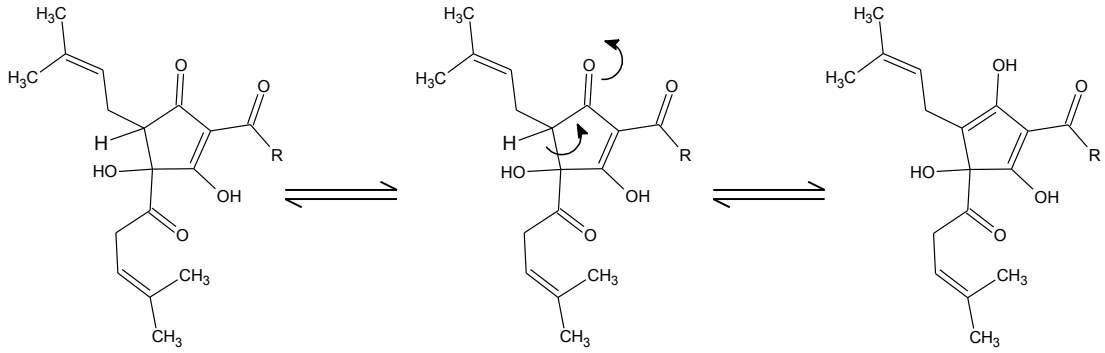


Fig. 39: Proposed keto-enol tautomerisation of iso-α-acids.

The additional acidic function can be expected to (partly) restore the weak acid function of methylated iso-α-acids and thus avouch for membrane potential generation in presence of a pH gradient.

In continuation to the presented ex situ experiments, live cell experiments were conducted to consolidate the findings in an *in vivo* approach. A resting cells assay was used to investigate the influence of iso-α-acids and modified iso-α-acids on the pH_{in} of a hop sensitive of *L. brevis* strain (*L. brevis* TMW 1.6). In consistence with previous results of this working group (Behr et al. 2006; Schurr et al. 2013), a comparison of the decrease of pH_{in} (ΔpH_{in}) in *L. brevis* TMW 1.6 showed that iso-α-acids and pure cis-iso-cohumulone caused similar ΔpH_{in} like a valinomycin/nigericin antibiotic combination, serving as positive control. Also, imino-iso-α-acids were found to be equally potent, providing evidence that manganese binding and/or a transmembrane redox reaction are not essentially required to trigger a decrease of pH_{in} in *L. brevis*. Although, the methylated forms of iso-α-acids and pure cis-iso-cohumulone led to a less pronounced decrease of pH_{in}, the detected ΔpH_{in} still supports the keto-enol tautomerisation and the involved formation of an acidic hydroxyl group that is proposed here. This is further substantiated by the results obtained for humulinic acids, for which ΔpH_{in}-values ranged between those of methyl-iso-α-acids and

methyl-cis-iso-cohumulone. It is therefore assumed here that privation of the acidic hydroxyl group can partly be restored by intramolecular rearrangement.

As the discussed mode of action studies are inextricably linked to the test compounds' capability to permeate cell membranes, distribution ratios ($D_{org/aq}$), which were described as a measure to characterise the relative solubility of an analyte in two immiscible phases (Fritz & Schenk 1989), were determined. By means of this control experiment, it was ensured that the interpretability and comparability of the obtained results could be guaranteed. Regarding the differences between distinct ΔpH_{in}-values, it can be stated that the differences cannot be attributed solely to differences in membrane solubility, as a comparison of iso-α-acids and imino-iso-α-acids illustrates. The respective substances caused similar decrease of ΔpH_{in}, although their membrane solubility was shown to be the most different of all test substances.

The antimicrobial effect of modified and original iso-α-acids was determined for four different *L. brevis* strains. There could be found a typical pattern of susceptibility of the test organisms towards the hop derivatives, which could be recovered for all test substances. This finding indicates, that the modifications' affected on the antimicrobial potential was equally pronounced for hop sensitive and hop resistant *L. brevis* strains. The most striking effect was observed for imino-iso-α-acids, as no antimicrobial activity could be detected within the experimental limits (c_{max} = 791.9 µM). Thus, in case of the hop sensitive strains, the MIC of imino-iso-α-acids is at least about 50 times higher than the MIC of iso-α-acids. Regarding the hop tolerant strains, the MIC must consequently be at least 14 times higher for imino-iso-α-acids. Imino-iso-α-acids are less membrane soluble as iso-α-acids. However, their $D_{org/aq}$-values merely differ by a factor of about eight, which clearly shows, that differing membrane solubility characteristics cannot be the reason for the completely abolished antimicrobial activity. Generally, the determined $D_{org/aq}$-values showed no consistency with the antimicrobial activity of the test substances. It can therefore be assumed that membrane solubility was not a limiting factor for the inhibitory effect of modified iso-α-acids. It has been

shown before by various researchers, that pH value and the concentration of monovalent and divalent cations strongly influence the antimicrobial activity of hop compounds (Shimwell 1937a; Shimwell 1937b; Simpson & Smith 1992; Simpson 1993a; Simpson 1993b).

This work is hitherto, to the best of my knowledge, the first to examine the antimicrobial activity of the same hop compound with and without manganese binding ability. The investigations revealed that the transmembrane redox reaction, which is connected with manganese binding, is a key prerequisite for the antimicrobial effect of iso-α-acids. It was shown in a proteomic research paper, that iso-α-acids/manganese complexes cause oxidative stress in *L. brevis* (Behr et al. 2007). Consequently, induction or overexpression of oxidative stress-associated proteins (e.g. formamidopyrimidine-DNA glycolase; (Behr et al. 2007)) must be regarded crucial for the survival of *L. brevis* under hop stress. Furthermore, experiments with C-1 modified iso-α-acids allow the conclusion that other mechanistic features like the ionophore activity of iso-α-acids are insufficient to prevent growth of hop sensitive and hop resistant *L. brevis* strains at µM concentrations. This conclusion is in line with the fact that weak acid food spoilage preventatives exert their antimicrobial activity only at millimolar levels (Salmond et al. 1984). According to standard literature, recommended application concentrations for e.g. benzoic acid and sorbic acid are approx. 8-9 mM (Baltes 2000).

Methylation of iso-α-acids and cis-iso-cohumulone and the concomitant loss of an acidic hydroxyl group in the C-3 position impeded the inhibitory potential, as well. Considering the unimpaired manganese complexation ability and the compounds' effect on pH_{in}, this finding is to some extent surprising, since it also cannot be attributed to the compounds' membrane solubility. However, it can be argued that in methyl-iso-α-acids, the ionophore activity and transmembrane redox reaction are dependent on the same molecular group, which can exist in two different chemical conditions. In the enol form, the C-1 bound hydroxyl group can exert its weak acid function, whereas in the keto form, redox activity is facilitated. The keto and enol form are present at an equilibrium, depending on pH, temperature and solvent

conditions (Mortimer & Müller 2003). Due to intramolecular hydrogen bonds between hydroxyl groups and the neighbouring carboxyl groups, in 1,3-diketones, the enolic form can sometimes be predominant. It could be shown that 43 % of 2 acetylcyclohexanone (a 1,3-diketone) exist in the enol form under acidic conditions in aqueous solutions (Iglesias 2003). Considering the experimental conditions (aqueous medium at pH 4.3), it is well likely that a good deal of methyl-iso-α-acids are present in the enolic form in the pH_{in} assay and growth challenge tests. Therefore, the antimicrobial effect of methyl-iso-α-acids might be reduced, since only part of the compound can impart redox stress on the cells.

The antimicrobial activity of humulinic acids was very pronounced and could be observed to even outperform the inhibitory effect of original iso-α-acids. In accordance with the pH_{in} assay, this finding underlines the obviously subordinate role of the isohexenoyl C-4 side chain in the antimicrobial activity of iso-α-acids. However, it seems to play a major role regarding sensory characteristics. Humulinic acids have been described as not bitter (Drewett & Laws 1970; Verzele et al. 1973; Schulze et al. 1981) or of strongly decreased bitterness (Diffor et al. 1972; Palamand & Aldanhoff 1973) in various scientific publications. The combination of antimicrobial potential and sensory characteristics makes humulinic acids appear to be virtually predestined for the use as preservative and there has been filed a patent in 2010 concerning the production of humulinic acids for the application as food additive and in pharmaceutical products (Taniguchi et al. 2012). Beyond the use in beer, the given features allow the use of humulinic acids in foods and beverages of various kinds without the necessity to mask bitterness with the help of sweeteners or the like. For instance, humulinic acids could be used as a preservative to prevent growth of e.g. *L. brevis* or *L. plantarum*, which can be found in spoiled soft drinks (Juvonen et al. 2001).

The results presented in this section allow for an enhanced evaluation of the antimicrobial activity of iso-α-acids regarding the influence of the compound's weak acid function and the potential to evoke oxidative stress. It could be confirmed that *L. brevis* exhibits distinct levels of resistance towards oxidative stress caused by a transmembrane redox reaction and the proton ionophore

effect of iso-α-acids as previously proposed by BEHR and VOGEL (Behr & Vogel 2010). In this respect, manganese binding induced oxidative stress appears to be of prime importance in iso-α-acids mediated growth inhibition of *L. brevis*, whereas subordinate emphasis is placed on the ionophore activity.

4.5 Concluding remarks

This work is the first to evaluate the antimicrobial potential (against *L. brevis*) of a wide range of hop compounds on various fractionation levels and additionally considering the influence of different hop varieties and production procedures. It could be shown that the antimicrobial potential of a given hop compound is dependent on numerous factors. The inhibitory potential can therefore solely be determined experimentally as it cannot be predicted precisely in a reliable manner. This conclusion also reflects the immanent variability embraced in natural products like hops. Furthermore, synergistic effects of hop compounds or hop constituents could be revealed. The inhibitory potential hence resembles a sensory perception in which only the entity of all components lead to the same flavour, even though not all components are sensory active themselves.

Beer spoiling *L. brevis* has been shown to have a broad variety of hop stress alleviating mechanisms at its disposal. These mechanisms comprise as different strategies as the employment of membrane bound transporters (HorA, HorC, Hit A) (Sami et al. 1997; Sami et al. 1998; Hayashi et al. 2001; Sakamoto et al. 2001; Suzuki, K. Iijima, et al. 2005), increased F_OF_1-ATPase activity (Sakamoto et al. 2002), increased intracellular ATP pools (Simpson 1993b), overexpression of ADI pathway related genes, increased LTA content in cell walls, cell membrane alterations (Behr et al. 2006), upregulation of manganese binding proteins, adjustment of the induction of energy generation associated enzymes and enzymes involved in DNA repair and protein degradation (Behr et al. 2007; Behr 2008).

