The role of plant-based antimicrobials in food and feed production with special regard to silage fermentation

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Die Bedeutung antimikrobieller Wirkstoffe pflanzlichen Ursprungs in der Lebens- und Futtermittelherstellung mit besonderer Betrachtung der Silageherstellung

1 Introduction

1.1 Plant-based Antimicrobials in Sugar Production

Since the early 1990ies plant components have been used under practical conditions for the control of infections in the extraction area of sugar factories. Due to several reasons, the industry voluntarily abstained from using formaldehyde, which was the most commonly used biocidal substance in sugar production at that time. However, in the first years after the ban of formalin, the sugar factories had to accept reduced yield along with operational problems in the extraction area caused by contaminating thermophilic lactic acid bacteria. At that time, it was discovered that higher lactic acid levels in the processed juices lead to better pressability of the beet pulp and eventually to savings in energy consumption (HOLLAUS et al., 1986; POLLACH et al., 1988). Also, some alternative substances to formalin were

Zusammenfassung

Das Ziel der vorliegenden Arbeit bestand in der Bestimmung der minimalen Hemmkonzentration natürlicher pflanzlicher Wirkstoffe gegen üblicherweise bei der Silageherstellung vorkommende Mikroorganismen mittels dreier mikrobiologischer Standardtestverfahren.

In den Ergebnissen zeigen die Wirkstoffe auf Basis von Hopfen-β-Säuren und Harzsäuren lediglich geringe Wirksamkeit gegen gramnegative Bakterien, Hefen und Schimmelpilze. Im Gegensatz dazu wurde eine Hemmung grampositiver Bakterien bereits in sehr niedrigen Konzentrationen erzielt. Interessanterweise zeigen einige Vertreter aus der Gruppe der Milchsäurebakterien eine wesentlich geringere Empfindlichkeit gegen die pflanzlichen Wirkstoffe als Verderbskeime wie Clostridien. Diese Ergebnisse bieten möglicherweise Potential für den Einsatz von Hopfen-β-Säuren und Harzsäuren als Silierzusätze, da sie offensichtlich in der Lage sind, bakteriellen Silageverderb zu hemmen ohne das Wachstum von Milchsäurebakterienstarterkulturen negativ zu beeinflussen.

Schlagworte: Antimikrobielle Wirkstoffe, Silageverderb, Hopfensäuren, Harzsäuren, minimale Hemmkonzentration.

Summary

The aim of this study was to determine the minimum inhibitory concentration of plant-based antimicrobials by means of three microbial standard test methods on a range of micro-organisms usually found in silage.

Results indicate that hop beta acids and rosin acids were not very effective against Gram-negative bacteria, yeasts and moulds. On the contrary, Gram-positive bacteria could be inhibited at very low concentrations. Interestingly, some representatives of the lactic acid bacteria group showed lower sensitivity against the plant-based antimicrobials than spoilage micro-organisms such as clostridia. This result offers some potential to apply hop beta acids and rosin acids as silage additives, as they are capable of inhibiting bacterial spoilage without negatively affecting the growth of lactic acid bacterial starters.

Key words: Antimicrobials, silage spoilage, hop acids, rosin acids, minimum inhibitory concentration.

considered to be of interest but were not effective enough or economically applicable. Subsequently, during continued studies on the improvement of the pressability of the beet pulp, in order to further reduce energy costs, interesting observations were made. It was discovered, that hop components can be utilised for the control of microbial activity in the sugar extraction (POLLACH et al., 1996; HEIN and POLLACH, 1997). These substances offered some major advantages to formalin as they were well known for their antimicrobial effect and possess a long history in the brewing industry. Furthermore, they are regarded as harmless for humans and animals. Rosin acids play a similar role as hop acids in the brewing industry, as they are used to prevent the spoilage of Retsina, a typical Greek wine. Fatty acids, on the other hand, are commonly found in plant oils, dairy products etc. and many applications have indicated their beneficial properties (NARZIß, 1986; BROCKMANN et al., 1987; JOHNSON et al., 1973; HORNSEY, 2007; BEUCHAT and GOLDEN, 1989; SÖDERBERG, 1990).

A milestone in the development of a marketable and easy-to-use product was achieved when cooperation was established with a well known hop company. Gradually, the application technique of the plant substances could be improved, thus finally resulting in an innovative "concept of natural antibacterials for the sugar industry". Corresponding details can be found in the studies by *Pollach* and *Hein* (POLLACH, 1995; POLLACH and HEIN, 2001; POLLACH et al. 2001, 2002, 2004; HEIN et al., 2006).

Meanwhile, the main focus of the application had shifted from combating thermophilic micro-organisms in the extraction area to other steps of the sugar production. In Hrusovany, a Czech sugar factory, operational problems due to heavy dextran formation caused by bacteria of the genus *Leuconostoc*, could be solved by the application of hop beta acids. Further, hop beta acids were successfully applied to repel a clostridia infection in an Austrian sugar factory thus establishing the wanted flora of lactic acid bacteria in that area. These observations as well as experiences in cooling water circuits, in the storage of thick juice and in beet storage trials have demonstrated that mesophilic micro-organisms can be also inhibited by applying these natural antibacterials (EMERSTORFER, 2005; HEIN et al. 2006).

