R. F. Vogel, P. Preissler and J. Behr

Towards an Understanding of Hop Tolerance in Beer Spoiling *Lactobacillus brevis*

Lactobacillus brevis **comprises strains with diverse metabolic capabilities. Some specialized strains can grow even in highly hopped pilsner beers without the need of long term adaptation. Other strains develop tolerance upon prolonged hop exposition or remain sensitive. In this communication genetic analyses, proteomics and physiological tests in beer as well as studies in membrane physiology and cell wall composition are reviewed, which are useful to differentiate *L. brevis* strains along their beer spoiling potential and to elucidate decisive marker traits for categorizing beer spoiling *L. brevis* along their relevance for the brewer. Hop adaptation in *L. brevis* TMW 1.465 is a multifactorial process, which results in changes in metabolism, protein profile, membrane and cell wall composition and intracellular manganese levels. It involves mechanisms to cope with intracellular acidification and divalent cation limitation, redox imbalance and oxidative damage and mechanisms for energy generation and economy, genetic information fidelity and enzyme functionality, and as a result enables beer spoilage. Differentiation along the beer spoiling capacity is possible by comparative proteomics, or by physiological tests employing manganese efflux or reduction of tetrazolium to formazan upon growth in lager beer. A simple test for manganese efflux provides a useful tool for categorization of beer spoiling *L. brevis*. On the other hand, genetic determinants potentially useful for PCR detection systems were widely spread in all strains with no reference to high hop tolerance. The further understanding of hop tolerance requires comparative genomic studies revealing critical strain differences and thus multiple (PCR) markers.**

Desriptors: *Lactobacillus brevis*, beer spoilage, hop tolerance, redox potential

1 Introduction

For most bacteria beer represents a bacteriostatic environment, which emerges from the lack of nutrients, presence of ethanol and antibacterial hop compounds, especially (iso)-humulones and lupulones. Therefore, the ability of lactic acid bacteria to grow in beer and thus cause beer spoilage requires intrinsic (lacking target) resistance or acquired hop tolerance. In theory, hop tolerance can be active or passive, i. e. energy consuming or target removal/shielding and consequently enable short or long-term survival in the presence of these bactericidal compounds. To understand hop tolerance, a deep mechanistic insight in the mechanism of hop inhibition on a molecular level is required, as any hypotheses and experimental designs targeted towards explanation of hop tolerance are based on the idea that tolerance should counteract the mechanism of hop action. This investigation enhances the view of hop inhibitory action from a currently accepted purely transport oriented mechanism to an additional transmembrane redox reaction and consequently addresses novel mechanisms of hop tolerance.

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Tables and figures see Appendix

2 Mechanism of hop inhibitory activity

The iso-α-acids, which are considered the major inhibitory compounds, are originally described as ionophores, which exchange H^+ for cellular divalent cations, namely Mn^{2+}, and thus dissipate ion gradients across the cytoplasmic membrane [1]. This generally leads to lowering of the intracellular pH and depletion of metabolic energy, which restrict several hop tolerance mechanisms. By using bilayer lipid membrane (BLM) measurements and growth challenges of hop sensitive and resistant *Lactobacillus brevis* strains in the presence of uncouplers of classes I and II or a H^+/Mn^{2+} exchanger we could revise and extend this view. While the antibacterial action of iso-α-acids as proton ionophores could be confirmed, the reported ionophore properties as electroneutral H^+/Mn^{2+} exchangers could not be verified [2]. Potentiometric measurements indicated a strongly manganese-dependent enhancement of transmembrane charge permeation [2]. The mode of antibacterial action of hop ionophores could be described as proton ionophores of classes I/II, which are capable of transporting protons within a wide range of pH due to their inherent complexity of chemical composition. Further investigation by using cyclic voltammetry in the BLM system identified a transmembrane redox reaction of hop compounds at low pH (as common in beer) and in the presence of manganese (present in millimolar levels in lactic acid bacteria [3]) [4]. The antibacterial action of hop compounds could be extended from the described proton ionophore activity, lowering the intracellular pH to pronounced redox reactivity, causing cellular oxidative damage [5]. Thus, we suggest that both ionophore- as well as oxidant tolerance is required for survival under hop stress conditions. This is corroborated by proteomic studies [6] demonstrating the respective