The role of cell membrane modifications as a response to hop mediated stress in a hop sensitive and hop tolerant *L. brevis* strain could be further

elucidated spotlighting the central role of ACP in the FAS II pathway. In this context, MALDI-TOF MS, as a high sensitivity tool for low molecular weight proteins, proved to be a fast and easy to apply tool to detect and characterise different stress situations.

The contribution and importance of the GAD system regarding hop tolerance could be demonstrated for the first time and was found to be one of the hop tolerance delineating factors. The ability to respond to altered environmental conditions turned out to be more important for microbial viability than the absolute GAD system activity.

Antimicrobial effects and the inhibitory mode of hops have been shown to be multifaceted. Reported effects, for instance are the reduced L-leucine uptake and L-leucine depletion, decrease of cellular ATP content (Simpson 1993b), formation of membrane leakages and subsequent glucose efflux and impaired respiratory chain activity and protein and RNA synthesis (in *B. subtilis*) (Teuber & Schmalreck 1973), ionophore activity leading to impeded pmf and decreased pH_{in} (Simpson & Smith 1992; Simpson 1993a; Behr & Vogel 2009), and a manganese dependent transmembrane redox reaction causing oxidative stress (Behr & Vogel 2010). These effects are known to depend on factors as pH, temperature and the presence of cations (e.g. Mn^{2+}) (Simpson & Smith 1992; Simpson 1993b; Behr & Vogel 2010).

Design and synthesis of different chemically modified iso-α-acids for this work, allowed for a hitherto lacking enhanced evaluation of the mechanism of iso-α-acids mediated growth inhibition on a molecular level. The investigations revealed the outstanding role of manganese binding induced oxidative stress. It could further be demonstrated that the ionophore activity is of subordinate importance.

Beyond, humulinic acids are suggested as a novel food preservative lacking undesired bitterness.

5 Summary

Beer represents a harsh environment for microorganisms (Back 1994; Sakamoto & Konings 2003) and only a very limited group of bacteria was found capable to overcome all growth limiting hurdles present in this milieu and to propagate and thus spoil beer. The most prominent representative of this group is *Lactobacillus* (*L.*) *brevis* as it is responsible for most detected beer-spoilage incidents (Back 1994; Sakamoto & Konings 2003; Thelen et al. 2006; Suzuki 2011). It is axiomatic that hop tolerance is a prerequisite for the ingenious circle of highly specialised beer spoiling bacteria.

This study has been designed to achieve a better understanding of the molecular mechanism of antimicrobial hop inhibition in *L. brevis*. The dissective approach chosen herein focused on microbial viability, physiology, metabolism and electrochemical membrane characteristics.

A screening of the antimicrobial activity of a broad spectrum of hop compounds was conducted by determination of individual MIC values to obtain an overview over general principals influencing the inhibitory potential of hops.

A comparison of crude hop extracts showed that the polarity of the extractant had no significant influence on the inhibitory effect. Also, extracts from different raw materials showed consistent MICs, except for pelleted hops. This might be attributed to the pelletising process, in which the hop bitter acids containing lupulin glands are damaged. Antimicrobial α-acids are degraded three times as fast in hop pellets as in green hops (Biendl et al. 2012). The soft resin fraction accounted for the greatest share of fractions' inhibitory potential. Amphiphilic compounds obtained from hard resin subfractionation also proved to be antimicrobially active. This clearly points towards a dependence of the inhibitory effect of these compounds on their membrane permeability. Several effects of hop compounds on cell membranes were demonstrated in scientific papers (Teuber & Schmalreck 1973; Behr & Vogel 2009; Behr & Vogel 2010).

Bioinformatic cluster analysis comparing hop fractions from different hop varieties and raw materials, showed that antimicrobial activity was mainly dependent the respective fraction. Thus, the choice of aroma or bitter hops appears to be more delicate regarding sensory properties and less important for the inhibitory effect. It must be kept in mind however, that differences between hop varieties might not have been detected due to the low solubility of α-acids (Spetsig 1955; Stevens 1966; Jaskula-Goiris et al. 2010), which is increased significantly upon isomerisation during the wort boiling process (Simpson & Smith 1992; García-Villalba et al. 2006).

A synergistic effect of hop constituents could be deduced, as some compounds were solely active in combination with others. Xanthohumol, for instance, did not have an inhibitory effect on the tested *L. brevis* strains. Yet, NATARAJAN et al. could demonstrate a synergistic effect of xanthohumol (and lupulone) and several antibiotics (Natarajan et al. 2008).

In this work, for the first time, the low molecular weight sub-proteome of two *L. brevis* strains was investigated using MALDI-TOF MS as a high sensitivity, fast and easy to apply tool to detect and characterise different stress situations over a prolonged period of investigation. The novel approach facilitated physiological examination of hop stress on a sub-proteomic level and guaranteed exclusion of immanent disadvantages of 2D gel electrophoresis.

In a hop shock experiment, strain specific stress responses could be found. For hop sensitive *L. brevis* TMW 1.6, a protein identified as acyl carrier protein (ACP) was only detected throughout a 48 h period under iso-α-acids induced stress. For hop tolerant *L. brevis* TMW 1.465, ACP could also be found under reference acid stress and in presence of xanthohumol.

ACP forms part of the fatty acid synthase II (FAS II) complex (Byers & Gong 2007) and acts like a shuttle for intermediates between the FAS II subunits during FA elongation (Heath et al. 2002; Zhang & Rock 2008). ACP is thus inextricably tied to biosynthesis of FAs and glycerol phospholipids, and consequently also to membrane de novo synthesis and structural alterations.

Numerous publications have reported the reorganisation of cell membranes as response to various stress conditions (Mykytczuk et al. 2007). Moreover, BEHR et al. observed hop induced altered membrane composition and fluidity (Behr et al. 2006).

The pattern of detected ACP under the respective stress condition can hence be regarded an indicator for ongoing cell membrane changes. The patterns of ACP detection allow the conclusion that *L. brevis* TMW 1.465 shows a more pronounced stress response. Considering the antimicrobial activity of xanthohumol (low) and iso-α-acids (high), it can be deduced that its tolerance mechanisms are activated by comparatively weaker stressors, which in turn could be one of the determining factors for lower susceptibility to hops. The findings are in line with a study showing a cell membrane FA composition accounting for lower fluidity in strains with higher beer-spoilage potential (Preissler 2011).

Moreover, these findings demonstrate that MALDI-OF MS can be used as an excellent tool for hop stress responses characterisation of *L. brevis* strains.

The role of the glutamic acid decarboxylase (GAD) system in hop tolerance of *L. brevis* was evaluated in this work. The acid stress tolerance mechanism GAD system, in *L. brevis* comprises a transcriptional regulator (Gad-tr), a glutamate/γ-aminobutyrate antiporter (GadC) and two glutamate decarboxylases ($GadB_1$, $GadB_2$) (Li et al. 2013).

The higher beer-spoilage potential of *L. brevis* TMW 1.465 was reflected by higher survival rates, lower final broth pH, when compared to *L. brevis* TMW 1.6. Further, it was able to increase γ-aminobutyrate (GABA) production by the factor four when exposed to iso-α-acids, whereas the sensitive strain constantly produced equally high amounts. These findings are supported by studies concluding that GAD system activity and GABA production are strain-specific characteristics and underlie variation depending on environmental conditions (Li & Cao 2010; Li et al. 2010). Investigation of the intracellular pH (pH_{in}) revealed that *L. brevis* TMW 1.465 was able to maintain a transmembrane pH gradient (and thus pmf) when supplied with

glutamic acid (Glu). This finding is in accordance with the elevated survival rates and stronger broth acidification.

On a transcriptional level, it could be found, that the expression of the assessed genes of *L. brevis* TMW 1.6 remained mainly unchanged, which clearly reflects the results of the metabolic analyses. It can therefore be concluded that additional hop stress could not be compensated for by improved maintenance of pH_{in}. For *L. brevis* TMW 1.465, a considerably different picture could be found, indicating a lower overall stress level. Induction or upregulation of the genes can therefore be held responsible for the detected fourfold higher GABA levels and the improved pH_{in} maintenance.

Accordingly, differences between strains were demonstrated not to arise from sheer quantity of produced GABA but more importantly from the ability to respond to given living conditions. It can also be deduced that the more hop tolerant strain is not forced to exploit all defence mechanisms at disposal under acid stress and can therefore react to additional stressors.

Taken together, the results demonstrate that the GAD system can play an important role in the alleviation of hop stress of some strains and can codetermine the beer-spoilage potential of *L. brevis* strains.

Chemically modified iso-α-acids were designed and synthesised to enhance the basic understanding of the molecular mechanism behind antimicrobial activity of iso-α-acids (cf. Fig. 38). To evaluate the share of the C-3 bound acidic hydroxyl group in the substance's ionophore activity and its overall antimicrobial effect, iso-α-acids were methylated in the C-3 position. Substitution of the C-1 carbonyl group facilitated the reassessment of the proposed manganese binding site (Simpson & Hughes 1993) and its influence on molecular characteristics. Accordingly, humulinic acids were prepared by cleavage of the C-4 bound isohexenoyl side chain.

It could be confirmed that the C-1 carbonyl group is involved in manganese binding by UV-Vis absorption scans and model BLM experiments determining membrane conductivity. Manganese dependent increased

transmembrane charge permeation has also been demonstrated by BEHR et al. (Behr & Vogel 2009). In this context, BEHR et al. further postulated a manganese dependent transmembrane redox reaction leading to oxidative stress (Behr & Vogel 2010). Transmembrane charge transfer furthermore also influences ΔΨ and the pmf (Berg et al. 2003; Foster 2004; Krulwich et al. 2005) and therefore the microbial viability. Consequently, (modified) iso-α-acids' effect on membrane conductivity can clearly be attributed to manganese binding ability. Assessment of membrane potential formation in presence of a pH or manganese gradient also confirmed the lack of manganese binding ability of imino-iso-α-acids (C-1 modification). BLM experiments with humulinic acids showed that the modification had no influence on either of the experiments. Also, methyl-iso-α-acids increased conductance and led to potential generation. The latter was rather unexpected as the C-3 bound hydroxyl group could not account for the ionophore activity. It is therefore suggested that the β-diketone structure of iso-α-acids or its methylated derivative can undergo keto-enol tautomerisation in aqueous solutions as shown before (Burdett & Rogers 1964; Otway & Rees 2000; Wu et al. 2002; Sojka 2006; Vigato et al. 2009). In the enol form, iso-α-acids exhibit an acidic hydroxyl group in the C-1 position which can be expected to (partly) restore the weak acid function (cf. Fig. 39).

The test substances' ionophore activity could also be verified in live cell experiments (Determination of pH_{in}). The proposed keto-enol tautomerisation is further substantiated by this finding.

