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Identification of Beer Bitter Acids Regulating Mechanisms of Gastric Acid Secretion

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ABSTRACT: Beer, one of the most consumed beverages worldwide, has been shown to stimulate gastric acid secretion. Although organic acids, formed by fermentation of glucose, are known to be stimulants of gastric acid secretion, very little is known about the effects of different types of beer or the active constituents thereof. In the present study, we compared the effects of different beers on mechanisms of gastric acid secretion. To investigate compound-specific effects on mechanisms of gastric acid secretion, organic acids and bitter compounds were quantified by HPLC-DAD and UPLC-MS/MS and tested in human gastric cancer cells (HGT-1) by means of a pH-sensitive fluorescent dye which determines the intracellular pH as an indicator of proton secretion. The expression of relevant genes, coding the H^+/K^+-ATPase, *ATP4A*, the histamine receptor, *HRH2*, the acetylcholine receptor, *CHRM3*, and the somatostatin receptor, *SSTR2*, was determined by qPCR. Ethanol and the organic acids succinic acid, malic acid, and citric acid were demonstrated to contribute to some extent to the effect of beer. The bitter acids comprising α-, β-, and iso-α-acids were identified as potential key components promoting gastric acid secretion and up-regulation of *CHRM3* gene expression by a maximum factor of 2.01 compared to that of untreated control cells with a correlation to their respective bitterness.

KEYWORDS: *beer, gastric acid secretion, hop-derived bitter acids, organic acids, HGT-1 cells*

INTRODUCTION

Beer is one of the most consumed alcoholic beverages worldwide. According to a report from the Japanese brewing company Kirin, the annual per capita consumption in 2004 ranged between 2.33 L in India and 158.6 L in the Czech Republic, with a maximum total annual consumption of almost 24 billion liters in the US. Beer is known to be a stimulant of gastric acid secretion.[1,2] An excessive secretion of gastric acid can promote the onset of diseases such as gastroenteritis, gastroesophageal reflux disease (GERD), stomach ulcers, and ultimately stomach cancer.[3,4] However, there is no data showing whether different types of beer have different effects on mechanisms of gastric acid secretion or which beer constituents are responsible for this effect. Therefore, we aimed at identifying key compounds that promote gastric acid secretion and understanding the underlying mechanisms of action.

Due to its preparation from water, malt, and hops, and the fermentation with yeast, beer has a complex composition that varies depending on the original ingredients, the production process, and the storage. Beers, except for alcohol-free beers, can have alcohol contents around 1.5% (light beer), 5.0% (regular beer), and more than 10% (strong beer). Findings on the effect of ethanol on gastric acid secretion are controversial. While some studies prove that ethanol has a mild stimulatory effect on gastric acid secretion in concentrations below 5% and no or a slight inhibitory effect in concentrations above 5%,[5,6] others found ethanol to be a potent stimulant.[7,8]

Teyssen et al. identified the products formed by yeast during the process of alcoholic fermentation of glucose as integral to promotion of gastric acid secretion.[6] The organic acids maleic and succinic acid have been evaluated as key compounds by means of fractionation.[9] Furthermore, a structure/effect hypothesis has been suggested, according to which a C4 body and two carboxyl groups are necessary to stimulate gastric acid secretion.[9] However, beer contains a large variety of substances that derive from ingredients other than fermented glucose. The effect of hop-derived bitter acids in beer is largely unexplored, although bitter taste is often associated with effects on the digestive system. Hop extracts have been shown to increase gastric juice volume but not gastric acid secretion in rats.[10] However, these studies did not take into account the formation of reaction products during the brewing process and storage, which constitute the majority of hop-derived compounds in finished beer.[11−13] In contrast, the effect of beer, hops, barley extract, and fractions thereof on the stimulation of pancreatic enzyme secretion have been described previously,[14−16] showing that there might be a relevance of various beer constituents for the biological activities of beer on the stomach physiology.