expression level of several published hop tolerance mechanisms involved in manganese binding/transport and intracellular redox balance, as well as proteins of oxidative stress under "highly reducing" conditions, i. e. anaerobic cultivation and "antioxidative" hop compounds in the growth medium. Figure 1 gives a summary on the categories of hop-inducible proteins. Strikingly, the largest number of identified proteins affected in their expression by hop stress is found within the group of proteins involved in energy generation and redox homeostasis. This clearly supports the role of manganese, which often is part of such enzymes or involved in their reactions. An example for this proteomic reaction [6] demonstrating the high amount of manganese-containing proteins is given in figure 2.

In consequence, this view enables new hypotheses and experimental approaches towards mechanisms of hop tolerance, which reach beyond counteractive transport mechanisms but address cellular redox homeostasis. For both mechanisms the protonation (pH)-dependent access of a hop compound to the bacterial membrane should also be considered.

3 Transport-counteracting hop tolerance

Paralleling the initially described hop action as proton ionophores and H^+/Mn^{2+} exchanger, hop tolerance has been reported to (consequently) reside in cellular transport functions. HorA and HorC are described or proposed, respectively, as multiple drug MDR transporters, which can remove hop compounds from the bacterial membrane in an ATP consuming mechanism [7–9]. HitA is suggested to counteract the ionophore action of hop compounds by its functionality as an Mn^{2+}/H^+ antiporter [10]. Further, bacterial ATPases generally can counteract a hop-mediated incoming H^+ stream by pumping them back again upon consumption of ATP. Indeed, HorA and HorC have been proposed as trans-species genetic markers for differentiating the beer-spoilage ability of lactic acid bacteria because of their presence in various species containing strains with the ability to grow in beer [11, 12]. However, all of these mechanisms are energy-consuming and thus limited in beer, where availability of ready to use small molecules (sugars, amino acids) is reduced as a result of the yeasts fermentation. It was also shown, that high hop tolerance can also be developed in mutant strains lacking transport of hop compounds [13]. Still, this should not be considered a contradiction, as the pH values and hop concentrations of the beers used in these studies were different as a result of personal preference of the consumers in various regions of the world. It is highly unlikely that all levels of hop tolerance in a variety of *L. brevis* strains and ability to grow in any variety of beers can be referred to a single marker gene. Taking this into account we suggest that transport-mediated hop tolerance should be considered as a mechanism to enable growth in moderately hopped beers (referring to ca. 16 ppm iso-α-acids), or in beers with comparably high pH, in which protonation of hop compounds and thus the concentration of active molecules entering the bacterial membrane is low [14]. We found that in beer with low hop content (ca. 8 ppm iso-α-acids), growth is even possible for strains without HorA, HorC and HitA. In highly hopped beers (32 ppm iso-α-acids) they may still provide a short-term survival strategy, providing enough time for the cell to develop alternative mechanisms of passive hop tolerance, which ensure long-term survival.

