MICROBIOLOGICAL METHODS

Importance of Tetrahydroiso-α-acids to the Microbiological Stability of Beer

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While beer provides a very stable microbiological environment, a few niche microorganisms are capable of growth in malt, wort, and beer. The production of off-flavors and development of turbidity in the packaged product are due to the growth and metabolic activity of wild yeast, certain lactic acid bacteria (LAB) and anaerobic Gram-negative bacteria. Beer also contains bitter hop compounds, which are toxic to Gram-positive and Gram-negative bacteria, and contribute to preventing the spoilage of this beverage. In the boiling process, the hop α-acids (humulones) are isomerized into iso-α-acids. These products are responsible for the bitter taste of beer, but they also play an essential role in enhancing foam stability. Antibacterial activity of iso a-acids and their hydrogenated derivates (rhoiso-α-acids and tetrahydroiso-α-acids) in MRS broth and beer have been evaluated against different LAB (*Lactobacillus* and *Pediococcus*) for the determination of their beer-stabilizing capabilities. Besides this, we have determined the minimum inhibitory concentration and the bacteriostatic effect of each compound against *Pediococcus*. We found that tetrahydroiso-α-acids (added directly to beer during production processes) are the compounds that present the greatest antibacterial activity against the main agents implicated in beer spoilage.

B eer has been recognized as a beverage with high microbiological stability offering a poor and rather hostile environment for most microorganisms. Its

ethanol concentration ranges from 0.5 to 10% (w/w) and is usually around 4.5%. These concentrations are high enough to make beer bacteriostatic or bactericidal. Beer is usually slightly acidic, with pH values ranging from 3.8 to 4.7, which is lower than most bacteria can tolerate for growth.

Beer also contains bitter hop compounds (approximately 17–55 ppm of iso-α-acids and derivates), which are toxic to Gram-positive bacteria. In fact, in the boiling process, the hop α-acids or humulones, which are almost tasteless, are isomerized into bitter-tasting iso-α-acids. The iso-α-acids comprise six major components: the *trans-* and *cis*-isomers of isocohumulones, isohumulones, and isoadhumulones (1). Currently, special attention is being paid to reduced iso-α-acids, among others, to make light-proof beers and beers with improved foam characteristics (2, 3).

The use of reduced, isomerized hop extracts (rhoiso-α-acids, tetrahydroiso-α-acids, and hexahydro iso-α-acids) became very popular during the last decade for achieving both better foam and light stability (Figure 1). Today some of the most common products are tetrahydroiso-α-acids (4). These compounds are very important in beer production since they are used to adjust the bitterness and flavor of the final product.

Only a few bacterial species are able to grow under such inhospitable conditions and spoil beer (Table 1). These bacteria include both Gram-positive and Gram-negative species. Gram-positive beer spoilage bacteria almost always belong to lactic acid bacteria (LAB; 6–9). Most bacterial species, including most other lactobacilli and pediococci, fail to grow in beer because hop compounds, which give beer its bitter flavor, are the major neutralizing agents for bacteria (10–12). Only a few Gram-negative bacteria are known to cause beer spoilage. Aerobic acetic acid bacteria, i.e., *Gluconobacter* and *Acetobacter* spp., were well-known as beer spoilage organisms in breweries, but the role of these bacteria in beer spoilage has been reduced significantly due to the much lower oxygen content during the brewing processes

Figure 1. Structure of -acids and *cis*-structures of iso a-acids and their hydrogenated derivatives.

and in packaged beer of modern breweries. Instead, the occurrence of strictly anaerobic bacteria in beer spoilage incidents has increased. These include the genera *Pectinatus, Megaspahera, Selenomonas*, *Zymomonas*, and *Zymophilus*. *Pectinatus* and *Megasphaera* species cause especially serious problems, mainly due to the production of the offensive "rotten egg" odor in finished beer (13). Hop compounds are reported to act as protonophores and dissipate the transmembrane pH gradient that prevents bacteria from growing in beer (10).

In previous studies, we have analyzed the chemical properties of humulone derivates and their influence on beer properties (14, 15). In this study, we evaluated the antibacterial activity of different -acids and derivates (iso -acids, tetrahydroiso -acids, and rhoiso -acids) against some Gram-positive bacteria described as beer spoilage agents to demonstrate the importance of the antibacterial activity of hop compounds in the prevention of beer spoilage.