To investigate the mechanism of action of beer, we studied the key mechanisms that control gastric acid secretion. The H^+/K^+-ATPase pumps the protons out of the parietal cell and, at the same time, chloride ions leave the cells through channels in exchange for hydrogen carbonate.[4,17] This function of the parietal cell is controlled by stimulating and inhibiting factors. The main stimulants are histamine, gastrin, and acetylcholine,

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while somatostatin is their antagonist. Gastrin and also acetylcholine stimulate the cAMP and Ca^{2+}-dependent release of histamine from the enterochromaffin-like cells of the stomach mucosa. Histamine binds on the histamine-2 receptor, a transmembrane receptor of the parietal cell, leading to an onset of a signal transduction pathway and finally resulting in the activation of the proton pump.

We showed in studies on coffee that processing of food has an impact on its ability to stimulate gastric acid secretion.[18−20] Our group has identified *N*-methylpyridinium, a product of the roasting process of coffee, to have an inhibitory impact on the proton secretion of human gastric cells (HGT-1). Because *N*-methylpyridinium is formed upon roasting, we could show that beverages prepared from light coffee roasts have a stronger stimulating effect on gastric acid production than those prepared from darker coffee roasts.[19,21] Furthermore, the effect of different coffees and coffee constituents on gastric acid secretion was shown in vitro.[18,22] The underlying mechanisms of action were studied on a gene regulatory level by qPCR.[18,20] These studies confirmed that the HGT-1 cells are a useful model system for the investigation of gastric acid secretion in vitro. Furthermore, HGT-1 cells express all four genes of interest, namely *ATP4A*, coding the H^+/K^+-ATPase, *HRH2*, coding the histamine receptor, *CHRM3*, coding acetylcholine receptor, and *SSTR2*, coding the somatostatin receptor, allowing the qPCR analysis of relevant parameters.

The aims of the here-presented study were to determine differences between different types of beers, to verify ethanol and succinic acid (Figure 1) to be active stimulants of gastric acid secretion,[23,24] also in beer-representative concentrations, and investigate the impact of other relevant organic acids such as malic and citric acid as well as the impact of hop-derived bitter acids (Figure 1) on mechanisms of gastric acid secretion. Therefore, the proton secretion and the expression of genes involved in gastric acid secretion were measured in order to gain insight into the cellular pathways stimulated by beer and beer components.

MATERIALS AND METHODS

Chemicals. Histamine was purchased from Sigma-Aldrich (Vienna, Austria) and dissolved at 1 mM in Krebs-HEPES buffer (KRHB). KRHB consisted of 10 mM HEPES, 11.7 mM D-glucose, 4.7 mM KCl, 130 mM NaCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 1.2 mM KH_2PO_4 brought to a pH of 7.4 with 5 M NaOH at 37 °C. The organic acids, succinic acid, maleic acid, malic acid, and citric acid were purchased from Sigma-Aldrich. All other chemicals were purchased from Roth (Karlsruhe, Germany). For the cell culture experiments, trypsin, glutamine, and penicillin/streptomycin were purchased from Sigma-Aldrich.

An iso-α-acid extract (30%) was prepared by preisomerization of a hop extract. Individual iso-α-acids were isolated from an iso-α-acid extract (30%; Hallertauer Hopfenveredelungsgesellschaft mbH, Mainburg, Germany), α-acids and β-acids were isolated from an ethanolic hop extract (Hallertauer Hopfenveredelungsgesellschaft mbH) following the protocol recently reported.[13]