To conclude the evaluation of modified iso-α-acids, their antimicrobial effect was assessed *in vivo*. The modifications' effect on the inhibitory potential was equally pronounced for all four test strains indicating that it did not depend on the overall susceptibility of a respective strain. The most striking effect was found for imino-iso-α-acids as no inhibitory effect could be detected. In consideration of the results discussed above, manganese binding and concomitant transmembrane redox reaction consequently are of utmost importance for microbial inhibition. Methylation of iso-α-acids decreased the antimicrobial activity. This may be explained by the fact that in methyl-

iso-α-acids, the ionophore activity and the transmembrane redox reaction depend on the same molecular group (C-1 bound). Consequently, only part of the molecules can be present in the apparently more toxic keto form. Humulinic acids managed to outperform original iso-α-acids in the MIC tests, underlining the subordinate role of the C-4 side chain in antimicrobial activity of iso-α-acids.

Due to the combination of the antimicrobial activity and their sensory characteristics (no/low bitterness, (Drewett & Laws 1970; Diffor et al. 1972; Palamand & Aldanhoff 1973; Verzele et al. 1973; Schulze et al. 1981)), humulinic acids appear virtually predestined for the use as food preservative. For instance, humulinic acids may be used without the necessity to mask undesired bitterness, to prevent growth of e.g. *L. brevis* or *L. plantarum*, which can be found in spoiled soft drinks (Juvonen et al. 2001).

The studies of chemically modified iso-α-acids hence allowed for an enhanced evaluation of the molecular mechanism behind the compounds' antimicrobial activity. Also, distinct levels of tolerance towards oxidative stress and the proton ionophore effect as presumed by BEHR and VOGEL (Behr & Vogel 2010) could be confirmed.

In conclusion, compared to the ionophore activity, manganese binding induced oxidative stress appears to be of greater importance in iso-α-acids mediated growth inhibition of *L. brevis*.

6 Zusammenfassung

Bier stellt eine unfreundliche Umgebung für Mikroorganismen dar (Back 1994; Sakamoto & Konings 2003) und nur eine sehr kleine Gruppe von Bakterien ist in der Lage alle in diesem Milieu vorhanden Hindernisse zu überwinden und sich zu vermehren. Der wichtigste Vertreter dieser Gruppe ist *Lactobacillus* (*L.*) *brevis*, da dieses Bakterium für die meisten erfassten Bierverderbsfälle verantwortlich ist (Back 1994; Sakamoto & Konings 2003; Thelen et al. 2006; Suzuki 2011). Es ist folglich unanzweifelbar, dass das Vorhandensein von Hopfentoleranz eine Grundvoraussetzung für diese erfinderische Gruppe von hoch spezialisierten Bierverderbern darstellt.

Diese Arbeit wurde konzipiert um ein besseres Verständnis des molekularen Mechanismus hinter der antimikrobiellen Wirkung von Hopfen zu erlangen. Der hierfür gewählte, in mehrere Ebenen gegliederte, wissenschaftliche Ansatz richtete sich auf die mikrobielle Überlebensfähigkeit, Physiologie und Stoffwechselprozesse, sowie strukturelle und elektrochemische Membrancharakteristika.

Es wurde ein Screeningverfahren zur Ermittlung der antimikobiellen Aktivität eines breiten Spektrums von Hopfeninhaltsstoffen durchgeführt, mit Hilfe dessen stammspezifische minimale Hemmkonzentrationen (MIC) ermittelt wurden. Es sollte so ein Überblick über Prinzipien, die das Hemmpotential von Hopfen beeinflussen erlangt werden.

Ein Vergleich kruder Hopfenextrakte zeigte, dass die Polarität des Extraktionsmittels keinen signifikanten Einfluss auf die antimikrobielle Aktivität hatte. Ebenso ergaben Extrakte, die aus unterschiedlichen Ausgangsmaterialien aufgearbeitet wurden, mit Ausnahme von Extrakten aus Hopfenpellets, übereinstimmende MIC-Werte. Dies könnte eine Folge des Pelletierungsprozesses sein, in dem die Lupulindrüsen, die die Hopfenbittersäuren enthalten beschädigt werden. Antimikrobiell wirksame α-Säuren werden in Pellets dreimal so schnell abgebaut, wie in Doldenhopfen (Biendl et al. 2012). Es wurde deutlich, dass hauptsächlich die

Weichharzfraktion für das antimikrobielle Potential verschiedener Hopfenfraktionen verantwortlich war. Amphiphile Komponenten, die aus einer Subfraktionierung der Hartharzfraktion gewonnen wurden erwiesen sich ebenfalls als antimikrobiell wirksam. Dies deutet auf eine Abhängigkeit des inhibitorischen Effektes von der Membrangängigkeit eines Hopfeninhaltsstoffes hin. In wissenschaftlichen Publikationen wurden verschiedene Effekte von Hopfenkomponenten auf Zellmembranen gezeigt (Teuber & Schmalreck 1973; Behr & Vogel 2009; Behr & Vogel 2010).

Der mittels einer bioinformatischen Clusteranalyse durchgeführte Vergleich der antimikrobiellen Aktivität von Hopfenfraktionen von verschiedenen Ausgangsmaterialien und verschiedenen Hopfensorten ergab, dass die Hemmwirkung hauptsächlich von der jeweiligen Fraktion abhängig war. Folglich erscheint die Wahl von Aroma- beziehungsweise Bitterhopfen von größerer Bedeutung hinsichtlich sensorischer Eigenschaften zu sein, und weniger Einfluss auf das inhibitorische Potential zu haben. Es muss hierbei jedoch bedacht werden, dass Unterschiede zwischen verschiedenen Hopfensorten möglicherweise aufgrund der geringen Löslichkeit der α-Säuren (Spetsig 1955; Stevens 1966; Jaskula-Goiris et al. 2010) nicht erfasst wurden. Die Löslichkeit von α-Säuren wird durch Isomerisierung während der Würzekochung erheblich verbessert (Simpson & Smith 1992; García-Villalba et al. 2006).

Aus dem Untersuchungsergebnis, dass einige Hopfeninhaltsstoffe nur in Kombination mit anderen Komponenten antimikrobiell aktiv waren, konnte ein synergistischer Effekt abgeleitet werden. Xanthohumol allein hatte beispielsweise keinen inhibitorischen Effekt auf die untersuchten *L. brevis* Stämme. NATARAJAN et al. gelang es jedoch synergistische Effekte zwischen Xanthohumol (und auch Lupulon) und verschiedenen Antibiotika nachzuweisen (Natarajan et al. 2008).

MALDI-TOF MS wurde als einfach und schnell anzuwendendes Instrument und wegen seiner hohen Sensitivität für diese Arbeit erstmals zur Untersuchung eines Subproteoms, das nur Proteine von geringem Molekulargewicht umfasste verwendet. Die Detektion und Charakterisierung

verschiedener Stresssituationen sollte so über einen längeren Untersuchungszeitraum ermöglicht werden. Dieser neuartige Ansatz erlaubte die physiologische Untersuchung von Hopfenvermitteltem Stress auf der Ebene eines Subproteoms und den gleichzeitigen Ausschluss von Fehlerquellen, die der 2D-Gelelektophorese immanenten sind.

In einem Hopfenschock Experiment konnten so stammspezifische Stressantworten beobachtet werden. Bei Untersuchungen mit dem hopfensensitiven Stamm *L. brevis* TMW 1.6 wurde ein Protein, das als Acyl-Carrier-Protein (ACP) identifiziert wurde, über einen Zeitraum von 48 h nur unter iso-α-Säuren induziertem Stress detektiert. Bei Untersuchungen mit dem hopfentoleranten Stamm *L. brevis* TMW 1.465 konnte ACP auch unter Säurestress (Referenzbedingung) und in Anwesenheit von Xanthohumol gefunden werden.

ACP ist Teil des Fettsäure-Synthase II (FAS II) Komplexes (Byers & Gong 2007) und fungiert als ein Shuttlemolekül für Stoffwechselzwischenprodukte zwischen den FAS II Untereinheiten der Kettenverlängerungsreaktion der Fettsäuresynthese (Heath et al. 2002; Zhang & Rock 2008). ACP ist demzufolge untrennbar mit der Biosynthese von Fettsäuren und Glycerophospholipiden und folglich auch der *de novo* Synthese und Mechanismen der strukturellen Veränderung von Membranen verbunden.

Eine Vielzahl wissenschaftlicher Publikationen berichtet von Restrukturierungen von Zellmembranen infolge von verschiedenen Stressbedingungen (Mykytczuk et al. 2007). Ferner beobachteten BEHR et al. hopfeninduzierte Veränderungen der Membranzusammensetzung und -fluidität (Behr et al. 2006).

Das Muster von detektiertem ACP unter der jeweiligen Stressbedingung kann demzufolge als Indikator für vorübergehend ablaufende Veränderungen der Zellmembran betrachtet werden. Die Muster der ACP Detektion erlauben den Schluss, dass *L. brevis* TMW 1.465 eine stärker ausgeprägte Stressantwort aufweist. Vor dem Hintergrund der antimikrobiellen Aktivität von Xanthohumol (gering) und iso-α-Säuren (hoch) erscheint die Folgerung, dass die Toleranzmechanismen dieses Stammes durch vergleichbar schwächere Stressoren aktiviert werden plausibel. Dieses Verhalten könnte

ein bestimmender Faktor für die geringere Empfindlichkeit von *L. brevis* TMW 1.465 gegenüber Hopfen sein. Diese Ergebnisse stimmen mit einer früheren Arbeit überein, in der gezeigt wurde, dass *L. brevis* Stämme mit hohem Bierverderbspotential, Zellmembranen mit einer Fettsäurezusammensetzung besitzen, die eine geringere Fluidität bedingen (Preissler 2011).

Des Weiteren verdeutlichen diese Forschungsergebnisse, dass MALDI-TOF MS ein ausgesprochen geeignetes Instrument zur Charakterisierung von hopfeninduzierten Stressreaktionen ist.

Die Rolle des Glutaminsäure Decarboxylase (GAD) Systems in der Hopfentoleranz von *L. brevis* war ebenfalls Gegenstand der Untersuchungen dieser Arbeit. Bei *L. brevis* umfasst das GAD System einen Transkriptionsregulator (Gad-tr), einen Glutaminsäure/γ-Aminobuttersäure Antiporter (Gad-C) und zwei Glutaminsäure Decarboxylasen ($GadB_1$ und $GadB_2$) (Li et al. 2013).