4 Synergisms of hop and pH tolerance

Hop compounds can be considered weak acids with pK values over a wide range [14] and are protonated depending on the pH of the beer. The protonated fraction can be considered to easily enter the bacterial membrane and/or enable by masking charged hop compounds. A passage through the membrane thus initiate transport and redox reactive functions. The transport functions of the hop compounds result in lowering of the intracellular pH, as protons are transported in the cell. The same effect results from other weak acids in the beer, which are protonated outside the cell at low pH, pass the cellular membrane as uncharged compounds and dissociate at the high intracellular pH. Therefore, any metabolic process compensating the acidification of the cytoplasm, no matter whether it was caused by hop mediated dissipation of the transmembrane gradient or intrusion of weak acids, should therefore help the cell to maintain intracellular pH and survive. In lactic acid bacteria various mechanisms for the maintenance of intracellular pH are described, which are overlapping in various species [15–17]. Proteomic analyses revealed that many strains of *L. brevis* induce arginine metabolism, when it comes to acid or hop stress [18]. This metabolic pathway forms neutralizing ammonia and furthermore generates ATP from carbamyl-phosphate, which may be used for energy-dependent, transport-based hop tolerance mechanisms [19]. Arginine may therefore significantly stimulate survival and also growth of lactobacilli in beer and provide an additional means to prolong the time for development of passive hop tolerance. Growth stimulation makes arginine a good addition to (beer based) detection media for beer spoiling bacteria, as its presence shortens the detection times significantly [13]. In other species of beer spoiling bacteria other strategy of acidification prevention may be found, ranging from re-routing the metabolism from lactic acid to acetoin/butanediol with concomitant diacetyl formation to decarboxylation of amino acids [6].

5 Passive hop tolerance and intrinsic resistance

It is anticipated that transport and acid-tolerance functions in beer-spoilage *L. brevis* strains provide the cells with time to develop mechanisms for hindering the entry of hop compounds through the cell membrane and for minimising dissipation of membrane proton motive force and drop in internal pH. Two potential mechanisms shall be considered. The access of protonated (lipophilic) hop compounds to the membrane may be hindered by changes in lipoteichoic acid (LTA) composition of the cell wall [20]. The finding fosters the hypothesis that hop-adapted cells have higher molecular weight and lower diversity of LTAs, leading to a hydrophilic cell surface. However, these structural improvements were unable to shield the hop resistant cells from penetration by hop compounds [13]. More importantly LTAs located in the cell wall provide a reservoir of divalent cations (including Mn^{2+}), which are otherwise scarce as a result of complexation with hop acids [1]. The altered LTAs also have an increased potential to bind Mn^{2+} and compete for these with the hop compounds, thus reducing

their detrimental effects towards the cell [13, 21]. Therefore, the inability of hop compounds to reach its target (the membrane) is not a major cause for hop resistance [1]. Instead, the hop resistant cells developed a strategy to survive in the presence of hop compounds. This can be an intrinsic cellular property resulting in an "obligate" beer spoiling strain or acquired upon adaptation in a "facultative" strain. Once established, maintenance of this tolerance mechanism is more or less passive. The second "passive" mechanism to limit hop stress can reside in the membrane itself. Upon adaptation to increasing hop concentrations, the membrane composition changes towards the incorporation of more saturated fatty acids rendering the membrane less fluid and protecting the cell against acid stress [13], which is synergistic to hop stress. This affects the functionality of the membrane and can only be tolerated to some extent, depending on the growth temperature and can also be an intrinsic strain dependent property.

6 Redox reactions in hop tolerance and the role of manganese

In the interplay of hop inhibitory mechanisms versus bacterial hop tolerance Mn^{2+} deserves a closer look as it serves antidromic functions. On one hand, the presence of Mn^{2+} strongly enhances hop-mediated electrogenic transport and induction of a lethal transmembrane redox reaction [4]. This redox reaction finally disrupts the cellular balance of the NAD^+/NADH pool required for many cellular reactions, because NADH is oxidized while Mn^{3+} is reduced to Mn^{2+}. In consequence, the cell dies upon metabolic exhaustion. On the other hand, survival and metabolism of lactic acid bacteria are highly dependent on high intracellular Mn^{2+} levels, because many key enzymes of these bacteria contain Mn^{2+} as an essential cofactor. In these bacteria Mn^{2+} frequently takes over the role described for iron in functionally related enzymes of other genera. This strong dependence on manganese generally prevents growth of acid and ethanol tolerant lactic acid bacteria in beer, i. e. their high intracellular manganese content makes them perfect targets for hop inhibition. As a consequence, specialists should be able to grow in beer, which can cope with low intracellular manganese levels thus minimizing hop inhibitory activity, even when it arrives at the cellular membrane. Obviously, some strains of *L. brevis* can adjust very fast to low intracellular manganese levels. These strains can keep up cellular functionality without the need of high amounts of manganese. Others need longer adaptation times and may die before adaptation is achieved. This may delineate "obligate" beer spoilers able to grow in beer without an extensive necessity of adaptation from other variants. Also, such a property need not be restricted to this species.