Samples and Sample Preparation. The beer samples, dark beer, wheat beer, lager beer, pilsener, and alcohol-free beer, were purchased from the Ottakringer Shop in Vienna, Austria. Except for the wheat beer (Passauer Weisse, Passau, Germany), all beers were produced by the Viennese brewery Ottakringer. The ethanol (EtOH) concentration and original wort in the beers, as published by the brewery, are shown in Table 1. The beers were degassed in an ultrasonic bath for 20 min. The bitter acid extract was made from 100 mL lager beer acidified with 2.5 mL of 37% HCl using ethyl acetate. After three extractions with 70 mL of ethyl acetate each, the solvent was evaporated (Rotavopar R210, Büchi, Essen, Germany) and the extract reconstituted with 100 mL of water. The individual bitter acids were dissolved using ethanol and diluted with water to concentrations found in beer with a final ethanol concentration below 0.03% so as not to interfere with the effect (α-acids: 3.57 mg/L; β-acids: 0.081 mg/L; iso-α-acids: 46.41 mg/L). The organic acids were dissolved at a concentration of 10 mg/mL in water and then diluted with KRHB to experimental concentrations quantified in lager beer. All further dilutions were prepared with KRHB.

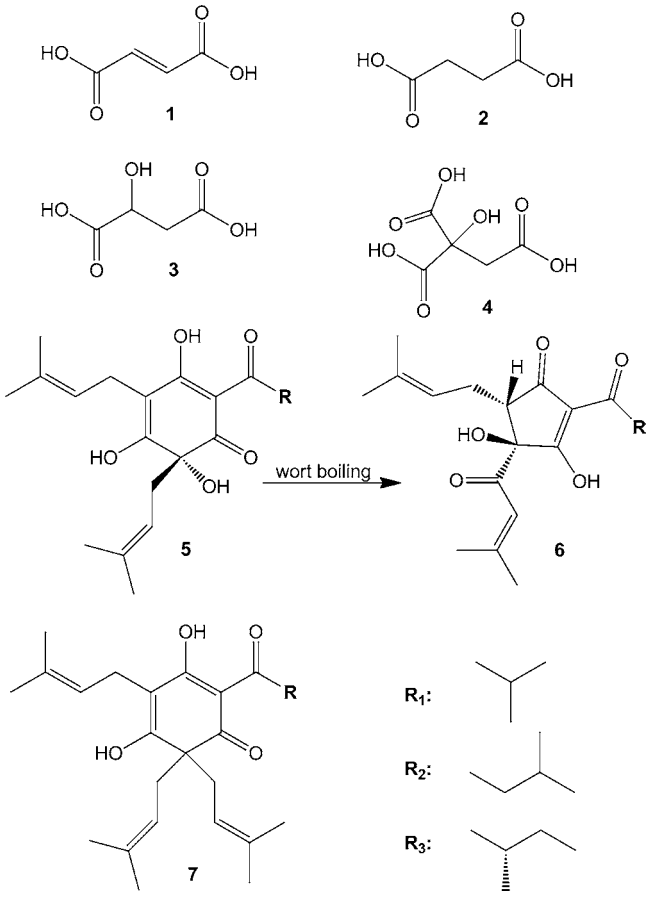

Figure 1. Beer constituents tested in this study: maleic acid (**1**), succinic acid (**2**), malic acid (**3**), citric acid (**4**), α-bitter acids (**5**), iso-α-bitter acids (**6**), and β-bitter acids (**7**). α-Bitter acids: R_1, cohumulone-derivatives; R_2, humulone-derivatives; R_3, adhumulone-derivatives. β-Bitter acids: R_1, colupulone-derivatives; R_2, lupulone-derivatives; R_3, adlupulone-derivatives.

Table 1. EtOH Concentration and Degree of Original Wort of the Test Solution

test solution	original wort (deg)	EtOH concn (%)
EtOH, 5.2%	–	5.2
alcohol-free beer	6.2	<0.5
lager beer	11.8	5.2

Cell Culture. HGT-1 cells (Dr. C. Laboisse, Nantes, France) were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich) containing 10% fetal bovine serum (FBS, Fisher Scientific, Vienna, Austria), 2% glutamine, and 1% penicillin/streptomycin and kept at 37 °C with 5% CO_2 and 95% humidity. Cells were harvested using trypsin at least 24 h prior to experiments.