Im Vergleich zu *L. brevis* TMW 1.6 spiegelte sich das höhere Bierverderbspotential von *L. brevis* TMW 1.465 in höheren Überlebensraten und geringerem pH-Wert des Mediums am Ende der Fermentation wider. Ferner war der Organismus in der Lage die γ-Aminobuttersäure (GABA) Bildung in Anwesenheit von iso-α-Säuren um den Faktor vier zu steigern, wohingegen der sensitivere Stamm gleichbleibend viel GABA bildete. Diese Ergebnisse stimmen mit Arbeiten überein, die die Aktivität des GAD Systems und GABA Bildung als stammspezifische und von Umweltbedingungen abhängige Charakteristika bezeichnen (Li & Cao 2010; Li et al. 2010). Messungen des intrazellulären pH-Wertes (pH_{in}) zeigten, dass *L. brevis* TMW 1.465 einen transmembranen pH Gradient (und somit die pmf) aufrechterhalten konnte, wenn dem Mikroorganismus Glutaminsäure zur Verfügung stand. Dies deckt sich mit den zuvor beschriebenen höheren Überlebensraten und stärker ausgeprägter Ansäurerung des Mediums.

Auf transkriptioneller Ebene, konnte beobachtet werden, dass die Expression der untersuchten Gene von *L. brevis* TMW 1.6 weitestgehend unverändert

blieb. Dies reflektiert eindeutig die Ergebnisse aus den Untersuchungen des mikrobiellen Stoffwechsels. Der Organismus war demzufolge nicht in der Lage zusätzlichen durch Hopfen induzierten Stress mittels verbesserter Aufrechterhaltung des pH_{in} zu kompensieren. Bei *L. brevis* TMW 1.465 bot sich ein deutlich anderes Bild, das auf insgesamt weniger ausgeprägten Stress hindeutete. Die Induktion und erhöhte Expression von Genen kann folglich für die detektierte vierfach höhere GABA Konzentration und die verbesserte Aufrechterhaltung des pH_{in} verantwortlich gemacht werden.

Es wurde demnach gezeigt, dass Unterschiede zwischen verschiedenen Stämmen nicht von deren bloßen GABA Produktion herrühren, sondern vielmehr von der Fähigkeit auf vorliegende Lebensbedingungen zu reagieren bestimmt werden. Es kann ferner hieraus abgeleitet werden, dass der Stamm mit der höheren Hopfentoleranz unter Säurestress nicht gezwungen war alle ihm zur Verfügung stehenden Abwehrmechanismen einzusetzen und daher noch auf weitere Stressoren reagieren konnte.

Insgesamt verdeutlichen diese Erkenntnisse, dass das GAD System eine wichtige Rolle in der Verminderung von Hopfenstress spielen kann und das Verderbspotential von *L. brevis* Stämmen mitbestimmt kann.

Im Rahmen dieser Arbeit wurden chemisch modifizierte iso-α-Säuren entworfen und synthetisiert um ein verbessertes Grundverständnis des molekularen Mechanismus hinter der antimikrobiellen Aktivität von iso-α-Säuren zu erlangen (cf. Fig. 38). Um den Anteil der C-3 gebundenen aciden Hydroxy-Gruppe an der ionophoren Aktivität und der gesamten antimikrobiellen Aktivität zu ermitteln, wurden iso-α-Säuren am C-3 Atom methyliert. Die Substitution der C-1 gebundenen Carbonyl-Gruppe ermöglichte die Neubewertung der von SIMPSON und HUGHES vorgeschlagenen Mangan-Bindestelle (Simpson & Hughes 1993) und deren Einfluss auf molekulare Charakteristika. Dementsprechend wurden Humulinsäuren durch Abspaltung der C-4 gebundenen Isohexenoyl-Seitenkette hergestellt.

Mittels UV-Vis Absorptionsscans und BLM-Modell Experimenten zur Bestimmung der Membranleitfähigkeit war es möglich die Beteiligung der C-1 Carbonyl-Gruppe an der Manganbindung nachzuweisen. Erhöhter transmembraner Ladungsübertritt in Abhängigkeit von Mangan konnte zuvor auch von BEHR et al. gezeigt werden (Behr & Vogel 2009). In diesem Zusammenhang postulierten BEHR et al. des Weiteren eine Manganabhängige transmembrane Redoxreaktion, die oxidativen Stress verursacht (Behr & Vogel 2010). Ferner beeinflusst der transmembrane Ladungsübertritt das Membranpotential ($\Delta\Psi$) und die pmf (Berg et al. 2003; Foster 2004; Krulwich et al. 2005) und somit auch die mikrobielle Überlebensfähigkeit. Der Einfluss von (modifizierten) iso-α-Säuren auf die Membranleitfähigkeit ist somit direkt auf die Manganbindefähigkeit zurückzuführen. Messungen zur Bildung von Membranpotentialen in Abhängigkeit eines Mangan- beziehungsweise pH-Gradienten bestätigten darüber hinaus den Verlust der Manganbindefähigkeit von imino-iso-α-Säuren (C-1 Modifikation). BLM-Modell Experimente mit Humulinsäuren ergaben, dass diese Modifikation keinen Einfluss auf die Messungen hatte. Ebenso führten methyl-iso-α-Säuren zu erhöhter Membranleitfähigkeit und zur Bildung von Membranpotentialen. Letzteres trat eher unerwartet auf, da die C-3 gebundenen Hydroxy-Gruppe nicht mehr zur ionophore Wirkung beitragen konnte. Es wird daher hierin angenommen, dass die β-Diketon Struktur der iso-α-Säuren und methyl-iso-α-Säuren in wässriger Lösung einer Keto-Enol-Tautomerisierung unterliegen kann, wie sie bereits in anderen Arbeiten gezeigt wurde (Burdett & Rogers 1964; Otway & Rees 2000; Wu et al. 2002; Sojka 2006; Vigato et al. 2009). In der Enol-Form, besitzen die iso-α-Säuren eine acide Hydroxy-Gruppe in der C-1 Position, von der anzunehmen ist, dass sie die Funktion der schwachen Säure (teilweise) wiederherstellen kann (cf. Fig. 39).

Die ionophore Aktivität der Testsubstanzen konnte auch in Lebendzell-Experimenten verifiziert werden (Bestimmung des pH_{in}). Durch diese Untersuchungsergebnisse wurde die Annahme der Keto-Enol-Tautomerisierung zusätzlich bekräftigt.

Um die Charakterisierung der modifizierten iso-α-Säuren abzuschließen wurde deren antimikrobielles Potential *in vivo* untersucht. Der Einfluss der Modifikationen auf das Hemmpotential der Substanzen erwies sich als für alle vier Teststämme gleich ausgeprägt. Dies deutet darauf hin, dass die Hemmwirkung unabhängig von der Ausprägung der Hopfentoleranz eines Stammes war. Der bemerkenswerteste Effekt zeigte sich bei den Experimenten mit imino-iso-α-Säuren, bei denen keine Hemmung festzustellen war. In Anbetracht der zuvor beschriebenen Ergebnisse kann man also folgern, dass Manganbindefähigkeit und die damit verbundene Redoxreaktion von entscheidender Wichtigkeit für die antimikrobielle Wirkung sind. Die Methylierung von iso-α-Säuren senkte ebenfalls die Hemmwirkung. Eine Erklärung hierfür könnte sein, dass bei methyl-iso-α-Säuren die ionophore Aktivität und transmembrane Redoxreaktion von derselben Molekülgruppe abhängig sind (gebunden an die C-1 Position). Es kann folglich nur ein Teil der Moleküle in der offensichtlich toxischeren Keto-Form vorliegen. Im Vergleich übertrafen die Humulinsäuren die unveränderten iso-α-Säuren in ihrer antimikrobiellen Wirkung. Es wurde dadurch die anscheinend untergeordnete Funktion der C-4 Seitenkette in der Hemmwirkung von iso-α-Säuren deutlich.

Aufgrund der antimikrobiellen Wirkung in Kombination mit ihren sensorischen Eigenschaften (geringe/keine Bitterkeit (Drewett & Laws 1970; Diffor et al. 1972; Palamand & Aldanhoff 1973; Verzele et al. 1973; Schulze et al. 1981)), scheinen Humulinsäuren geradezu prädestiniert für die Verwendung als Konservierungsstoff in Lebensmitteln zu sein. Zum Beispiel könnten Humulinsäuren ohne die Notwendigkeit zur Maskierung unerwünschter Bitterkeit verwendet werden um das Wachstum von *L. brevis* und *L. plantarum* zu verhindern, die zum Beispiel in verdorbenen alkoholfreien Erfrischungsgetränken gefunden werden können (Juvonen et al. 2001).

Die Untersuchungen mit chemisch modifizierten iso-α-Säuren ermöglichten somit eine verbesserte Analyse des Wirkmechanismus antimikrobiell wirksamer iso-α-Säuren. Ferner konnten die von BEHR und VOGEL vermuteten individuell ausgeprägten Grade der Resistenz gegenüber oxidativem Stress und protonophorem Effekt bestätigt werden.

Zusammenfassend kann festgestellt werden, dass durch Manganbindung induzierter oxidativer Stress, im Vergleich zu ionophorer Aktivität, von größerer Bedeutung für die iso-α-Säuren vermittelte Wachstumshemmung von *L. brevis* ist.

7 Resumen

La cerveza representa un ambiente hostil para los microorganismos (Back 1994; Sakamoto & Konings 2003) y sólo un grupo de bacterias muy reducido es capaz de hacer frente a los obstáculos presentes en este medio y multiplicarse. El representante más importante de este grupo es *Lactobacillus* (*L.*) *brevis*, el cual es responsable de la mayoría de los casos detectados del deterioro de la cerveza (Back 1994; Sakamoto & Konings 2003; Thelen et al. 2006; Suzuki 2011). Por lo tanto, es indiscutible que la tolerancia al lúpulo es un prerrequisito fundamental para que este grupo especializado de bacterias logre deteriorar la cerveza.

El presente trabajo ha sido diseñado con el objetivo de lograr una mejor comprensión del mecanismo molecular de la inhibición antimicrobiana del lúpulo sobre *L. brevis*. El enfoque sistemático que ha sido elegido, se centra en viabilidad microbiana, fisiología, metabolismo y características electroquímicas de la membrana celular.

Para determinar qué factores influyen en la actividad antimicrobiana del lúpulo, se analizó una gran variedad de compuestos derivados de éste y se determinaron las concentraciones inhibitorias mínimas (MIC, del inglés minimum inhibtory concentration) individuales.