Until that time, investigations have almost exclusively been focusing on solving problems of microbial contaminations in the process and on questions concerning intermediate and end-products of the sugar production such as white sugar, molasses, and thick juice (POLLACH et al., 1999; HEIN et al., 2002). Expectedly, there was some need to ensure that an application of hop bitter acids does not interfere with the taste of white sugar. Thus, sensory tests were carried out to dispel these concerns. Results from residual studies also indicated no negative effects on the subsequent application of molasses in fermentation. At the same time it was assumed that the major part of the products applied in the extraction area finally ends up in the pressed beet pulp. Due to the fact that farmers did not report about problems with the antibacterial remnants in the pressed pulp in the course of silage production, no further research activities were needed in this direction. However, already in 1999, *Pollach* et al. assumed positive effects linked to the substance-remnants in the pressed pulp as inhibition of unwanted groups of micro-organisms was observed (POLLACH et al. 1999, POLLACH, 2002; GUDMUNDSON, 1998).

Against this background, considerations to expand the fields of application for the substances concentrated on repeatedly observed effectiveness of hop beta acids on clostridia. Several studies on "natural antibacterials" revealed that Gram-positive bacteria – obviously due to their specific cell wall structure – can be inhibited even at low concentrations, while Gram-negatives, yeasts, and moulds are rather resistant. As a matter of fact, research activities concentrated on areas where Gram-positive contaminants need to be eliminated or even pathogens of this group may cause some economic impact. For example, bacterial sources of mastitis in dairy cattle and the production of silages as a feed stock could be identified as promising fields of application (WILKINSON and TOIVONEN, 2003; DEUTZ et al., 2004). The latter topic will be dealt with in this study.

1.2 Plant-based Antimicrobials in Silage Fermentation

Apart from yeasts and moulds – which are not discussed in detail in this study – clostridia are one of the major disturbing factors in silage fermentation, especially in the field of grass silage. The individual properties of the plant raw material exert some pronounced influence on the development of the microbial ecology in silage. Practically, it is absolutely necessary to comply with certain rules in silage making (DLG, 2006).

It is well-known that inhibition of clostridial growth during the ensilage process is achieved if a fast decline in pH can be obtained by a strong metabolic activity of lactic acid bacteria that naturally occur in the plant material. In silage production, different minimum pH-values are needed to stabilise the silage, depending on the dry matter content

(WEISSBACH, 2002). Under certain circumstances (contamination with soil during harvesting, low initial cell counts of lactic acid bacteria in the source material, low carbohydrate content and high buffering capacity, low dry matter content, lack of natural inhibitory substances, etc.) the desired pH-reduction will be delayed leading to instable conditions during fermentation. In such a case, clostridia can proliferate and spoilage of the silage by these anaerobes is induced.

As stated in several studies the nitrate content in the plant raw material also strongly influences the fermentation process (POLIP, 2001; WEISS, 2000). In case of a slow decrease in pH and in presence of nitrate in the initial phase of the process, formed lactic acid is rather converted to acetic acid in a first step, and after complete reduction of nitrate, further metabolised to butyric acid. Inhibition of clostridia by nitrate depends on the fermentation coefficient (FC-value) of the feedstock. German authors specify a minimum nitrate concentration of 1.3 – 5.8 g/kg dry matter necessary in order to get sustained suppression of clostridial growth in grass silage (DLG, 2006). If no nitrate is present in the plant raw material, lactic acid is directly converted to butyric acid immediately after start of the fermentation – an issue that has increasingly been observed in the last years due to reduced use of nitrogen fertilizer, even though silages contain rather high dry matter contents. This malfermentation leads to increased pH-values and to extensive production losses due to subsequent proteolysis by clostridia. These silages can be regarded as spoiled and do not meet the qualitative requirements of animal feeds.

Apart from the above described spoilage problems, silages containing butyric acid also bear another hygiene problem. Associated with clostridia, diverse pathogenic bacteria may be found in such products as there is insufficient acidity to inhibit this micro-flora (DRIEHUIS et al., 2000). Contaminated silage therefore may, for example, subsequently contaminate the milk in primary production, and contaminated milk is an improper source for the production of dairy products, even if several steps of processing and heating are applied. It is a well-known fact that Swiss-type cheese can only be produced from raw milk of high quality and free of clostridial endospores. Otherwise quality problems as well as economic losses will result for farmers as well as the dairy industry (WILKINSON and TOIVONEN, 2003; ERNST, 2005; ROBINSON and WILBEY, 1998; ADLER, 2002).

Under practical conditions, the plant material available is not always of optimum quality, it may lack fermentable sugars, also high buffering capacity or low dry matter concentration are of disadvantage. To support the natural preservation process, silage additives are increasingly used in order to prevent the development of unwanted contaminants. A commonly used method directly applies organic acids (e.g., formic acid) in order to rapidly achieve a low pH-value that forms a barrier for clostridial growth. However, the application of acids bears some disadvantages like aggressiveness, corrosion of equipment and safety measures for the user. As a consequence, neutral silage additives such as salts and salt solutions have gained in importance over the years. These products exert inhibitory effects not only due to their ability to reduce the pH of the silage material, but also – depending on the achieved pH-level and the number of carbon atoms of the applied substance – to a bacteriostatic or even microbicidal effect which originates from the undissociated proportion of the acid.