Proton Secretion. Proton secretion was determined using the pH-sensitive fluorescent dye Carboxy-SNARF-1 AM (Invitrogen, Vienna, Austria) and a fluorescence plate reader (Infinite M200 Plate Reader, Tecan, Männedorf, Switzerland), measuring the intracellular pH as a marker of proton secretion. An increase in the intracellular pH

indicates that protons were transported out of the cell; thus, the determination of the intracellular pH correlates directly with the proton secretion.[21] The dye exhibits a pH-dependent emission shift from 580 nm under acidic conditions to 640 nm under basic conditions. Thus, the ratio between the emissions measured at these two wavelengths can be used to calculate the pH when using a standard curve. For the experiments, HGT-1 cells were seeded in a 96-well plate at 100 000 cells per well and incubated for 24 h to grow adherent. The cell culture medium was removed, and cells were washed once with KRHB before they were loaded with 3 μM Carboxy-SNARF-1 AM in KRHB for 30 min. The dye was removed, and the cells were washed twice with KRHB. Then the test substances were applied for 10 min, which was shown previously to be the optimal incubation time for the positive control histamine,[21] and the cells were washed once with KRHB prior to the fluorescence detection at 580 and 640 nm after excitation at 488 nm. All samples were measured in sextuplicate. On each plate, a calibration curve for the intracellular pH was recorded with a buffer containing 2 μM nigericin (Sigma-Aldrich) and consisting of 20 mM NaCl, 110 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 18 mM D-glucose, and 20 mM HEPES that was set to different pH levels (6.8−8.0) by titration with NaOH. The intracellular proton concentration in nmol/L was calculated and related to nontreated control cells in KRHB as follows: Intracellular proton index (IPX) (%) = ((proton concentration$_{sample}$/proton concentration$_{control}$) × 100) − 100. The lower the intracellular proton concentration, the higher the proton secretion by the cell.

Gene Expression. Cells were sown at a density of about 30 000 cells per well in a six-well plate and grown to confluence for 72 h. Medium was removed, and the cells were washed once with KRHB. After treatment with test substances for 5, 10, 15, 20, 25, or 30 min, the cells were washed once with cold PBS and then harvested for RNA extraction and cDNA transcription. KRHB was used as control treatment. All applications were 1.5 mL in volume. RNA was extracted using the SV Total RNA Isolation Kit (Promega, Mannheim, Germany). For cDNA synthesis, the High Capacity RNA-to-cDNA Mastermix (Applied Biosystems, Vienna, Austria) was used with a 20 μL reaction setup. Real-time qPCR was conducted with 100 ng of cDNA and the Power SYBR Green PCR Master Mix (Applied Biosystems) in a 10 μL reaction setup. Primer design was taken from previous studies at a concentration of 100 nM each.[18] Measurements were performed on a StepOnePlus Realtime PCR System (Applied Biosystems). Target genes were *ATP4A, HRH2, CHRM3*, and *SSTR2* (primer sequences see Table 2). *PPIA* (peptidylproyl isomerase A) was used as a reference gene.[25] Efficiencies and N_0 values were calculated per reaction setup using LinregPCR software.[26] Efficiency outliers were defined outside of 5% per gene.

Table 2. Primer Sequences of the Four Target Gene Primer Pairs and the Reference Gene Primer Pair Used for qPCR Analysis

gene	direction	sequence
ATP4A	sense	5′-CGG CCA GGA GTG GAC ATT CG-3′
	antisense	5′-ACA CGA TGG CGA TCA CCA GG-3′
HRH2	sense	5′-TGG GAG CAG AGA AGA AGC AAC C-3′
	antisense	5′-GAT GAG GAT GAG GAC CGC AAG G-3′
CHRM3	sense	5′-AGC AGC AGT GAC AGT TGG AAC-3′
	antisense	5′-CTT GAG CAC GAT GGA GTA GAT GG-3′
SSTR2	sense	5′-TCC TCC GCT ATG CCA AGA TGA AG-3′
	antisense	5′-AGA TGC TGG TGA ACT GAT TGA TGC-3′
PPIA	sense	5′-CCA CCA GAT CAT TCC TTC TGT AGC-3′
	antisense	5′-CTG CAA TCC AGC TAG GCA TGG-3′