When such passive tolerance is developing upon hop adaptation, cells are depleting their cytoplasm from manganese, which apparently is stored in form of a counter ion in the negatively charged LTAs of the cell surface. This way little intracellular manganese is available for hop-mediated electrogenic charge permeation through the cell membrane. This view is supported by a comparative proteomic study including hop-adapted and non-adapted strains as well as "obligate" beer spoilers versus strain with adaptive potential and intrinsically sensitive strains of *L. brevis* [18]. In these investigation it was shown, that beyond general stress proteins and chaperones, which are mainly induced by acid stress, a large group of manganese dependent enzymes is overexpressed in the presence of hop. Obviously, hop tolerant strains, no matter whether this were an acquired or intrinsic property, try to compensate for low intracellular manganese levels by overexpression of these enzymes. As a result of this "mechanism" the intracellular manganese content is further minimized fostering hop tolerance and escaping oxidative damage.

In consequence, hop tolerance of bacteria can be described as the multiple tolerance against a heterogeneous mixture of compounds comprising different known and yet unknown charge transport mechanisms, which were dependent on several factors as e.g. compounds concentrations, cation composition and pH value. Thus, only specialists as some *L. brevis* strains, which can cope with unusually low intracellular manganese levels, can survive hop stress. Accordingly, a cross resistance to single proton ionophores or H^+/Mn^{2+} exchangers was not detectable and cannot be expected.

7 Approaches for categorization of beer spoilers

In theory, any difference in growth behaviour of a bacterium should either be referred to its genetic setting, enzymatic activities and/or its metabolism. These differences should therefore be detectable on the gene/genome (presence/absence/functionality), proteome (expression/regulation) or metabolome (basically described by physiology) level. However, the "gold standard" for a brewery will always remain the ability to grow in a given beer, and therefore this behaviour must also be the reference for any other differentiation method. A set of carefully evaluated strains showing different growth behaviour in various beers is therefore the mandatory prerequisite for the development of any less time-consuming, easier to handle test. To establish such a set of characterized bacteria with intrinsically different properties, which are not the result of adaptation to hop, we have grown strain in the absence of hops for many generations. Subsequently we used these in growth tests in beers with various hop and ethanol contents [22]. Figure 3 shows an example of the growth curves obtained with various strains of *L. brevis* in a regular German lager beer. Only strain TMW 1.313 was able to grow without delay, while some strains expressed a prolonged lag-phase, and a third group never acquired the ability to grow in this environment. The results of this investigation also showed that differences in growth behaviour are not detectable in every beer, and sorting of strains to fixed categories is restricted to a specific beer [18]. While virtually all strains were able to grow in alcohol free wheat beer, only a few strains were able to grow in a highly hopped Pilsner beer [23–25]. Categories of "wheat-beer-*L. brevis*" strains (containing "non-adaptive" strains only) *versus* "Pilsner-*L. brevis*" strains (containing "constitutive" and "adaptive" strains) can be delineated. This categorization fits with practical experiences in breweries. By the use of non-adapted stains obtained upon prolonged growth in laboratory media the category of "adaptive" strains, which can develop hop tolerance upon longer lag phases they exhibit in stronger hopped beers, can be delineated from the "constitutive" strains. Based on the results other differentiation methods can be developed along hypotheses derived from the described hop action/hop tolerance mechanisms.