A biological alternative is the use of lactic acid bacteria as silage additives to intensify the natural production of lactic acid, especially in the crucial stages of the fermentation. The application of lyophilised starter cultures ensures a high initial number of lactic acid bacteria and an accelerated conversion of plant sugars to organic acids. Concomitantly the pH falls to disadvantageous levels for clostridia species and many pathogens. In practice, combined silage inoculants containing homo- and heterofermentative strains are very common, whereas the latter are intended to form acetic acid which has an antimycotic effect (KRAMER, 2002). Lactic acid starters can also be combined with other supplements to achieve a further drop in pH, for example with enzymes that are added to support the generation of fermentable substrate for the bacteria. Also, additives such as molasses have been widely applied in the past. Common to these measures is the aim to raise the content of fermentable sugars in the plant material that can be converted to organic acids by active lactic acid bacteria (DLG, 2006; THAYSEN et al., 2007).

One of the most effective and commercially available silage additives is based on nitrite and hexamethylenetetramine. In sausage manufacture, nitrite is part of the curing salt mixture which is well-known for its clostridia-inhibiting properties. In silage, nitrite is converted to nitrous gases in the early phase of fermentation where especially clostridia species are inhibited. Lactic acid bacteria, on the other hand, are not negatively affected by nitrous gases although they are highly toxic to animals and humans. Therefore, appropriate precautions have to be taken during handling. Hexamethylenetetramine, the second active ingredient of the combination, is hydrolysed during pH drop progression, liberates formaldehyde and therefore delivers

sustained protection against clostridia proliferation, also at later stages of the fermentation process (LÜCK, 1985).

More recent studies suggest the combination of starter cultures with active ingredients of various origins for inhibiting clostridia. For example, bacteriocin-synthesizing bacteria can be seen as a promising alternative to nitrite. However, only limited effort has been made so far to implement these strains in commercial products (CZECH, 2003; TÜRK, 2005; FLYTHE et. al, 2004; KRAMER, 2002).

The objective of this work was to assess the inhibition characteristics of naturally derived plant ingredients (hop beta acids, rosin acids, myristic acid) against a selection of practically relevant micro-organisms common in silage production (SZALAY, 2007).

2 Materials and Methods

2.1 Test Methods for the Assessment of the Minimum Inhibitory Concentration (MIC)

Microbial strains

In Table 1 the microbial strains tested in this study are summarized. The table features the origin, isolation source and cultivation conditions of the investigated bacteria, yeasts and moulds.

Antimicrobial Plant Components and Standard Solutions

BetaStab 10A® produced by BetaTec GmbH (Nuremberg, Germany), provided by Zuckerforschung Tulln GmbH; an aqueous, alkaline solution (pH 10.0–11.5) containing approx. 10 % (w/w) of a mixture of resins and resin acids of hops, principle component being hop beta acids (lupulone) (BETATEC, 2005).

PineStab 20A, provided by Zuckerforschung Tulln GmbH (Tulln, Austria); an aqueous, alkaline solution (pH 10.0–11.5) containing approx. 20 % (w/w) of a mixture of pine resin acids, principle components being isomers of abietic acid and dehydroabietic acid (ZFT, 2004).

PileStab 20A, provided by Zuckerforschung Tulln GmbH (Tulln, Austria); an aqueous, alkaline solution (pH 10.0–11.5) containing approx. 12 % (w/w) of a mixture of pine resin acids, principle components being isomers of abietic acid and dehydroabietic acid and 8 % (w/w) of myristic acid (ZFT, 2004).

Hydrogen peroxide, caustic potash solution: In the experiments dilutions of a 3 % (v/v) H_2O_2 stock solution were used as a reference for the plant components (dilution series were freshly prepared every two weeks using distilled water);

KOH (pH 13) was used as a control to ensure that micro-organisms were inhibited by the active ingredients and not by acidity.

Culture Media

Broth Media

Tryptic Soy Broth (Oxoid CM 129), RCM Broth (Reinforced Clostridial Medium) (Merck 1.05411), Brain Heart Infusion Broth (Oxoid C 0225), M17 Broth acc. to Terzaghi (Merck 1.15029), MRS Broth (Lactobacillus Broth acc. to de Man, Rogosa and Sharpe) (Merck 1.10661.500), Wort Broth (Merck 1.05449), Meat Broth (typical composition).

Agar Media

Tryptic Soy Agar (Oxoid CM 0131), Brain Heart Infusion Agar (Oxoid CM 0375), Wort Agar (Merck 1.05447), M17 Agar acc. to Terzaghi (Merck 1.15108), Wilkins-Chalgren Anaerobe Agar (Oxoid CM 0619), MRS Agar (Lactobacillus agar acc. to de Man, Rogosa and Sharpe) (Merck 1.10660.0500), YGC Agar (Yeast Extract – Glucose – Chloramphenicole Agar) (Merck 1.16000.0500).