Al comparar extractos crudos de lúpulo, se observó que la polaridad del agente de extracción no influye significativamente sobre el efecto inhibitorio. Igualmente, aparte de pellets de lúpulo, los extractos preparados de diferentes materias primas mostraron MICs consistentes. Esto podría ser atribuido al proceso de peletización, durante el cual las glándulas de lupulina resultan dañadas. Los α-ácidos antimicrobianos son degradados tres veces más rápido en los pellets que en los conos de lúpulo (Biendl et al. 2012). La fracción de resina blanda aportó la mayor proporción del potencial inhibidor de varias fracciónes. Los compuestos anfifílicos, obtenidos en el subfraccionamiento de la resina dura, también presentan actividad antimicrobiana. Este resultado indica que el efecto inhibitorio de estos

compuestos depende de la permeabilidad de la membrana. Los efectos de los compuestos del lúpulo sobre la membrana celular han sido demostrados en variados artículos científicos (Teuber & Schmalreck 1973; Behr & Vogel 2009; Behr & Vogel 2010).

Al comparar fracciones de lúpulo provenientes de diferentes variedades y materias primas utilizando análisis bioinformático cluster, se observó que la actividad antimicrobiana depende, principalmente, de las fracciones respectivas. Por lo tanto, la selección del tipo de lúpulo (lúpulo aromático o lúpulo amargo) pareciera ser más importante respecto a las características organolépticas que al efecto inhibitorio. Sin embargo, no hay que olvidar que las diferencias entre las variedades de lúpulos, podrían no haber sido detectadas debido a la baja solubilidad de los α-ácidos (Spetsig 1955; Stevens 1966; Jaskula-Goiris et al. 2010). La solubilidad de los α-ácidos aumenta significativamente debido a la isomerización durante el proceso de cocción del mosto de la cerveza (Simpson & Smith 1992; García-Villalba et al. 2006).

Se pudo deducir un efecto sinérgico entre ciertos compuestos de lúpulo, ya que algunos de ellos sólo muestran un efecto inhbitorio en combinación con otros. El xanthohumol, por ejemplo, no afecta a las cepas estudiadas de *L. brevis*, mientras que NATARAJAN et al. demostraron un efecto sinérgico entre xanthohumol (y lupulona) y varios antibióticos (Natarajan et al. 2008).

En el presente trabajo, por primera vez, un subproteoma de bajo peso molecular de dos cepas de *L. brevis* ha sido investigado utilizando MALDI-TOF MS para detectar y caracterizar diferentes situaciones de estrés durante un período de análisis prolongado. El MALDI-TOF MS es una herramienta de alta sensibilidad y fácil aplicación. Este novedoso enfoque ha facilitado la exploración fisiológica de estrés inducido por el lúpulo a nivel proteómico, evitando las desventajas propias de la electroforesis en gel bidimensional.

En un experimento de choque en presencia de lúpulo, se observaron respuestas al estrés cepa-específicas. Para *L. brevis* TMW 1.6, la cepa más

sensible al lúpulo, se detectó la proteína identificada como proteína transportadora de acilo (ACP, del inglés acyl carrier protein), durante un periodo de 48 h en presencia de iso-α-ácidos. En la cepa más tolerante al lúpulo (*L. brevis* TMW 1.465), la ACP también pudo ser detectada bajo condiciones de etsrés ácido de referencia y en presencia de xanthohumol.

La ACP forma parte de la ácido graso sintetasa tipo II (FAS II, del inglés fatty acid synthase) (Byers & Gong 2007) y actúa como un medio de transporte para los metabolitos intermediarios entre las subunidades de la FAS II durante la elongación de las cadenas de ácidos grasos (Heath et al. 2002; Zhang & Rock 2008). De esta manera, la ACP está estrechamente relacionada con la biosíntesis de los ácidos grasos y los glicerofosfolípidos, y en consecuencia, con la síntesis *de novo* de membranas celulares y las alteraciones de su estructura. Numerosas publicaciones han informado sobre la reorganización de las membranas celulares en respuesta a varias condiciones de estrés (Mykytczuk et al. 2007). Además, BEHR et al. observaron alteraciones en la composición y la fluidez de la membrana inducidas por la exposición al lúpulo (Behr et al. 2006).

El modelo de la ACP detectado bajo las condiciones de estrés respectivas, puede ser utilizado como un indicador de las alteraciones temporales de las membranas celulares. La detección de la ACP permite concluir que *L. brevis* TMW 1.465 muestra una respuesta al estrés más pronunciada. Teniendo en cuenta la actividad antimicrobiana del xanthohumol (baja) y de los iso-α-ácidos (alta), se puede deducir que los mecanismos de tolerancia de esta cepa son activados por agentes estresantes comparativamente menos fuertes, lo que podría ser un factor determinante de su sensibilidad al lúpulo. Estos resultados concuerdan con los reportados de PREISSLER, en los que se demostró que las cepas con un mayor potencial de deterioro de la cerveza presentan una composición de ácidos grasos de membrana tal que resulta en una menor fluidez (Preissler 2011).

Además, estos resultados demuestran que MALDI-TOF MS puede ser utilizado como una excelente herramienta para caracterizar las respuestas al estrés de *L. brevis*.

En el presente trabajo, se evaluó el papel del sistema ácido glutámico descarboxilasa (GAD, del inglés glutamic acid decarboxylase) en la tolerancia al lúpulo de *L. brevis*. En *L. brevis*, el mecanismo de ácido-tolerancia, llamado sistema GAD, incluye un regulador de la transcripción (Gad-tr), un antiportador ácido glutámico / ácido γ-aminobutírico (GadC), y dos descarboxilasas ($GadB_1$ y $GadB_2$) (Li et al. 2013).

El mayor potencial de deterioro de la cerveza de *L. brevis* TMW 1.465, en comparación con *L. brevis* TMW 1.6, se refleja en tasas de supervivencia más altas y niveles de acidificación del medio más bajas. Además, esta cepa fue capaz de producir ácido γ-aminobutírico (GABA, del inglés γ-aminobutyric acid) cuatro veces más en presencia de iso-α-ácidos, mientras que la cepa sensible produjo altas candidades de GABA en forma constante. Estos hallazgos se sustentan en otros estudios, en los que se demostró que la actividad del sistema GAD y la formación de GABA son características cepa-específicas y que las variaciones se deben a las condiciones ambientales (Li & Cao 2010; Li et al. 2010). Un estudio del pH intracelular (pH_{in}) reveló que *L. brevis* TMW 1.465 es capaz de mantener un gradiente de pH transmembrana (y por eso la fuerza motriz de protones; pmf, del inglés proton motive force) en presencia de ácido glutámico (Glu, del inglés glutamic acid). Lo cual se relaciona con las tasas de supervivencia más altas y la mayor acidificación del medio observadas.

A nivel transcripcional se pudo observar que la expresión de los genes analizados de *L. brevis* TMW 1.6 permaneció estable, lo cual se refleja en los resultados del análisis metabólico. Por lo tanto, se puede concluir que no habriá sido posible compensar un estrés adicional inducido por lúpulo con una mejora en el mantenimiento del pH_{in}. En el caso de *L. brevis* TMW 1.465, el escenario es considerablemente distinto, indicando un nivel de estrés más bajo. Por lo tanto la inducción o la expresión aumentada de estos genes puede ser considerada como responsable de los niveles cuatro veces más altos de GABA y de la mejora en el mantenimiento del pH_{in}.

Por consiguiente, se ha demostrado que las diferencias entre cepas no derivan de la cantidad de GABA formado, sino que de la capacidad de responder a las condiciones ambiental. Igualmente se puede llegar a la

conclusión que en presencia de estrés ácido, la cepa más tolerante al lúpulo no necesita hacer uso de todos los mecanismos de defensa disponibile y por lo tanto, puede responder a un estrés adicional.

Tomados en su conjunto, estos hallazgos demuestran que el sistema GAD puede jugar un papel importante en la reducción del estrés inducido por el lúpulo y puede ser un factor determinante del potencial de las cepas de *L. brevis* para alterar la cerveza.

Los iso-α-ácidos modificados quimicamente se diseñaron y sintetizaron con el objetivo de mejorar la comprensión del mecanismo molecular asociado con la actividad antimicrobiana de iso-α-ácidos (cf. Fig. 38). Los iso-α-ácidos fueron metilados en C-3 para evaluar el papel del grupo hidroxilo ácido (en posición C-3) en la actividad ionóforo y en el efecto antimicrobiano general de la sustancia. La sustitución del grupo carbonilo en la posición C-1 facilitó la revaloración del sitio de ligación de manganeso propuesto (Simpson & Hughes 1993) y su influencia en las características moleculares. Igualmente, se preparó ácidos humulinicos por clivaje de la cadena lateral de isohexenoilo en C-4.

Se pudo confirmar que el grupo carbonilo de C-1 participa en la ligación de manganeso utilizando análisis de absorción UV/Vis y determinando la conductividad de la membrana mediante experimentos utilizando el modelo doble membrana lipídica (BLM, del inglés bilayer lipid membrane). BEHR y VOGEL también demostraron un aumento de la transferencia transmembrana de carga eléctrica dependiente del manganeso (Behr & Vogel 2009). Asimisimo, BEHR y VOGEL han postulado una reacción redox transmembrana dependiente del manganeso que provoca estrés oxidativo (Behr & Vogel 2010). La transferencia transmembrana de carga eléctrica además influye en $\Delta\Psi$ y en pmf (Berg et al. 2003; Foster 2004; Krulwich et al. 2005) y, en consecuencia en la viabilidad microbiana. Por consiguiente, el efecto de iso-α-ácidos (modificados) sobre la conductividad de la membrana puede ser atribuido directamente a la capacidad de ligar el manganeso. La comprobación de la formación de potenciales de membranas, también confirmó la incapacidad de ligar el manganeso en imino-iso-α-ácidos

(modificación en C-1). Experimentos con ácidos humulinicos utilizando el modelo BLM mostraron que la modificación no influeye en ninguno de los experimentos. Igualmente, metil-iso-α-ácidos aumentaron la conductividad permitiendo la formación de potenciales. Esto último fue inesperado debido a que el grupo hidroxilo en C-3 no pudo contribuir a la actividad ionóforo. Por lo tanto, se propone que la estructura de la β-dicetona de los iso-α-ácidos o metil-iso-α-ácidos puede estar sujeta a la tautomería ceto-enólica en soluciones acuosas como se demostró anteriormente (Burdett & Rogers 1964; Otway & Rees 2000; Wu et al. 2002; Sojka 2006; Vigato et al. 2009). En la forma enólica, los iso-α-ácidos exhiben un grupo hidroxilo ácido en la posición C-3 que podría regenerar (en parte) el mecanismo del ácido débil (cf. Fig. 39).

La actividad ionóforo de las sustancias también fue verificada en experimentos con células vivas (determinación de pH_{in}). La tautomería ceto-enólica propuesta fue reconfirmada por estos hallazgos.