Quantification of Relevant Beer Constituents. *Organic Acids.* Quantification of organic acids was performed in duplicate as described by Montanari et al.[27] on an HPLC system (Ultimate 3000RS Standard LC Systems, Dionex, Vienna, Austria) equipped with a binary pump (Dionex UltiMate 3400RS Binary Pump, Dionex) and a diode array detector (Dionex Diode Array Detector DAD-3000RS, Dionex) recording at 210 nm. Data were collected on a Chromeleon 6.8 system (Dionex). The analysis was performed isocratically at 0.5 mL/min with a Phenomenex Luna 5 μm C18 100 Å LC Column 250 × 3 mm (Phenomenex, Aschaffenburg, Germany) at 30 °C. The mobile phase consisted of 97% 10 mM phosphate buffer at pH 2.54 and 3% methanol filtered through 0.2 μm regenerated cellulose (Whatman GmbH, Dassel, Germany). All standards were analytical grade (Roth). Samples were degassed for 20 min in an ultrasonic bath. The organic acids were extracted through anion exchange using Strata-X-A SPE columns (Phenomenex). Columns were activated and equilibrated with 5 mL of methanol and 5 mL of water prior to being loaded with 10 mL of beer sample at pH 6−7. Samples were washed with 10 mL of water and 10 mL of methanol and eluted with 5 mL of 0.1 M HCl. The eluate was passed through a 0.2 μm nylon filter (Sigma-Aldrich), and 30 μL was injected directly onto the Phenomenex Luna C18 column. For calibration, a six-point calibration curve was recorded for each analyte by diluting a stock solution of 10 mg/mL 1:10, 1:20, 1:40, 1:100, 1:200, and 1:400. The efficiency of the extraction was determined by adding 1 mg of each organic acid to 10 mL of water at pH 6−7 and treating the solution in the same manner as a sample.

Bitter Acids. For quantification of bitter acids, beer samples (5 μL) were degassed by ultrasonification and, after membrane filtration (0.45 μm, Sartorius, Goettingen, Germany), directly injected into a Dionex UltiMate 3000 series UHPLC system consisting of a pump, a degasser, a column compartment, and an autosampler (Dionex, Idstein, Germany) connected to an API 4000 Q-TRAP mass spectrometer (AB Sciex Instruments, Darmstadt, Germany) which was equipped with an electrospray ionization (ESI) source and operated in negative ionization mode. The temperature of the autosampler was set to 5 °C and of the column compartment to 20 °C. Quantitative analysis was performed by means of multiple reaction monitoring (MRM) mode using the fragmentation parameters and retention times of pure reference compounds obtained using protocols reported by Haseleu, Intelmann, and co-workers.[13,28,29] The MS/MS parameters (declustering potential (DP), the cell exit potential (CXP), and the collision energy (CE)) were optimized for each substance to induce fragmentation of the pseudomolecular ion $[M - H]^-$ to the corresponding target product ions after collision-induced dissociation. The ion spray voltage was set to −4500 V, and dwell time for each mass transition was 3.3×10^{-3} s. Nitrogen was used as the collision gas (4×10^{-5} Torr). To enable quantification of the analyzed compounds, six-point external matrix calibration curves were determined by means of UHPLC-MS/MS, revealing correlation coefficients of >0.999 for all reference compounds in unhopped beer. Data processing and integration was performed by means of Analyst software version 1.5 (AB Sciex Instruments). As stationary phase, a Synergi 4 μm Hydro-RP column (150 × 2.0 mm) (Phenomenex) was used. The mobile phase consisted of acetonitrile (MeCN) + 0.1% formic acid (HCOOH) as solvent A and H_2O + 0.1% HCOOH as solvent B. Using a flow rate of 0.25 mL/min, chromatographic separation was achieved by gradient elution increasing solvent A from 20% to 60% within 20 min and further increased to 70% in 15 min, to 92% during 28 min, and, finally, to 100% within 2 min. It was maintained at 100% for 5 min, followed by readjustment to 20% within 1 min and re-equilibrated for 5 min prior to the next injection.