Other Agents and Media

Agar Bacteriological (Agar No. 1) (Oxoid LP 0011), Lab. Lemco Powder (Oxoid L 29).

Neutralized Bact. Peptone (Oxoid L 34), distilled water, Glycerol (Merck 1.04093), Sodium Chloride.

Microbiological Test Methods

Trials were performed using conventional laboratory equipment and a Laminar Flow (Steril Gemini) anaerobic workstation (MACS-VA 500 micro-aerophilic Workstation).

Well Diffusion Assay

The well diffusion assay is used to evaluate the effectiveness of a test substance on the surface of an agar medium. The test substance is pipetted into wells where the agar has been displaced and diffuses into the agar. Micro-organisms grow on the agar surface, but not in the surroundings of wells which at least contain the inhibitory concentration of the substance (TAGG and MCGIVEN, 1971, TAGG et al., 1976).

After solidification of the agar medium in the Petri dish, 0.1 mL of cell suspension (24-h culture in nutrient broth) are evenly distributed on the agar surface. Afterwards, agar holes are punched out with the back end of a sterile Pasteur

Table 1: Bacteria, yeasts and moulds used in this study (for further explanations see text)
Tabelle 1: Übersicht über untersuchte Bakterien, Hefen und Schimmelpilze (weitere Erläuterungen im Text)

Internal Code	Strain	Collection	Official Code	Culture Conditions
G Lb 35	Lactobacillus brevis	DSMZ[a]	DSM 2647	MRS, 37°C, anaerobic
G Lb 129	Lactobacillus brevis	IFA[b]	LAC172, IFA508	MRS, 37°C, anaerobic
G Lb 128	Lactobacillus brevis	IFA	LAC280, IFA599	MRS, 37°C, anaerobic
G Lb 37	Lactobacillus rhamnosus	Medipharm[c]	ATCC7469, M2	MRS, 37°C, anaerobic
G Lb 120	Lactobacillus rhamnosus	IFA/Lactosan[d]	LAC259, IFA589	MRS, 37°C, anaerobic
G Lb 121	Lactobacillus rhamnosus	IFA/Lactosan	LAC161, IFA496	MRS, 37°C, anaerobic
G Lb 77	Lactobacillus plantarum	Lactosan	DSM 12837	MRS, 37°C, anaerobic
G Lb 69	Lactobacillus plantarum	Medipharm	LSI	MRS, 37°C, anaerobic
G Lb 143	Lactobacillus plantarum	DSMZ	DSM 2648	MRS, 37°C, anaerobic
G Lb 180	Lactobacillus buchneri	Lactosan	DSM 12856	MRS, 37°C, anaerobic
G Lb 170	Lactobacillus coryneformis	Medipharm	S 13	MRS, 37°C, anaerobic
G Lb 74	Lactobacillus coryneformis coryneformis	DSMZ	DSM 20001	MRS, 37°C, anaerobic
G Lb 75	Lactobacillus coryneformis coryneformis	DSMZ	DSM 20007	MRS, 37°C, anaerobic
G Lc 4	Lactococcus lactis	Medipharm	SR 3.54 555	MRS, 37°C, aerobic
G Lc 12	Lactococcus lactis	Lactosan	NCIMB 30160 Wcb5/12/01	MRS, 37°C, aerobic
G Pd 17	Pediococcus pentosaceus	Lactosan	DSM 16244 Lac 973	MRS, 30°C, aerobic
G Pd 18	Pediococcus acidilactici	Lactosan	DSM 16243 Lac 055	MRS, 30°C, aerobic
G En 2	Enterococcus faecalis	DSMZ	DSM 20478 ATCC 19433	BHI, 37°C, aerobic
G En 61	Enterococcus faecium	Medipharm	M 74	BHI, 37°C, aerobic
G En 24	Enterococcus faecium	Lactosan	DSM 7134	BHI, 37°C, aerobic
G En 19	Enterococcus casseliflavus	DSMZ	DSM 20680 ATCC 25788	BHI, 37°C, aerobic
G Bc 6	Bacillus licheniformis	DSMZ	DSM 13 ATCC 14580	TS broth, 37°C, aerobic
G Bc 7	Bacillus subtilis	DSMZ	DSM 10 ATCC 6051	TS broth, 37°C, aerobic
G Bc 45	Bacillus cereus	SLALW[e]	(498) wild	TS broth, 37°C, aerobic
G Bc 46	Bacillus pumilus	DFST[f]	-	TS broth, 30°C, aerobic
G Li 1	Listeria innocua	DFST	BCCM LMG 11387 ATCC 33090	BHI broth, 30°C, aerobic
G Li 2	Listeria ivanovii	DFST	BCCM	BHI broth, 30°C, aerobic
G Li 3	Listeria monocytogenes	DFST	BCCM	BHI broth, 30°C, aerobic
G Cl 2	Clostridium butyricum	DFST	University Graz 8260	RCM broth 37°C, anaerobic
G Cl 3	Clostridium beijerinckii	DFST	NCDO 1759	RCM broth 37°C, anaerobic
G Ec 1	Escherichia coli	DSMZ	DSM 613 ATTC 11303	TS broth, 37°C, aerobic
G Ec 11	Escherichia coli	DFST	-	TS broth, 37°C, aerobic
Ps 1	Pseudomonas fluorescens	DFST	-	TS broth, 30°C, aerobic
Ps 2	Pseudomonas fluorescens	DFST	-	TS broth, 30°C, aerobic
Ps 3	Pseudomonas maltophilia	DFST	-	TS broth 30°C, aerobic
Ps 4	Pseudomonas marginalis	DSMZ	DSM 50276	TS broth, 30°C, aerobic
K 66	Pichia fermentans	DFST	-	wort broth, 25°C, aerobic
M 136	Candida parapsilosis	DFST	-	wort broth, 25°C, aerobic
Sy 17	Saccaromyces cerevisiae	DFST	-	wort broth, 25°C, aerobic
F 104	Rhodotorula mucilaginosa	DFST	-	wort broth, 25°C, aerobic
Z 12	Zygosaccharomyces rouxii	DSMZ	DSM 70841	wort broth, 25°C, aerobic
AS 1	Aspergillus niger	DFST	-	YGC agar, 25°C, aerobic
Mu 1	Mucor	DFST	-	YGC agar, 25°C, aerobic
Pe 1	Penicillium roqueforti	DFST	-	YGC agar, 25°C, aerobic
Rh 1	Rhizopus	DFST	-	YGC agar, 25°C, aerobic