As a result of the demand for easy detection methods currently the differentiation along presence/absence of genes by PCR techniques has been mostly followed (not only for beer spoilers). Obviously, detection on the species level, based on the 16 S rDNA Sequences of bacterial species cannot differentiate *L. brevis* strains along their beer spoiling capabilities. A promising candidate was proposed with HorC [9, 12] following the hypothesis, that beer spoilage ability was mainly determined by transport. As recent investigations rather revealed a multifactorial process, selection of a single genetic marker is generally limited, which is also unable to quantify a beer spoiling potential [26]. We have tested various strains of *L. brevis*, which were carefully checked for their beer spoiling potential for the presence or absence of several genes, which were identified via reverse genetics in a comparative proteome approach. However, at least with the genes selected so far (transporters, genes of the arginine pathway, stress proteins), we only found a very limited congruence with the ability of the strains to grow in beer [18]. While figure 4 and table 1 demonstrate the potential of proteome-based differentiation of beer spoiling *L. brevis* strains, table 2 shows that the same markers are not useful as single markers on a genetic level. This finding is not restricted to the example proteins shown here. For example, HorA was also found in some non-beer spoilers by us and other groups [27]. On the other hand, HorA PCR-positive strains did not always express functional HorA. This may result from co-amplification of lmrA sequences. LmrA is a homologous MDR transporter sharing the same ATP binding cassette, and is found in many lactobacilli. Another reason for false PCR positives results from the presence of mutations in some of the HorA genes, which cannot be detected by PCR approaches. Indeed, sequencing of several HorA genes from various strains, revealed such mutations (unpublished data). Thus, the detection of false positives by PCR is an apparent general limit, not restricted to HorA.

To date, comparative proteomics is an advanced analytical technique reserved for research laboratories with highly educated personnel, rather than a routine technology feasible for a brewery. Nevertheless, strains with different capabilities to grow in beer can be clearly distinguished by this technique. In comparative analyses of different *L. brevis* strains we could demonstrate significant differences between beer spoiling and non-beer spoiling strains. Ongoing analyses revealed a large number of differentially expressed proteins to manganese containing/dependent or pointing to manganese as a general regulator when it comes to the defense of oxidative stress (as induced by hop components) [4, 6, 28, 29].

Finally, the repeated hints to the importance of manganese levels in beer spoiling bacteria, the different capabilities of *L. brevis* strains to cope with low intracellular manganese, and the ability of hop tolerant strains to decrease intracellular manganese levels can be used to simple physiological differentiation of strains: a high beer spoiling potential should be indicated by a fast release of manganese upon hop stress. Indeed, preliminary experiments show that a simple test based on manganese efflux can differentiate *L. brevis* strains along their ability to grow in the presence of hop (unpublished data). Figure 5 demonstrates the differentiattive ability of such tests. While strains belonging the the category of "wheat-beer-*L. brevis*" can be distinguished from "Pilsner-*L. brevis*", the latter cannot be sub-categorized to "constitutive" versus "adaptive" strains (compare Figure 3 and respective text).

8 Summary and outlook

Hop tolerance developing during adaptation appears as a multifactorial dynamic property summarized in figure 6. A basic (low level) hop tolerance is provided by various mechanisms of transport, some of which are general properties of lactic acid bacteria, while others are pronounced in strains of *L. brevis*. This basic tolerance provides the cell enough time to express a second level of hop and acid defence. Arginine catabolism contributes to energy and proton motive force generation and, together with rerouting of lactic acid fermentation towards butanediol formation and changes in the cellular membrane composition, minimizes acid stress. Concomitantly, high molecular weight LTAs replace a large variety of smaller ones and pick up manganese ions released from the cell, rendering them unavailable for the hop compounds to initiate enhanced electrogenic transport and a transmembrane redox reaction. While some strains do not express these properties and thus are unable to grow in beer, others can adapt to quite high hop concentrations upon long term adaptation. A third group of strains, which is comparably rare, expresses intrinsic high hop tolerance. These strains deserve more attention to be studied.