Además, se realizó un estudio *in vivo* para evaluar el efecto antimicrobiano de los iso-α-ácidos modificados. El efecto de las modificaciónes sobre el potencial inhibitorio fue igualmente pronunciado en las cuatro cepas estudiadas, indicando que el efecto no depende de la susceptibilidad general de las cepas respectivas. El efecto más destacado fue observado para los imino-iso-α-ácidos ya que no se pudo detectar un efecto inhibtorio. Teniendo en cuenta los resultados analizados anteriormente, se puede deducir que la ligación de manganeso y la concomitante reacción redox transmembrana son cruciales para la inhibición microbiana. La metilación de los iso-α-ácidos redujo la actividad antimicrobioana, lo cual se puede atribuir a que en los metil-iso-α-ácidos, la actividad ionóforo y la reacción redox transmembrana dependen del mismo grupo molecular (en la posición C-1). En consecuencia, sólo una parte de las moléculas puede estar presente en la forma aparentemente más tóxica, la forma ceto. En comparación, los ácidos humulinicos sobrepasaron a los iso-α-ácidos en el potencial inhibitorio, lo que resalta el papel subordinado en la actividad antimicrobiana de la cadena lateral en C-3.

Debido a la combinación de la actividad antimicrobiana y las características organolépticas (no/poco amarga, (Drewett & Laws 1970; Diffor et al. 1972; Palamand & Aldanhoff 1973; Verzele et al. 1973; Schulze et al. 1981)), los ácidos humulinicos aparecen virtualmente predestinado para el uso como conservantes alimentarios. Por ejemplo, los ácidos humulinicos prodrían ser utilizados sin la necesidad de enmascarar la amargura indeseada, para impedir el crecimiento de (por ejemplo) *L. brevis* o *L. plantarum*, los cuales pueden encontrarse en refrescos deteriorados (Juvonen et al. 2001).

Los estudios de los iso-α-ácidos modificados quimicamente, por consiguiente, permitieron una mejor evaluación del mecanismo molecular asociado con la actividad antimicrobiana del compuesto. Igualmente, se lograró confirmar distintos niveles de tolerancia al estrés oxidativo y al efecto protón ionóforo, como propusieron BEHR Y VOGEL antoriamente (Behr & Vogel 2010).

En conclusion, el estrés oxidativo inducido por la ligación de manganeso parece ser más importante en la inhibición del crecimiento de *L. brevis* mediado por iso-α-ácidos que la actividad ionóforo.

8 References

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9 Appendix

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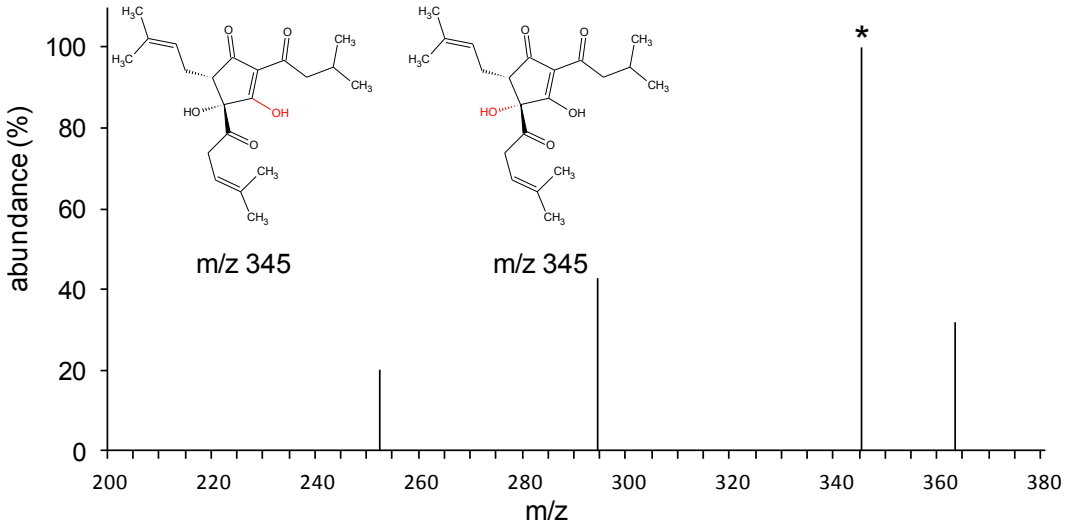
Appendix Tab. 1: Individual minimum inhibitory concentrations (MIC) and standard deviations (sd) of hop compounds (µg/mL) determined according to 2.2.13. na; not available, value above experimental limit (286.3 µg/mL). Hop varieties: M, HALLERTAUER MAGNUM; Tr, HALLERTAUER TRADITION; Ts, HALLERTAUER TAURUS; Pe, PERLE. Raw materials: gh, green hops; rh, raw hops; pl, pellets type 90; et, EtOH-extract; df, hop draff. Hop fractions: tr total resin; sr soft resin; hr hard resin; dr, δ-resin; er, ε-resin; F01-11, er subfractions 1 - 11, cEt, crude EtOH extract; cHex, crude hexane extract; cTHF, crude tetrahydrofuran extract.

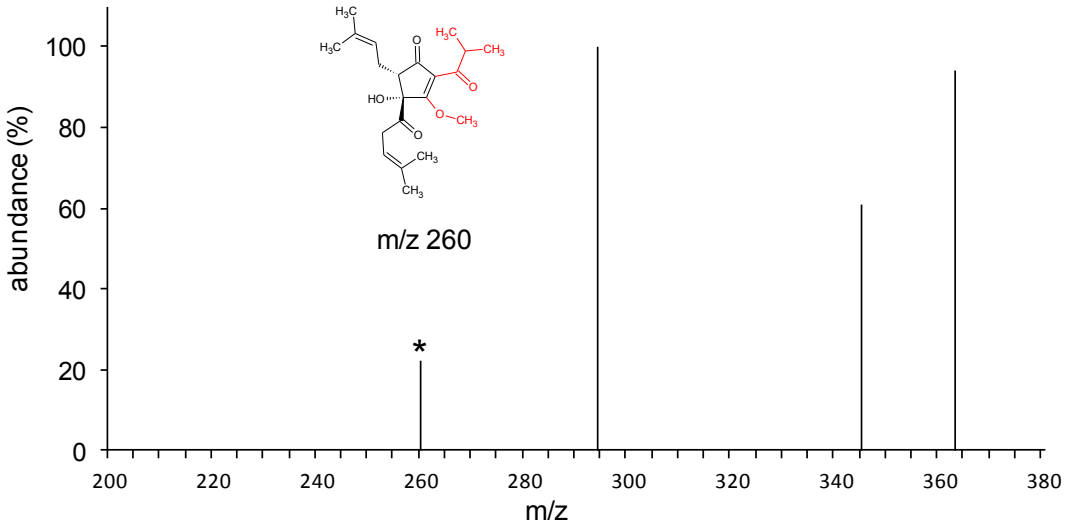
Strain	**TMW1.6**		**TMW1.1369**		**TMW 1.465**		**TMW 1.313**	
Hop compound	**MIC**	**sd**	**MIC**	**sd**	**MIC**	**sd**	**MIC**	**sd**
5-prenylxanthohumol	na	na	na	na	na	na	na	na
6-prenylnaringenine	171.8	0	na	na	na	na	na	na
8-prenylnaringenine	na	na	na	na	na	na	na	na
cis-iso-cohumulone	12.5	2.5	12.5	2.5	30.4	2.5	35.8	0
co-humulone	121.7	10.1	na	na	85.9	0	85.9	0
co-lupulone	28.6	0	na	na	43.0	20.3	43.0	20.3
humulinic acids	1.8	0	1.8	0	3.6	0	3.6	0
imino-iso-α-acids	na	na	na	na	na	na	na	na
Isoxantho-Flav™ 85	na	na	na	na	na	na	na	na
isoxanthohumol	na	na	na	na	na	na	na	na
isoxanthohumol 95 %	na	na	na	na	na	na	na	na
iso-α-acids	3.6	0	3.6	0	16.1	2.5	19.7	2.5
iso-α-acids 90 %	3.9	1.0	4.1	1.1	11.6	3.3	16.1	2.9
M-et-dr	38.2	14.8	38.2	14.8	138.4	52.5	152.7	35.8
M-et-er	34.3	12.8	45.8	15.7	163.2	7.8	209.0	27.9
M-et-er-F01	na	na	na	na	na	na	na	na
M-et-er-F02	na	na	na	na	na	na	na	na
M-et-er-F03	229.1	40.5	200.4	0	229.0	0	na	na
M-et-er-F04	100.2	20.2	128.8	0	186.1	0	286.3	0
M-et-er-F05	50.1	10.2	28.6	0	128.8	20.2	157.5	20.3
M-et-er-F06	28.6	0	28.6	0	71.6	20.2	107.4	10.1
M-et-er-F07	28.6	0	28.6	0	71.6	20.2	85.9	0
M-et-er-F08	28.6	0	28,6	0	57.3	0	85.9	0
M-et-er-F09	28.6	0	28,6	0	71.6	20.2	100.2	20.2

Strain	TMW1.6		TMW1.1369		TMW 1.465		TMW 1.313	
Hop compound	**MIC**	**sd**	**MIC**	**sd**	**MIC**	**sd**	**MIC**	**sd**
M-et-er-F10	43.0	20.3	42,9	0	128.8	20.2	171.8	0
M-et-er-F11	28.6	0	28,6	0	143.2	40.5	250.5	50.6
M-et-hr	57.3	20.3	60.1	21.2	89.7	65.6	171.8	23.4
methyl-iso-cohumulone	46.5	5.1	46.5	5.1	93.0	8.3	93.0	8.3
methyl-iso-α-acids	10.7	1.1	14.6	2.1	51.9	12.7	57.3	10.1
M-et-sr	28.6	0	45.8	15.7	66.8	34.3	120.2	12.8
M-et-tr	28.6	0	42.9	16.5	43.0	16.6	114.5	23.4
M-gh-cEt	38.2	12.7	42.9	13.3	109.7	17.5	164.6	45.9
M-gh-cHex	21,5	8,3	21.5	7.2	51.9	10.8	96.6	42.2
M-gh-cTHF	37.6	13.6	45.3	10.9	75.2	21.5	128.8	43.0
M-gh-hr	na	na	na	na	na	na	na	na
M-gh-sr	31.5	6.4	37.2	12.8	74.4	25.6	114.5	0
M-gh-tr	28.6	0	31.0	5.8	74.0	26.3	105.0	14.8
M-pl-hr	112.1	54.6	131,3	72.7	na	na	na	na
M-pl-sr	45.8	15.7	48.7	12.8	154.6	32.7	206.1	37.3
M-pl-tr	40.1	15.7	54.4	29.3	163.2	49.2	217.6	52.0
M-rh-hr	na	na	na	na	na	na	na	na
M-rh-sr	28.6	0	28.6	0	55.3	35.3	120.2	12.8
M-rh-tr	28.6	0	28.6	0	55.3	35.3	120.2	23.9
n-humulone	57.3	0	na	na	128.9	60.7	128.9	60.7
n-lupulone	28.6	0	28.6	0	71.6	20.2	71.6	20.2
Pe-df-hr	na	na	na	na	na	na	na	na
Pe-df-sr	57.3	0	57.3	0	143.1	0	171.8	0
Pe-pl-hr	na	na	na	na	na	na	na	na
Pe-pl-sr	28.6	0	28,6	0	85.9	0	114.5	0
trans-iso-n-humulone	28.6	0	na	na	28.6	0	28.6	0
Tr-et-hr	46.5	13.7	64.4	14.3	153.9	54.0	221.9	41.3
Tr-et-sr	33.4	8.3	47.9	16.5	124.0	8.3	186.1	14.3