a: DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany;
b: IFA Tulln, Department for Agrobiotechnology, Tulln, Austria;
c: Medipharm AB, Kageröd, Sweden;
d: Lactosan GmbH & Co. KG, Kapfenberg, Austria;
e: SLALW: Sächsische Landesanstalt für Landwirtschaft, Fachbereich Landwirtschaftliche Untersuchungen (LUVA), Leipzig, Germany;
f: DFST Department of Food Science and Technology, University of Natural Resources and Applied Life Sciences, Vienna, Austria

pipette and 15 µL of the test substance are pipetted into the resulting wells. The testing procedure for each strain was carried out in triplicate. After incubation for 24 h at optimum temperature, the zone of inhibition was measured.

Spot Test

The spot test is a method in which the test substance is dotted onto the agar surface and then diffuses into the agar. Influenced micro-organisms grow on the surface, but not on the dotted spots that contain at least the minimum inhibitory concentration of the test substance (DE VUYST et al., 1996).

After solidification of the agar medium in the Petri dish, 5 µL portions of each test substance are dotted onto 5 spots on the agar surface. Afterwards, 0.3 µL of cell suspension (24-h culture in nutrient broth) are evenly distributed on the agar surface. The testing procedure for each strain was carried out in triplicate. After incubation for 24 h at optimum temperature, the zone of inhibition was measured.

Micro-dilution Test

This submerse test is used to determine the growth behaviour of micro-organisms in nutrient broth containing the inhibitory test substance at different concentrations. The growth progression is determined via optical density measurements in a photometer in standardised time intervals (Spectronic Genesys 10 Bio, Thermo Electron Corporation). All readings were carried out by means of a microtiter plate reader (Bioscreen C), with the exception of clostridial strains, which were incubated in test tubes (JORGENSEN and FERRARO, 1998; WHITE et al., 2001).

150 µL of the test substance and 150 µL of diluted cell suspension (1:10 or 1:100) with adjusted optical density (based on McFarland 0.5) as well as blanks and controls are pipetted into the wells of a microtiter plate and placed into the apparatus for recording of growth curves. Test settings for the experiments were as follows: pre-heating 15 min; temperature 25°C/30°C/37°C, depending on the micro-organisms; time interval 1 d (bacteria)/2 d (yeasts, *Listeria*); measurement intervals 30 min (1 d), 50 min (2 d); shaking before measurement 20 s; wave length 420-580 nm; The micro-dilution test was carried out in five replicates for each strain. Optical density values for growth curves were measured in 12-hour intervals.

For the tests with clostridia an anaerobic working bench was used. As these strains did not grow in the small well volumes of the microtiter plates, clostridial tests were performed in larger test tubes. 2 mL of the substance and 2 mL of bacteria suspension were pipetted into each vial. The bacteria suspension was diluted by 1:10 with RCM broth and adjusted to a McFarland value of 3.0 which is equivalent to an optical density of 0.480–0.700 at 625 nm. For measurement of bacterial growth based on optical density, an aliquot of 200 µL of each test tube was pipetted into the wells of a microtiter plate and placed into the optical reader at 620 nm. The testing procedure was carried out in five replicates for each strain. Optical density values for the growth curve were measured in 12-hour intervals.

3 Results and Discussion

The results of the inhibitory experiments are depicted according to the groups of micro-organisms. The minimum inhibitory concentrations (MIC) shown in the tables refer to the active plant-based ingredient and are displayed in mg substance per kg solvent (distilled water). MIC-values for the spot test and well diffusion assay were calculated from arithmetical mean values of zones of inhibition from triplicates. MIC-values for the micro-dilution test were calculated from arithmetical mean values of five growth curve replicates. MIC variations were within the expected range of ± 1 dilution variation of MICs. According to CLSI (2009) this variation is intrinsic to the microbiological testing systems used. MIC-values exceeding 10.000 mg/kg indicate no inhibition and therefore are referred to **n. i.** (no inhibition).