The specific detection of strains with the constitutive or adaptive potential to grow in beer by PCR-detection of genes is tempting, as PCR-based tests are a wide-spread standard in the food industry and can be performed in many breweries. While a single gene marker approach can currently not be achieved, the development of a genome-based genetic barcode delineating biotypes of *L. brevis* is promising. Also, upcoming methods of second generation proteomics employing MALDI-TOF MS techniques replacing gel-based electrophoretic approaches enable exploitation of the discriminative potential of proteomics in fast, robust analyses.

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Appendix

strain	ADI	ClpL	ADH	
L. brevis TMW 1.313	+	+	+	spoilage
L. brevis TMW 1.485	-	+	+	
L. brevis TMW 1.230	-	+	+	
L. brevis TMW 1.465	+	-	+	
L. brevis TMW 1.315	+	+	+	
L. brevis TMW 1.6	-	-	-	nonspoilage
L. brevis TMW 1.302	-	-	-	
L. brevis TMW 1.1369	-	-	-	
L. brevis TMW 1.1370	-	-	-	
L. brevis TMW 1.1371	-	-	-	

Table 1 **Expression of the potential marker proteins shown in figure 4 in strains of *L. brevis* with different abilities to grow in lager beer. Arginine deiminase (ADI), chaperonen/protease (ClpL) and zinc-dependent alcohol dehydrogenase (ADH)**

strain	ADI	ClpL	ADH	
L. brevis TMW 1.313	+	+	+	spoilage
L. brevis TMW 1.485	+	+	+	
L. brevis TMW 1.230	+	+	+	
L. brevis TMW 1.465	+	-	+	
L. brevis TMW 1.315	+	+	+	
L. brevis TMW 1.6	+	-	+	nonspoilage
L. brevis TMW 1.302	+	-	+	
L. brevis TMW 1.1369	+	-	-	
L. brevis TMW 1.1370	+	-	+	
L. brevis TMW 1.1371	+	-	-	

Table 2 **Potential of PCR detection to differentiate beer spoiling *Lactobacillus brevis* strains. Presence of arginine deiminase (ADI), chaperonen/protease (ClpL) and zinc-dependent alcohol dehydrogenase (ADH) in the genome of different *L. brevis* strains. See text for an explanation of the seeming contradictions as compared to the proteomic analyses**

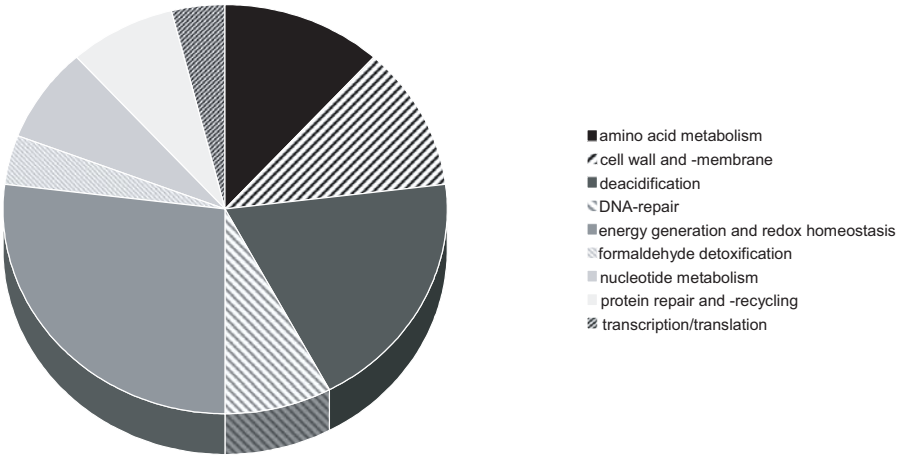

Fig. 1 **Enzyme categories induced upon hop stress. Categories are listed in clockwise direction and delineated with shades**