METHOD

Materials

(**a**) *Microorganisms.*—All the species used in this research work are deposited in the Spanish Type Culture Collection (CECT). The tested strains were *Lactobacillus brevis* CECT 216, *L. buchneri* CECT 4111, *L. lindneri* CECT 815, *Pediococcus inopinatus* CECT 4784, *P. pentosaceus* CECT 4695, and *P. damnossus* CECT 793.

All bacterial species were grown anaerobically at 30 C in MRS broth (Oxoid Ltd, Hampshire, UK; No. CM0359). Anaerobic conditions were generated by AnaeroGen Compact paper sachets (Oxoid Ltd, AN010C).

(**b**) *Hop compounds.*—Two different samples of isomerized hop compounds were obtained from Hopsteiner (New York, NY; Iso-extract 30%, Rho-iso-extract 35%, and Tetra-iso-extract 10%) and from Botanix (Rona Inc., Boucherville, QC, Canada; Isohop, Redihop, and Tetrahop Gold) as a concentrated hop extract (aqueous solution of the potassium salts of iso- rho-, and tetrahydroiso -acids). A sample of humulone (92% w/w; Hopsteiner) was also analyzed.

(**c**) *Obtaining bacterial suspensions.*—Bacterial suspensions of each strain were prepared in physiological saline (0.85% NaCl). Suspensions were adjusted to a density of McFarland No. 1 standard (approximately 3 10^8 CFU/mL).

Evaluation of Antibacterial Capacity of Hop Compounds by an Agar Diffusion Test

Bacterial susceptibility to antimicrobial agents was measured in vitro by using the principles of agar diffusion (16). Standard solutions of 30 ppm of each hop compound were prepared and stored in the dark at 4 C. MRS agar was selected as the medium for in vitro susceptibility tests. Aseptically, 1 mL of the cell suspension was pipetted into a labeled Petri dish. Then approximately 20 mL of melted

Table 1. Beer spoilage bacteria[a]

Rod-shaped	Cocci
Gram-positive bacteria	
Lactobacillus spp.	*Pediococcus* ssp.
L. brevis	P. inopinatus
L. brevisimilis	P. pentasaceus
L. buchneri	P. damnossus
L. casei	
L. coryneformis	*Micrococcus* ssp.
L. curvatus	M. kristinae
L. lindneri	
L. malefermentans	
L. parabuchneri	
L. plantarum	
Gram-negative bacteria	
Pectinatus ssp.	*Megasphaera* ssp.
P. cerevissiphilus	M. cerevisiae
P. frisingensis	
Selenomonas ssp.	*Zymomonas* ssp.
S. lacticifex	Z. mobilis
Zymophilus ssp.	
Z. raffinosivorans	

[a] Ref. 5.

MRS agar that had been cooled at 44–45 C was added. Plates with bacteria and agar mixture were mixed well by slight rotation. Subsequently, the agar was allowed to solidify to trap bacteria within the media. Using a sterile glass tube (6 mm id), small plugs were cut from the agar substrate to obtain wells. Wells were filled with 200 L of one of the hop compounds under study. Plates were incubated anaerobically at 30 C for two days. After the incubation period, the diameters of growth inhibition were measured and expressed in mm.

The antimicrobial agent deposited in the wells diffused through the agar, resulting in a gradient of the hop compound concentration. No growth appeared in the area where inhibitory concentrations of the products are present. Three replications were carried out for the agar diffusion test.

Evaluation of Minimum Inhibitory Concentration (MIC) in MRS Broth

In each test, 1 mL of a standard solution of 5, 10, 15, 20, 25, or 30 ppm of a hop bitter acid (-acids, iso -acids, tetrahydroiso -acids, or rhoiso -acids) was added to test tubes containing 8 mL MRS broth. To this, 1 mL of the bacterial suspension was added to be tested in sterile physiological saline from recent cultures (approximately 3 10^3 CFU/mL).

Tubes were incubated anaerobically at 30 C for two days. The tubes were tested regarding the emergence of turbidity, or lack thereof, as a consequence of bacterial growth. The lowest concentration that completely inhibited visible growth of the microorganism as detected by the unaided eye was recorded as the MIC. Before reading and recording MIC results for the test strains, growth controls were examined for viability. Growth was indicated by turbidity throughout the tube or by a single sediment button of 2 or more mm in diameter or several buttons with smaller diameter, in the tube bottom.

An aliquot from tubes that presented turbidity was plated on an agar plate and incubated to ensure that the emergence of turbidity was due to microbial growth.