Main attention is paid to results achieved from the micro-dilution test rather than to results from the two surface tests (well diffusion assay, spot test) as this test method is better reproducible and as the cell suspensions used were always adjusted to standardised McFarland values. In addition OD-measurement was performed automatically. For the surface tests, 24-h old cultures in nutrient broth were used, which may have slightly differed in their micro-population density.

Table 2 summarises the results of Gram-positive bacteria that are found and/or used in silage production. Generally, all investigated Gram-positives were sensitive to the tested plant antimicrobials. In comparison to the spot test and well diffusion assay, very low MIC-values were found in the micro-dilution test, which is obviously due to an optimum distribution of the plant antimicrobial in the test format.

All testing procedures have shown that hop beta acids (B) were most effective, followed by rosin acids (R) and the mixture of rosin acids and myristic acid (R/M). In the

Table 2: Inhibitory test results obtained with Gram-positive bacteria; mean values derived from triplicates (spot test, well diffusion assay) or five replicates (micro-dilution test); minimum inhibitory concentrations displayed as mg/kg of active ingredient: B … hop beta acids, R … rosin acids, R/M … mixture of rosin acids/myristic acid, H_2O_2 … hydrogen peroxide; n.i. = no inhibition at 10,000 mg/kg; for strain codes see Table 1.

Tabelle 2: Hemmwirkungsergebnisse bei grampositiven Bakterien; Mittelwerte gebildet aus 3 (Well Diffusion Test, Tüpfeltest) bzw. 5 Wiederholungen (Microdilution-Test); minimale Hemmkonzentration angegeben in mg/kg Wirkstoff: B … Hopfen-β-Säuren, R … Harzsäuren, R/M … Mischung aus Harzsäuren/Myristinsäure, H_2O_2 … Wasserstoffperoxyd; n.i. = keine Hemmung bei 10.000 mg/kg; Stammbezeichnungen in Tabelle 1.

Strain	Micro-dilution Test				Spot Test				Well Diffusion Assay			
	B	R	R / M	H_2O_2	B	R	R / M	H_2O_2	B	R	R / M	H_2O_2
G Lb 35	10	100	100	150	400	6000	5000	**n.i.**	40	3000	4000	3000
G Lb 129	15	300	250	150	500	5000	9000	**n.i.**	150	5000	5000	3000
G Lb 128	12.5	100	200	150	600	4000	5000	**n.i.**	50	7000	1000	3000
G Lb 37	5	100	100	150	400	5000	6000	**n.i.**	40	3000	900	3000
G Lb 120	5	100	100	150	300	5000	8000	**n.i.**	150	3000	1500	3000
G Lb 121	5	100	100	150	400	5000	4000	**n.i.**	20	800	5000	3000
G Lb 77	10	400	400	150	1000	5000	7000	**n.i.**	150	4000	3000	3000
G Lb 69	15	200	250	150	500	9000	**n.i.**	**n.i.**	80	4000	600	3000
G Lb 143	10	100	200	150	300	5000	6000	**n.i.**	150	4000	1500	3000
G Lb 180	15	50	200	150	800	**n.i.**	8000	**n.i.**	150	4000	5000	3000
G Lb 170	12.5	100	100	150	400	3000	6000	300	50	3000	4000	3000
G Lb 74	15	100	100	150	400	2000	4000	300	80	300	3000	3000
G Lb 75	20	200	100	150	300	6000	**n.i.**	**n.i.**	60	3000	1500	3000
G Lc 4	5	30	25	150	300	3000	4000	**n.i.**	150	500	4000	3000
G Lc 12	10	15	25	150	400	2000	4000	**n.i.**	200	600	800	3000
G Pd 17	2.5	200	450	150	500	**n.i.**	**n.i.**	**n.i.**	70	3000	1500	3000
G Pd 18	2.5	1000	1000	150	600	9000	**n.i.**	**n.i.**	50	8000	5000	3000
G En 2	50	25	250	1500	5000	4000	600	**n.i.**	200	600	900	3000
G En 61	15	250	250	1500	300	5000	2000	**n.i.**	400	600	900	3000
G En 24	10	150	200	1500	400	2000	2000	**n.i.**	90	200	800	3000
G En 19	35	25	250	1500	300	2000	3000	**n.i.**	70	600	900	3000
G Bc 6	5	15	15	150	100	1000	3000	**n.i.**	40	200	300	3000
G Bc 7	5	20	10	1500	80	500	1500	**n.i.**	20	300	500	3000
G Bc 45	5	10	10	150	200	1000	700	**n.i.**	30	200	200	3000
G Bc 46	5	10	25	1500	70	1500	900	**n.i.**	20	300	300	3000
G Li 1	0.25	20	35	1500	500	750	2000	3000	75	700	600	3000
G Li 2	0.25	20	30	150	300	900	4000	3000	60	500	900	3000
G Li 3	0.25	20	35	1500	300	800	3000	3000	45	300	1000	3000
G Cl 2	10	10	15	150	800	500	900	3000	150	50	300	3000
G Cl 3	5	10	10	150	400	800	600	3000	250	300	90	3000

micro-dilution test, H_2O_2 inhibits lactobacilli, lactococci and pediococci even at lower MICs than R and R/M except for enterococci, bacilli, listeriae and clostridia. In the well diffusion assay a similar pattern for H_2O_2, R and R/M was obtained. With the spot test, the plant antimicrobials as well as H_2O_2 gave much higher MIC-values than with the other two testing procedures, whereas for H_2O_2, Gram-positives were able to grow at the highest concentration tested (10,000 mg/kg).