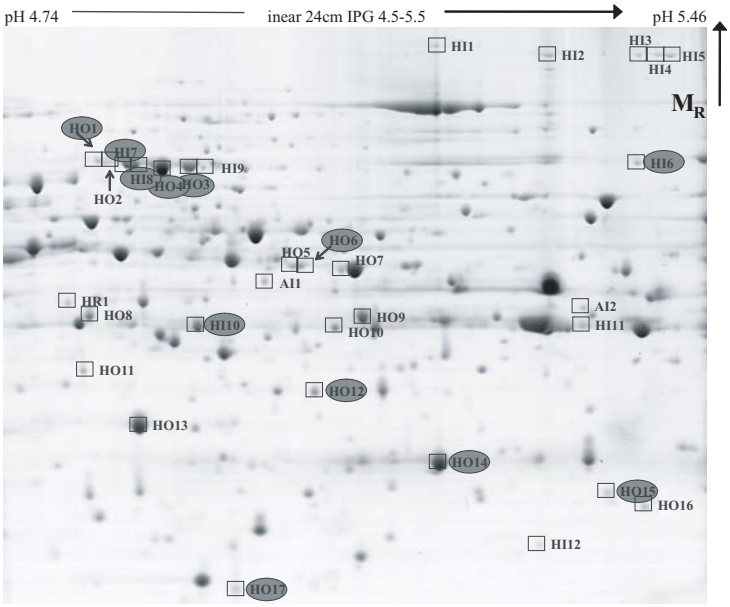

Fig. 2 **Hop-inducible proteins in *Lactobacillus brevis* TMW 1.465A.**

IPG= immobilized pH gradient,

M_R= molecular weight.

Proteins induced upon hop and acid stress are marked "H" and "A", respectively. Manganese-dependent enzymes are marked with a grey oval shade

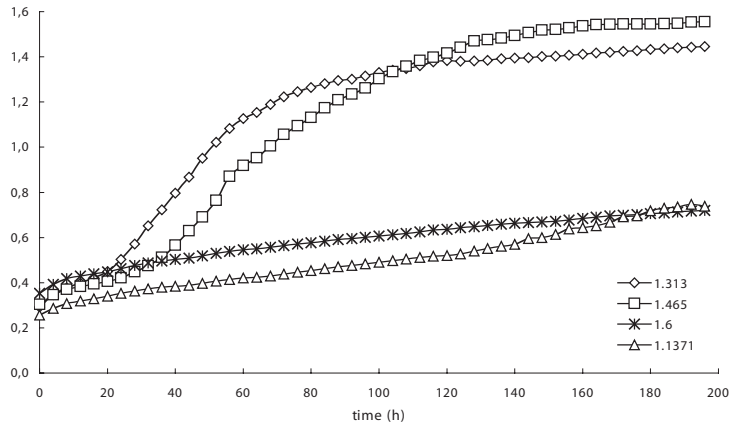

Fig. 3 **Growth behaviour of representative members of non-adapted *Lactobacillus brevis* strains in lager beer with medium hop content. *L. brevis* strains are considered as**

(i) "constitutive beer spoilers" growing in every beer without lag phase (1.313),

(ii) "adaptive beer spoilers" exhibiting longer lag phases in stronger hopped (lager, pilsner) beers (1.465) or

(iii) "non-adaptive beer spoilers" only able to grow in non-hopped or weakly hopped (wheat) beers (1.1371, 1.6)

Fig. 4 **Potential of differential proteomics to differentiate beer spoiling *Lactobacillus brevis* strains.**

HopstressinducesADI(argininedeiminase)=1, ClpL (chaperone/protease) = 2 and (ADH (zinc-dependent alcohol dehydrogenase) = 3 (compare Table 1).