Evaluation of Beer Spoilage Risk

As hop-free beers cannot be found in the market, the beer with the lowest concentration of hop bitter acids and commercialized in Spain was chosen to carry out these studies. In each test, 1 mL of a standard solution of 5, 10, 15, 20, 25, or 30 ppm of hop bitter acids (-acids, iso -acids, tetrahydroiso -acids, or rhoiso -acids) was added to test tubes containing 8 mL of degassed commercial beer. To this, 1 mL of the bacterial suspension was added to be tested in sterile physiological saline from recent cultures
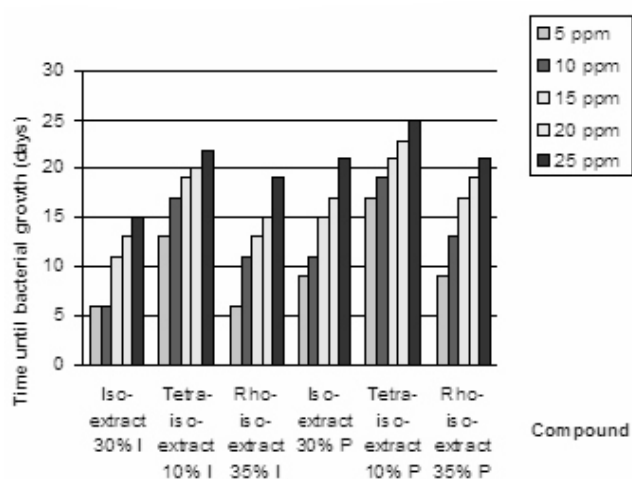
Table 2. Diameter of halos and MIC obtained for *P. pentosaceus* and *P. inopinatus* against -acids, iso -acids, tetrahydroiso -acids, and rhoiso -acids

Compound	Diameter of halo, mm		MIC, ppm	
	P. pentosaceus	P. inopinatus	P. pentosaceus	P. inopinatus
Iso-extract, 30%	12	10	>30	15
Tetra-iso-extract, 10%	21	18	10	5
Rho-iso-extract, 35%	11	9	25	15
Isohop	11	10	>30	20
Tetrahop Gold	17	15	15	5
Redihop	10	9	30	15
-Acid	—[a]	—	>50	>50

[a] — = Without inhibition.

Figure 2. Bacteriostatic effect of iso -acids, tetrahydroiso -acids, and rhoiso -acids against *P. inopinatus* (I) and *P. pentosaceus* (P) in beer.

(approximately 3 10^3 CFU/mL). Target tests were carried out in parallel to determine development of the strains in beer that did not contain the hop compounds. In order to evaluate whether hop compounds evidenced bacteriostatic or bactericidal activity, all of the inoculated beers were incubated anaerobically at 30 C and examined regularly for visible growth for up to 30 days.

Results and Discussion

The following results were obtained in the study on the antibacterial activity of isohumulones: The *L. brevis* CECT 216, *L. buchnerii* CECT 4111, *L. lindneri* CECT 815, and *P. damnosus* CECT 793 strains were found to be resistant to the assayed hop compounds; and the growth of *P. pentosaceus* CECT 4695 and *P. inopinatus* CECT 4784 was inhibited in several tests. Details are shown in Table 2.

Tetrahydroiso -acids were the compounds that showed the greatest diameters of growth inhibition against *P. pentosaceus* and *P. inopinatus*. Therefore, it is to be expected that they will be the compounds that will present the greatest inhibition levels against both bacteria and consequently minor MIC.

The MIC of different derivatives of iso -acids against *P. pentosaceus* CECT 4695 and *P. inopinatus* CECT 4784 when inoculated in MRS broth was determined by analyzing the results obtained in the previous experiments. Results are detailed in Table 2. It was observed that the value of MIC decreased when the hydrogenation of the analyzed compounds increased. Tetrahydroiso -acids were the compounds that showed the lowest MIC value.

According to the information supplied in Figure 2, there was a bacteriostatic effect; bacterial growth and multiplication was prevented for several days, but bacteria were not killed because they could start their growth again after more or fewer days of incubation, depending on the strain. For instance,

when *P. pentosaceus* was cultivated in beer with added hop compounds, its growth was observed for the first time after six days of incubation, while the growth *of P. inopinatus* was inhibited until the ninth day.

Hop compounds are weak acids that can cross cytoplasmatic membranes in undissociated form in response to the transmembrane pH gradient (17). Due to the higher internal pH, these compounds dissociate internally, thereby dissipating the pH gradient across the membrane (18).