Statistics. Statistical analysis was performed using the software programs Apple Numbers 09, Microsoft Excel 2007, and Systat software SigmaPlot 11. Comparisons between data sets were calculated by applying the two-tailed Student's *t* test for equal variances for the comparison of histamine to the control. To compare all test samples with the control and among each other, a variance analysis (ANOVA) with a Holmes−Sidak posthoc test was performed. Numbers of replicates for each experiment are stated in the figures (n = number of biological replicates, including three to six technical replicates).

RESULTS AND DISCUSSION

The aim of the here-presented study was to investigate the influence of beer on gastric acid secretion with a focus on compositional differences among different types of beer. To prove our hypothesis of various prosecretory compounds besides the identified active constituents ethanol and succinic acid being present in beer, we chose HGT-1 cells to analyze the effects of different beers. To prove the activity of structurally promising constituents, the amounts of organic and bitter acids were quantified and then tested for their prosecretory potential.

Comparison of Effect of Different Beer Types on Proton Secretion. The five types of beer tested differed in their alcohol content, the original wort, and the types of hops used for brewing. Therefore, a comparison of the effects of different types of beer on proton secretion in vitro was conducted. Tables 1 and 3 show the beer ingredients and differences in original wort and ethanol concentrations of the tested beers, as given by the brewery. Samples were lager beer, dark beer, wheat beer, pilsener, and alcohol-free beer, which were compared to 5.2% ethanol (Figure 2). All samples were

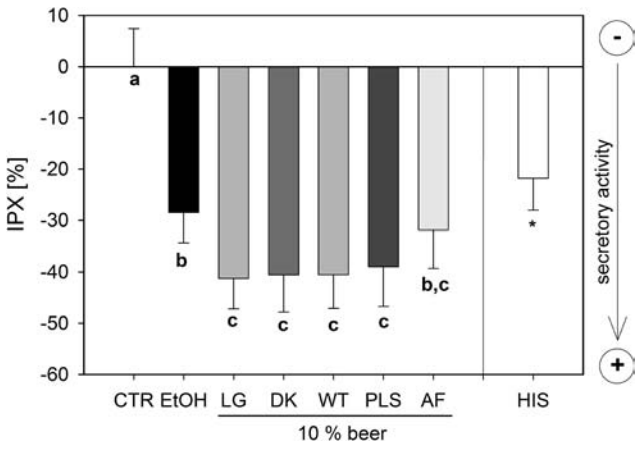

Figure 2. Effect of different types of beer on proton secretion of HGT-1 cells. Treatment with a 1:10 dilution of 5.2% ethanol (EtOH), lager (LG), dark beer (DK), wheat beer (WT), pilsener (PLS), and alcohol-free beer (AF) for 10 min. Positive control was histamine (HIS) 1 mM, $^{**}p \leq 0.01$. Data represents mean ± SEM of $n = 6$. Significant differences ($p \leq 0.05$) among the samples are indicated by the letters a to c.

diluted 1:10 and therefore relate to a 10% beer solution. Histamine, a known stimulant of proton secretion, was used as a positive control at a concentration of 1 mM. The use of histamine in the test system has been established in our group in previous studies.[18−21]

All tested beers showed an effect significantly higher than that of the untreated control ($p \leq 0.001$). Ethanol itself had a distinct effect ($p \leq 0.001$), but this was significantly weaker than the effect of all alcoholic beers ($p \leq 0.05$). On the other hand, the effect seen for the alcohol-free beer was weaker than that for the alcoholic beers without being significant. In contrast to the results of Singer et al.,[2] who studied the effect of beer, beer constituents, and ethanol on gastric acid secretion, the findings of the here-presented in vitro experiments indicate that ethanol promotes gastric acid secretion in concentrations found in beer.