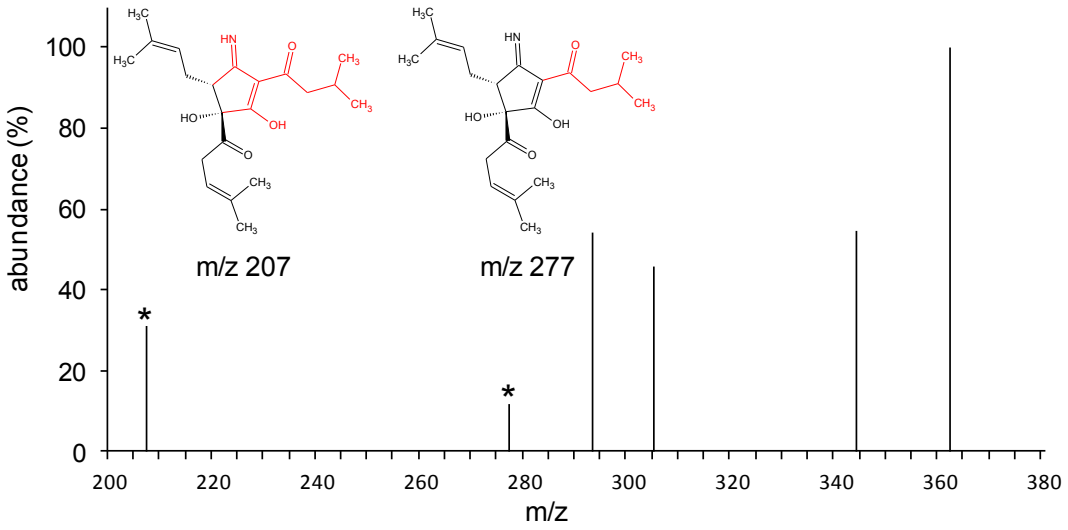
Strain	TMW1.6		TMW1.1369		TMW 1.465		TMW 1.313	
Hop compound	**MIC**	**sd**	**MIC**	**sd**	**MIC**	**sd**	**MIC**	**sd**
Tr-et-tr	35.8	8.3	42.9	16.5	107.3	54.2	171.8	57.3
Ts-df-hr	57.3	0	42.9	0	257.7	0	286.3	0
Ts-df-sr	28.6	0	28.6	0	114.5	0	114.5	0
Ts-pl-hr	114.5	0	85.9	0	na	na	na	na
Ts-pl-sr	28.6	0	28.6	0	85.9	0	114.5	0
Xantho-Flav™	na	na	na	na	na	na	na	na
xanthohumol	na	na	na	na	na	na	na	na
xanthohumol C	na	na	na	na	na	na	na	na
xanthohumol H	257.7	0	257.7	0	na	na	na	na
xanthohumol I	na	na	na	na	na	na	na	na
xanthohumol L	na	na	na	na	na	na	na	na
α-acids	28.6	0	43.0	20.3	93.1	10.1	100.2	20.2
β-acids	28.6	0	28.6	0	71.6	20.2	100.2	20.2



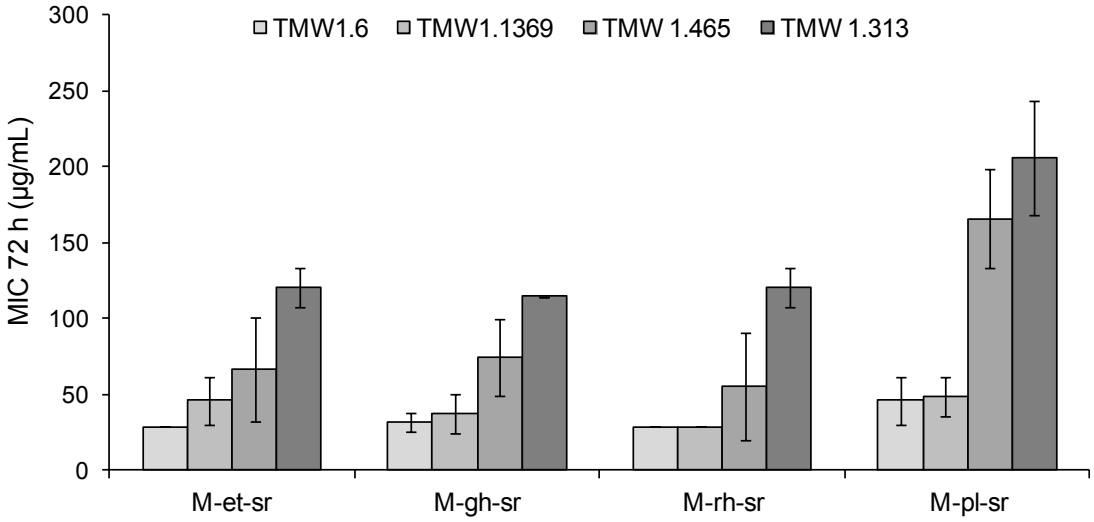
Appendix Fig. 1: Mass spectrum and characteristic fragmentation behaviour of iso-n-humulone obtained by LC-ESI MS/MS and subsequent data processing. Arbitrary abundance units were normalised using the peak of highest intensity as reference. Molecule parts marked in red indicate loss. Asterisk indicates depicted fragments.



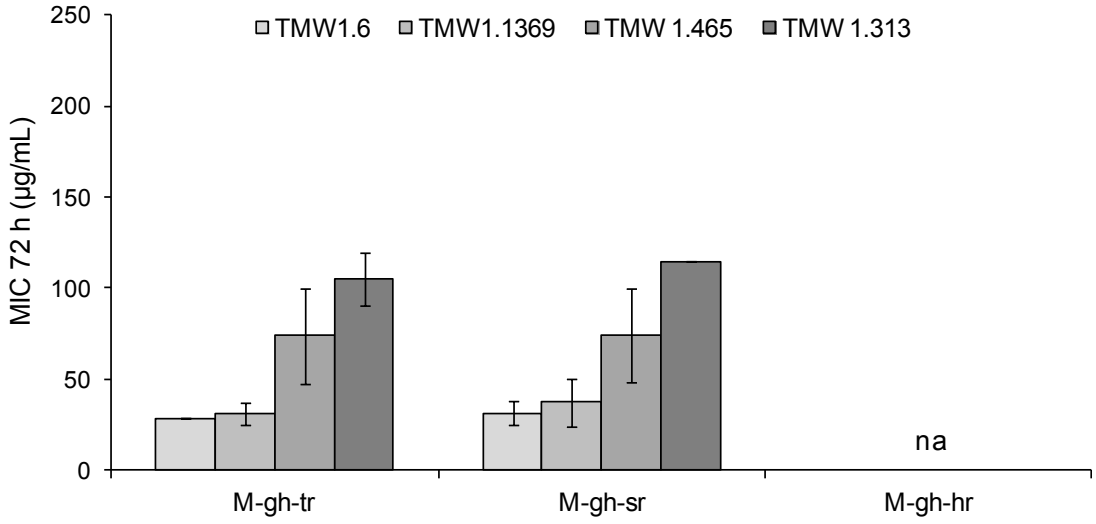
Appendix Fig. 2: Mass spectrum and characteristic fragmentation behaviour of methyl-iso-cohumulone obtained by LC-ESI MS/MS and subsequent data processing. Arbitrary abundance units were normalised using the peak of highest intensity as reference. Molecule parts marked in red indicate loss. Asterisk indicates depicted fragment.



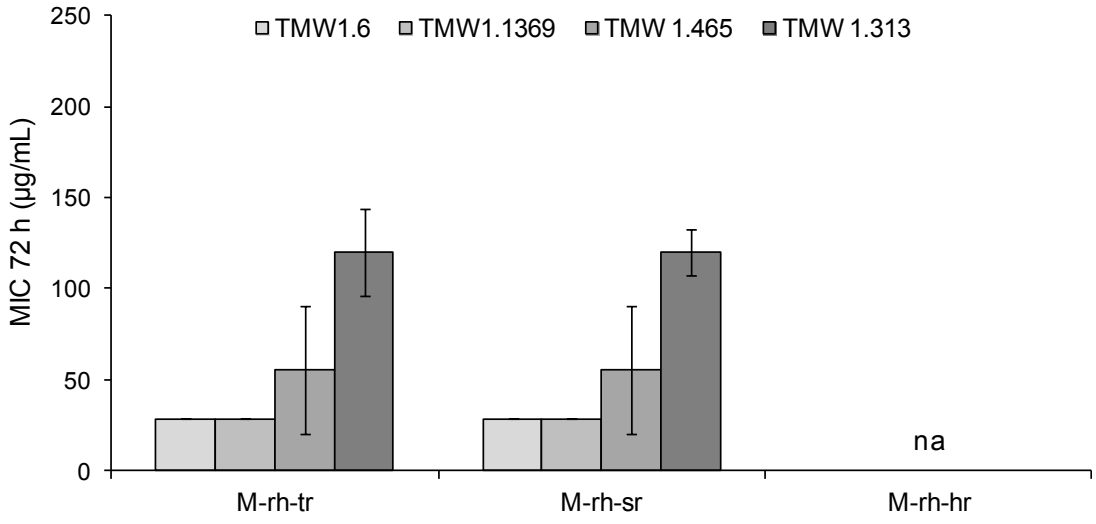
Appendix Fig. 3: Mass spectrum and characteristic fragmentation behaviour of imino-iso-n-humulone obtained by LC-ESI MS/MS and subsequent data processing. Arbitrary abundance units were normalised using the peak of highest intensity as reference. Molecule parts marked in red indicate loss. Asterisks indicate depicted fragments.