It has been observed that increased hydrophobicity (lipophilicity) leads to a greater antimicrobial activity. The most hydrophobic reduced iso -acids are more antimicrobial than their naturally occurring analogs, in addition to which the degree of reduction is important. Increased hydrophobicity renders a compound more prone to interaction with the cell membrane, thus explaining the observed effects.

Several studies (19, 20) have shown that tetrahydroiso -acids are the hop compounds that confer the greatest degree of bitterness to beer and are the most stable compounds during storage (regarding both light and temperature). Our research demonstrated that they also present the highest antibacterial activity, thus enabling them to be regarded as the preferred compounds to be added to beer with the aim of preserving its properties until the moment of consumption. The results also highlight that the most saturated forms display the greatest antibacterial activity. It has also been observed that the relative increase of antimicrobial activity of tetrahydroiso -acids, when compared to rhoiso -acids, is considerably higher than that of rho forms when compared to iso -acids.

Among iso- -acids potentially present in beer, hexaderivatives present the longest diameter, although their length does not exceed the threshold 1.5 nm to cross bacterial membranes (15). Furthermore, hexahydroiso -acid presents a partition coefficient almost 10 times higher than that of iso -acid, which would make this hexahydrogenated derivative a rather good bacteriostatic agent when present in beer. This leads us to suggest that the hexa forms would display greater antimicrobial activity than the tetra forms if applied in the beer industry.

Acknowledgments

References

(1) Briggs, D.E., Boulton, C.A., Brookes, P.A., & Stevans, R. (2004) *Brewing Science and Practice,* CRC Press, Boca Raton, FL

(2) De Cooman, L., Aerts, G., & Overmeire, H. (2000) *J. Inst. Brew.* **106**, 169–178

(3) Simpson, W.J., & Hughes, P.S. (1994) *Cerevisia Biotechnol.* **19**, 39–44

(4) Vanhoenacker, G., De Keukeleire, D., & Sandra, P. (2004) *J. Chromatogr. A* **1035**, 53–61

(5) Weiss, A., Schönberger, C., Mitter, W., Biendl, M., Back, W., & Krottenthaler, M. (2002) *J. Inst. Brew.* **108**, 236–242

(6) Back, W. (1994) *Brauwelt Int.* **4**, 326–333

(7) Back, W. (1994) *Farbatlas und Handbuch der Getränkebiologie,* Verlag Hans Carl, Nürnberg, Germany

(8) Hollerová, I., & Kubizniaková, P. (2001) *J. Inst. Brew.* **107**, 2355–2358

(9) Sakamoto, K., & Konings, W.N. (2003) *Int. J. Food Microbiol.* **89**, 105–124

(10) Simpson, W.J. (1993) *J. Gen. Microbiol.* **139**, 1041–1045

(11) Simpson, W.J. (1993) *J. Inst. Brew.* **99**, 405–411

(12) Priest, F.G. (1996) in *Brewing Microbiology*, 2nd Ed., F.G. Priest & I. Cambell (Eds), Chapman and Hall, London, UK

(13) Van Vuuren, H.J.J. (1996) in *Brewing Microbiology*, 2nd Ed., F.G. Priest & I. Cambell (Eds), Elsevier, Amsterdam, The Netherlands

(14) Blanco, C.A., Rojas, A., Caballero, P., Ronda, F., Gómez, M., & Caballero, I. (2006) *Trends Food Sci. Technol.* **17**, 373–377

(15) Blanco, C.A., Rojas, A., & Nimubona, D. (2007) *Trends Food Sci. Technol.* **18**, 144–149

(16) Barry, A.L., & Thornsberry, C. (1991) in *Manual of Clinical Microbiology*, 5th Ed., A. Balows, W.J. Hausler, Jr, K.L. Herrmann, H.D. Isenberg, & H.J. Shadomy (Eds), American Society for Microbiology Press, Washington, DC

(17) Sakamoto, K., Margolles, A., Van Veen, H.W., & Konings, W.N. (2001) *J. Bacteriol.* **183**, 5371–5375

(18) Sakamoto, K., Van Veen, H.W., Saito, H., Kobayashi, H., & Konings, W.N. (2002) *Appl. Environ. Microbiol.* **68**, 5374–5378

(19) De Keukeleire, D. (2000) *Quím. Nova* **23**, 108–112

(20) Sakamoto, K. (2002) Doctoral Thesis, University of Groningen, The Netherlands