IPG= immobilized pH gradient

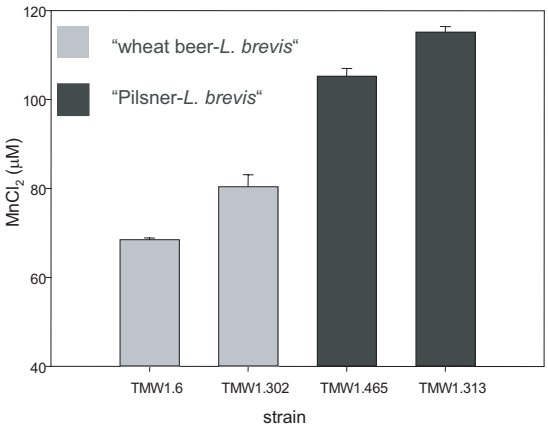

Fig. 5 **Manganese efflux in different *Lactobacillus brevis* strains. *L. brevis* TMW 1.6 and 1.302 release little manganese, whereas strains 1.465 and 1.313 release high amounts upon 3-h hop challenge in beer. This enables them to minimize hop-induced redox reactions and thus survive in beer (compare also Fig. 3 and respective text for categorization of beer spoiling *L. brevis*.)**

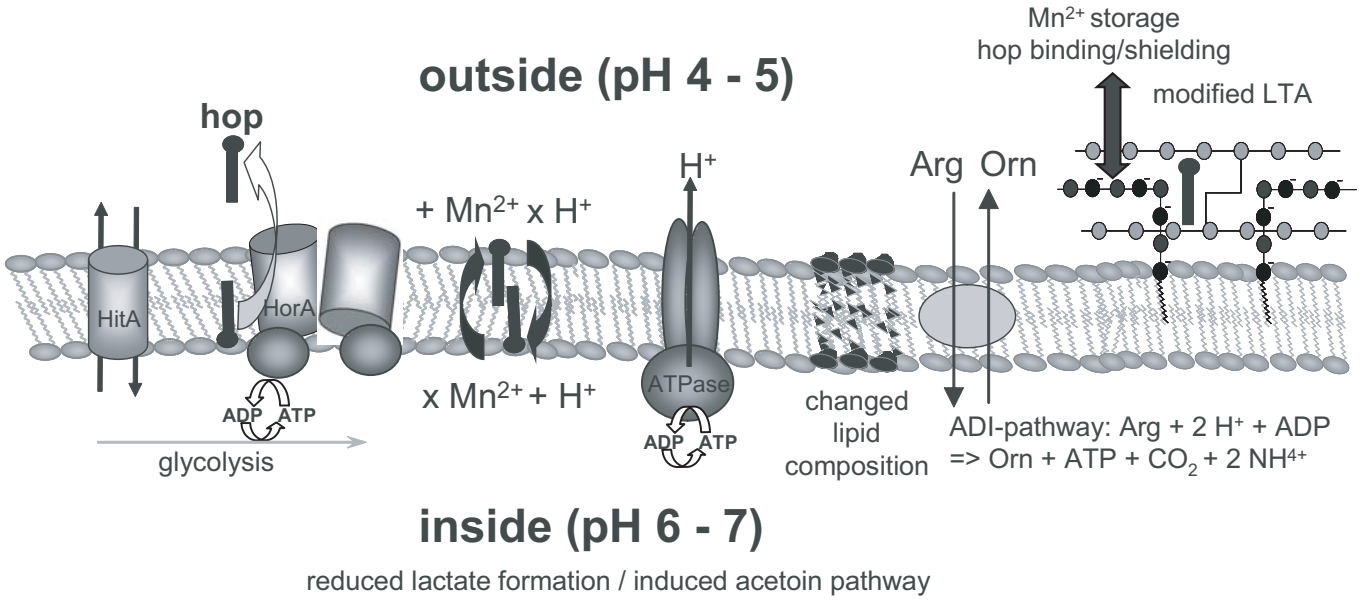

Fig. 6 **Transport, barrier and acid stress minimization-based hop tolerance functions in *Lactobacillus brevis***