Among the bacteria tested, *Listeria* strains show a very low MIC (0.25 mg/kg) for B (micro-dilution test). Also R and R/M show low MIC-values ranging from 20–35 mg/kg. The efficacy of B against *Listeria monocytogenes* has previously been reported. (LARSON et al.). The authors found very low concentrations of a range of hop acid preparations to be effective in trypticase soy broth. The best effect was achieved with a preparation containing 41 % (w/w) beta acids, which revealed a MIC of 10 mg/L for the given test settings.

Clostridia, which are frequently responsible for silage spoilage, were inhibited at MIC-values ranging from 5–10 mg/kg by B and 10–15 mg/kg by R and R/M. The effect of B against clostridial strains is also subject of a patent application (JOHNSON and HAAS, 1999). Bacilli were inhibited at 5 mg/kg by B and at 10–25 mg/kg by R and R/M. It should be noted that lactic acid bacteria are inhibited by B at

roughly the same MIC-levels as silage-spoiling or pathogenic bacteria but not by R and R/M. These plant antimicrobials inhibit clostridia, listeriae, and bacilli and bacilli at a concentration which is 5 to 10 times lower than that observed with lactic acid bacteria.

Table 3 shows the results collected with Gram-negative bacteria common for silage production. In contrast to the above described Gram-positives, MIC-values of hop beta acids (B), rosin acids (R) and the mixture of rosin acids/myristic acid (R/M) were much higher or could not be determined, especially with the micro-dilution test. Although B is most effective, the lowest MIC-values achieved in the micro-dilution test were at least 1,000 mg/kg, which is much higher than observed with H_2O_2 as the reference substance. Both surface tests have shown that all bacterial strains tested were able to grow at concentrations exceeding 10,000 mg/kg. This observation differs largely from the results obtained with the Gram-positives.

Table 4 summarises results of the inhibitory tests of the plant antimicrobials against yeast strains commonly found in silage. Similarly to the Gram-negative bacteria – MIC-values of yeasts were only detectable when B and R/M were applied in the micro-dilution test. In contrast to R, the R/M mixture may have had an effect, probably due to the myristic acid, which is known to have some antifungal properties (BEUCHAT and GOLDEN, 1989). Again, hop beta acids (B) were shown to be most effective, although they do not display similar MIC-values as H_2O_2. The surface tests did not indicate any inhibition even at the highest concentration of plant antimicrobials tested (10,000 mg/kg). Hence, it must be stated that in this case the substances were simply ineffective. These results are not surprising, since hop acids and rosin acids were originally introduced to beer and Retsina production as additives to allow alcoholic fermentation by yeasts and prevent the spoilage by bacteria.

Table 5 gives an overview on the effectiveness of hop beta acids, rosin acids and the mixture of rosin acids/myristic acid

Table 3: Inhibitory test results obtained with Gram-negative bacteria; mean values derived from triplicates (spot test, well diffusion assay) or five replicates (micro-dilution test); minimum inhibitory concentrations displayed as mg/kg of active ingredient: B … hop beta acids, R … rosin acids, R/M … mixture of rosin acids/myristic acid, H_2O_2 … hydrogen peroxide; n.i. = no inhibition at 10,000 mg/kg; for strain codes see Table 1.

Tabelle 3: Hemmwirkungsergebnisse bei gramnegativen Bakterien; Mittelwerte gebildet aus 3 (Well Diffusion Test, Tüpfeltest) bzw. 5 Wiederholungen (Microdilution-Test); minimale Hemmkonzentration angegeben in mg/kg Wirkstoff: B…Hopfen-β-Säuren, R … Harzsäuren, R/M … Mischung aus Harzsäuren/Myristinsäure, H_2O_2 … Wasserstoffperoxyd; n.i. = keine Hemmung bei 10.000 mg/kg; Stammbezeichnungen in Tabelle 1.

Strain	Micro-dilution Test				Spot Test				Well Diffusion Assay			
	B	R	R / M	H_2O_2	B	R	R / M	H_2O_2	B	R	R / M	H_2O_2
G Ec 1	1000	**n.i.**	2500	150	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**
G Ec 11	1000	**n.i.**	2500	150	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**
Ps 1	1000	**n.i.**	**n.i.**	150	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	3000
Ps 2	1000	**n.i.**	**n.i.**	150	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	3000
Ps 3	1000	**n.i.**	**n.i.**	150	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	3000
Ps 4	1000	**n.i.**	**n.i.**	150	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	3000

Table 4: Inhibitory test results obtained with yeasts; mean values derived from triplicates (spot test, well diffusion assay) or five replicates (micro-dilution test); minimum inhibitory concentrations displayed as mg/kg of active ingredient: B … hop beta acids, R … rosin acids, R/M … mixture of rosin acids/myristic acid, H_2O_2 … hydrogen peroxide; n.i. = no inhibition at 10,000 mg/kg; for strain codes see Table 1.