The alcoholic beers did not differ from each other in their ability to promote proton secretion. However, the alcohol-free beer also had a substantial effect on gastric acid secretion. Supporting the findings of previous studies on rats that showed an effect of both an alcohol-free beer and a beer containing 4.9% (v/v) EtOH,[30] the results of the in vitro tests also show the difference between 5.2% EtOH and the alcoholic beers as well as a prosecretory effect of alcohol-free beer, suggesting the relevance of other beer compounds for the stimulation of gastric acid secretion.

Quantification of Organic and Bitter Acids in Lager and Alcohol-Free Beer. To investigate the effect of individual beer compounds in concentrations representative in beer on mechanisms of gastric acid secretion, it was necessary to quantify the beer constituents. Although the effects of the alcoholic types of beer did not differ significantly from each other in the proton secretion assay, there was a clear trend for the lager beer having the strongest effect, which prompted us to choose it as a representative of alcoholic beers. Its constituents were quantified in comparison to those in alcohol-free beer to determine their relevance for mechanisms of gastric acid secretion. Previous studies by Teyssen et al.[9] on the promotion of gastric acid secretion by fermented beverages showed that organic acids are an important class of compounds with regard to a prosecretory potential. Therefore, known organic acids were quantified by HPLC-DAD (Table 3). Maleic acid could be

Table 3. Quantitative Data of Organic Acids and Hop-Derived Bitter Compounds in Lager Beer and Alcohol-Free Beer[a]

	lager	alcohol-free beer	limit of detection
organic acids			
maleic acid (mg/L)	<LOD	<LOD	25
succinic acid (mg/L)	350.8	161.2	25
malic acid (mg/L)	108.1	43.1	25
Citric acid (mg/L)	90.0	104.6	25
bitter acids			
α-acids (mg/L)	4.812	0.057	0.005
β-acids (mg/L)	0.142	0.012	0.005
iso-α-acids (mg/L)	51.37	34.04	0.010

[a]LOD: limit of detection.

quantified neither in the lager nor in the alcohol-free beer. In contrast, succinic acid was the most predominant organic acid quantified in both beers, with 350 mg/L in the lager and 161 mg/L in the alcohol-free beer, respectively. A ratio similar to that for succinic acid, which was determined in a 2.2 times higher concentration in lager beer than in alcohol-free beer, was found for malic acid with 2.5 times more malic acid in the lager than in the alcohol-free beer. However, the alcohol-free beer contained 15% more citric acid than the lager beer.

The kind and amount of organic acid generated during fermentation is highly dependent on the type of yeast and overall process control.[31] Accordingly, the absence of maleic acid as well as the rather high amount of succinic acid compared to previous findings can be explained.[9,27] Concentrations determined for malic and citric acid are well within the range determined in other studies, 40 to 220 mg/L and 50 to 150 mg/L, respectively.[27] The higher amount of organic acids found in lager beer than in alcohol-free beer might be attributed to the fact that alcohol-free beer is generally produced under shorter fermentation times.

The hop-derived bitter acids are largely unknown for their effect on gastric acid secretion, but their contribution to the

bitter taste of beer and the fact that bitter substances are commonly regarded to have an effect on the digestive system makes them constituents of high interest. Thus, the bitter compounds were quantified in the classes α-, β-, and iso-α-acids in lager and alcohol-free beer (Table 3).[12]

The amount and ratio of bitter acids found in beer is determined by the amount and kind of hops added during wort boiling and the time of addition. During wort boiling, the α- and β-acids, which are the major phytochemicals in hops, are isomerized to give the corresponding iso-α-acids exhibiting the highest contribution to the bitter taste of beer.[12] The findings on bitter acids are comparable to previous studies.[12,13] Lager beer usually has a more bitter taste than alcohol-free beer, which is reflected in the respective amount of bitter acids. The difference in the concentration of the bitter acids between the lager and the alcohol-free beer could be due to a better solubility of the bitter compounds in ethanol than in water. However, for the commercial samples studied here, it is not known whether the same amount of hops was used to make lager and the alcohol-free beer.