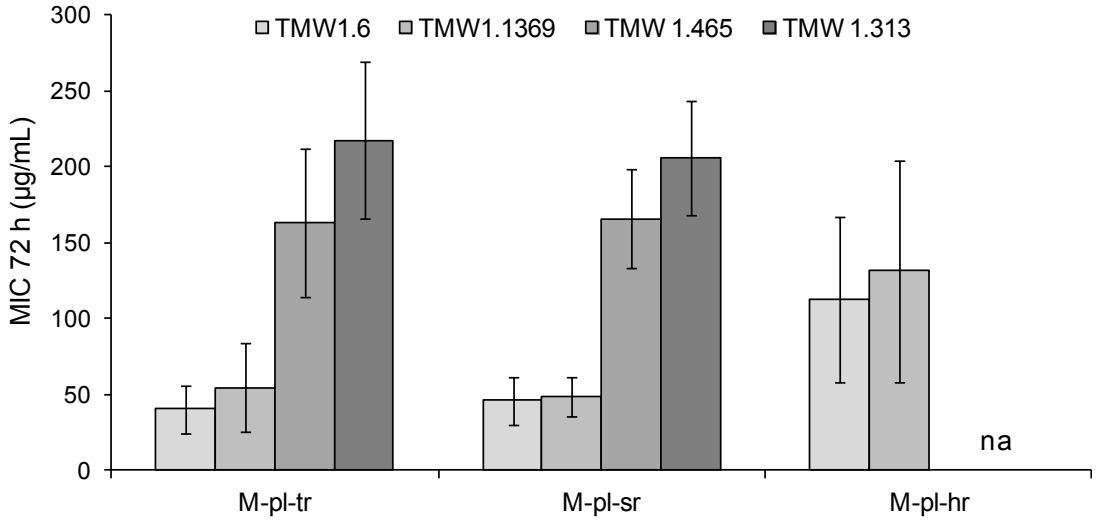
Appendix Fig. 4: MIC of soft resins prepared from different HALLERTAUER MAGNUM hop raw materials. MIC determined after 72 h.



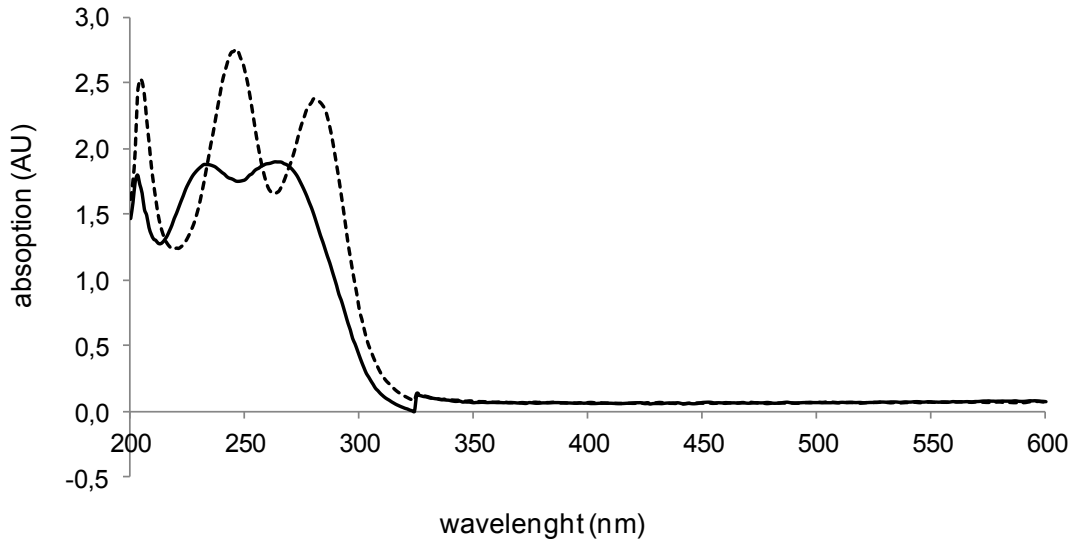
Appendix Fig. 5: MIC of total, soft and hard resin prepared from HALLERTAUER MAGNUM green hops. MIC determined after 72 h. na, MIC above experimental limit (286.3 µg/mL).



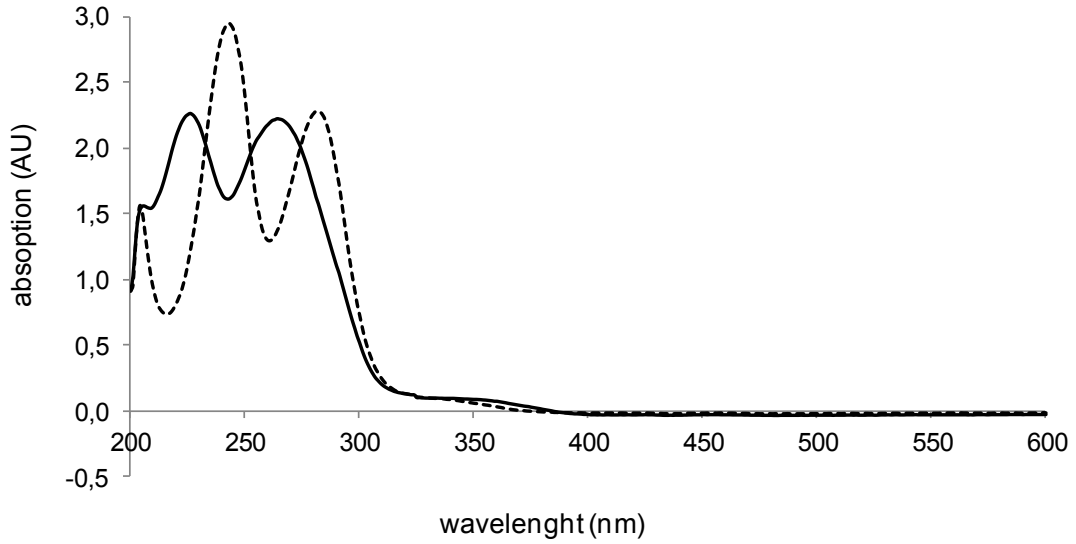
Appendix Fig. 6: MIC of total, soft and hard resin prepared from HALLERTAUER MAGNUM raw hops. MIC determined after 72 h. na, MIC above experimental limit (286.3 µg/mL).



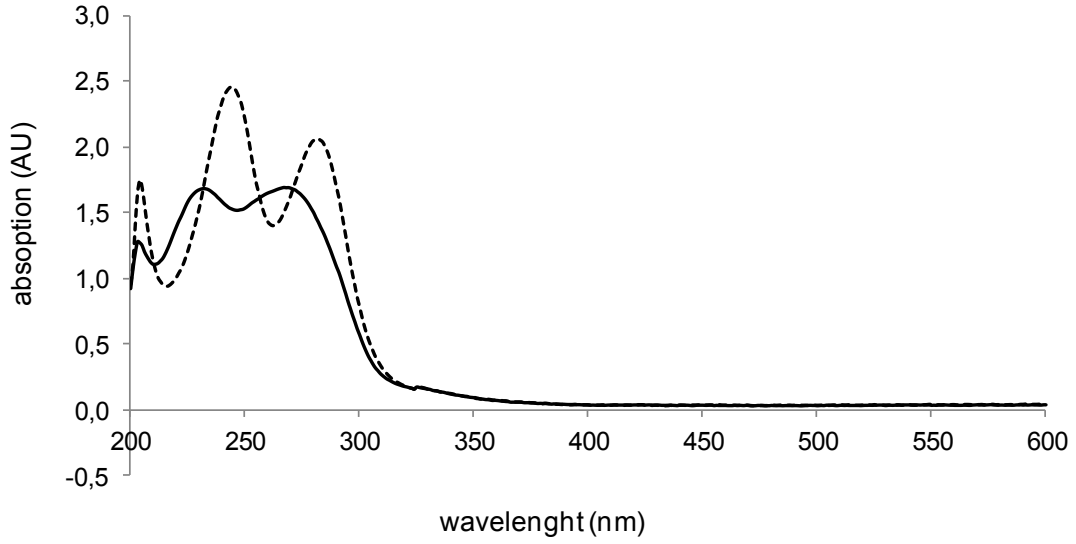
Appendix Fig. 7: MIC of total, soft and hard resin prepared from HALLERTAUER MAGNUM pellets type 90. MIC determined after 72 h. na, MIC above experimental limit (286.3 µg/mL).



Appendix Fig. 8: UV-Vis absorption spectra of cis-iso-cohumulone (solid line) and cis-iso-cohumulone in presence of 10 mM manganese (dotted line) determined in methanol.



Appendix Fig. 9: UV-Vis absorption spectra of humulinic acids (solid line) and humulinic acids in presence of 10 mM manganese (dotted line) determined in methanol.



Appendix Fig. 10: UV-Vis absorption spectra of methyl-iso-α-acids (solid line) and methyl-iso-α-acids in presence of 10 mM manganese (dotted line) determined in methanol.

List of publications that resulted from this dissertation

Original papers

Schurr, B.C., Hahne, H., Kuster, B., Behr, J. & Vogel, R.F.; Molecular mechanisms behind the antimicrobial activity of hop iso-α-acids in *Lactobacillus brevis*. Food Microbiology, 2015, 46, 553-563. doi:10.1016/j.fm.2014.09.017

Schurr, B.C., Behr, J. & Vogel, R.F.; Detection of acid and hop shock induced responses in beer spoiling *Lactobacillus brevis* by MALDI-TOF MS. Food Microbiology, 2015, 46, 501-506. doi: 10.1016/j.fm.2014.09.018

Schurr, B.C., Behr, J., & Vogel, R.F.; Role of the GAD system in hop tolerance of *Lactobacillus brevis*. 2013. European Food Research and Technology, 237(2), 199–207

Oral presentations

„Role of the GAD system for hop tolerance of *Lactobacillus brevis*". Schurr, B.C., Behr, J., Vogel, R.F.; 2012, 3rd International Young Scientists Symposium for Brewing, Distilling and Malting Sectors, Nottingham, UK

„Model organisms in beer spoilage research allow categorization of hop fractions along their inhibitory potential on beer spoiling *Lactobacillus brevis* strains". Schurr, B.C., Behr, J., Preissler, P., Vogel, R.F.; 2010, 2nd International Symposium for Young Scientists and Technologists in Malting, Brewing and Distilling, Freising, Germany

Poster presentations

„Influence of raw materials and extraction methods on the antimicrobial potential of hops". Schurr, B.C., Behr, J., Vogt, C., Dresel, M., Hofmann, T., Vogel, R.F.; 2011, 33rd congress of the European Brewery Convention, Glasgow, UK

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Ausbildung

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