Tabelle 4: Hemmwirkungsergebnisse bei Hefen; Mittelwerte gebildet aus 3 (Well Diffusion Test, Tüpfeltest) bzw. 5 Wiederholungen (Microdilution-Test); minimale Hemmkonzentration angegeben in mg/kg Wirkstoff: B … Hopfen-β-Säuren, R … Harzsäuren, R/M … Mischung aus Harzsäuren/Myristinsäure, H_2O_2 … Wasserstoffperoxyd; n.i. = keine Hemmung bei 10.000 mg/kg; Stammbezeichnungen in Tabelle 1.

Strain	Micro-dilution Test				Spot Test				Well Diffusion Assay			
	B	R	R / M	H_2O_2	B	R	R / M	H_2O_2	B	R	R / M	H_2O_2
K 66	1000	**n.i.**	5000	150	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	3000
M 136	1000	**n.i.**	5000	1500	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	3000
Sy 17	1000	**n.i.**	5000	150	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	3000
F 104	1000	**n.i.**	5000	1500	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	3000
Z 12	1000	**n.i.**	5000	1500	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	3000

Table 5: Inhibitory test results obtained with moulds; mean values derived from triplicates (spot test, well diffusion assay) or five replicates (micro-dilution test); minimum inhibitory concentrations displayed as mg/kg of active ingredient: B … hop beta acids, R … rosin acids, R/M … mixture of rosin acids/myristic acid, H_2O_2 … hydrogen peroxide; n.i. = no inhibition at 10,000 mg/kg; for strain codes see Table 1.

Tabelle 5: Hemmwirkungsergebnisse bei Schimmelpilzen; Mittelwerte gebildet aus 3 (Well Diffusion Test, Tüpfeltest) bzw. 5 Wiederholungen (Microdilution-Test); minimale Hemmkonzentration angegeben in mg/kg Wirkstoff: B…Hopfen-β-Säuren, R … Harzsäuren, R/M … Mischung aus Harzsäuren/Myristinsäure, H_2O_2 … Wasserstoffperoxyd; n.i. = keine Hemmung bei 10.000 mg/kg; Stammbezeichnungen in Tabelle 1.

Strain	Micro-dilution Test				Spot Test				Well Diffusion Assay			
	B	R	R / M	H_2O_2	B	R	R / M	H_2O_2	B	R	R / M	H_2O_2
AS 1	-	-	-	-	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	3000
Mu 1	-	-	-	-	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**
Pe 1	-	-	-	-	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**
Rh 1	-	-	-	-	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**

against typical moulds relevant to silage production. In these test series, the micro-dilution test as a submerse test procedure was not applicable and therefore excluded. The MIC-values for the spot test and the well diffusion assay could not be clearly determined as the moulds considered even grew at the highest concentration tested (10,000 mg/kg). Also, H_2O_2 did not yield interpretable results. Moulds are obviously not inhibited by the plant antimicrobials applied.

The inhibitory effect of hop beta acids and rosin acids found in this study is very congruent with findings of the application of plant antimicrobials in food production where they are known to sustainably inhibit Gram-positive bacteria, but only seldom Gram-negatives, yeasts and moulds.

Rosin acids from pine trees have been used for more than two-thousand years in the production of the traditional Greek wine Retsina in order to prevent wine spoilage by acetic acid fermentation under Mediterranean climate conditions. However, the results obtained indicate that lactic acid bacteria are less susceptible to rosin acids than clostridia or listeriae. Therefore, they could be useful to suppress spoilage bacteria in silage fermentation without affecting lactic acid starter cultures. The mixture of rosin acids and myristic acid was included in the test series because it is known from the sugar industry that the combination of the two plant components exerts technical advantages. Minor addition of myristic acid improves the solubility of the concentrated alkaline rosin acids solution, even at low temperatures and this eases handling of the mixture. Also, myristic acid is known to possess some antimicrobial effect, mainly against Gram-positives but also yeasts (BEUCHAT and GOLDEN, 1989).

The antimicrobial properties of hop bitter acids (alpha acids/humulones and beta acids/lupulones and both their corresponding homologues) have been used in beer brewing for centuries. Naturally, many publications in brewing science deal with the antimicrobial properties of hop compounds (SIMPSON, 1991; SIMPSON and SMITH, 1992). Results obtained in this study indicate that hop acids seem to be unsuitable as supplements to silage production, as they affect the growth of lactic acid bacteria. However, there are several reports about lactic acid bacteria in brewing that have developed resistance mechanisms protecting them from the actions of hop bitter acids (SAKAMOTO and KONINGS, 2003; SUZUKI et al, 2006). Consequently, the hop beta acids may be applicable in silage production in combination with hop resistant lactic acid bacterial strains to inhibit spoilage bacteria such as clostridia.

The development of nature-based supplements is therefore an interesting alternative to commonly used but disputed substances like formaldehyde in sugar industry on the one hand and to nitrite and hexamethylenetetramine for silage production on the other.

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