Effect of Organic Acids and Bitter Acids on Proton Secretion. The quantified organic acids, succinic acid, which was identified as a stimulant of gastric acid secretion in previous studies,[9] malic acid, which conforms to the structure−effect hypothesis of requiring a C4 body and two carboxyl groups,[9] and citric acid, which is another quantitatively relevant organic acid found in beer, were used to analyze their secretory potential. Test substances were compared to the effect of lager beer with an IPX of −47.9 ± 4.1%. All samples were diluted 1:10 unless indicated otherwise, and histamine (1 mM) was used as a positive control.

Figure 3 shows that all three tested organic acids have an effect significantly higher than that of nontreated control at concentrations found in beer and dilutions thereof ($p \leq 0.001$) in a dose-dependent manner. In concentrations comparable to those found in lager beer, succinic acid stimulated proton secretion with an IPX of −32.7%, malic acid with an IPX of −40.1%, and citric acid with an IPX of −31.2% (Figure 3). These effects should probably not be presumed to be additive,

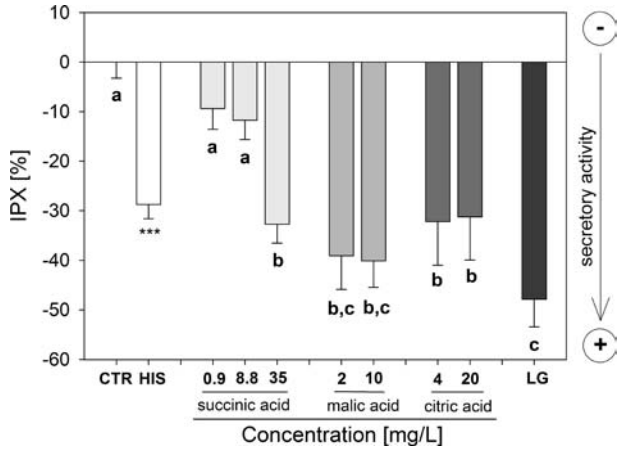

Figure 3. Effect of organic acids on proton secretion of HGT-1 cells. Treatment of cells with 1:10 to 1:400 dilutions of succinic acid (350.8 mg/L), malic acid (100 mg/L), and citric acid acid (200 mg/L) compared to a 1:10 dilution of lager beer for 10 min. Positive control was histamine (HIS) 1 mM, ***$p \leq 0.001$. Data represent mean ± SEM of $n = 5-8$. Significant differences ($p \leq 0.05$) are indicated by the letters a to c.

since linearity of the impact is not to be expected, given the physiological limitations of parietal cells in proton output. The maximum in proton output of the parietal cells might have been reached with the 1:10 diluted beer already. No differences between the highest tested concentrations of the organic acids were detectable. This also means that it cannot be excluded that there are other substances besides the organic acids that might contribute to the effect of the beer.

Succinic acid was confirmed to promote gastric acid secretion, but additionally, citric acid and malic acid showed an effect as well. For malic acid, the structure−effect hypothesis from a previous study would fit.[9] In contrast, for citric acid, which does not match these criteria, a rather substantial effect could be observed. In the study by Teyssen et al., fermented glucose was used to identify the active organic acids, not beer. Beer is known to consist of more than the six quantified organic acids formed during fermentation of glucose.[9,32−34] These findings strongly suggest that the spectrum of prosecretory substances found in beer is broader than previously assumed.

In addition to the organic acids, hop-derived α-, β-, and iso-α-acids (Figure 1) were quantified in beer and tested as purified substances in their natural concentrations on their potential to stimulate proton secretion, an indication for a stimulation of gastric acid secretion in concentrations quantified in lager beer (Figure 4). Their effect was compared to 5.2% ethanol and

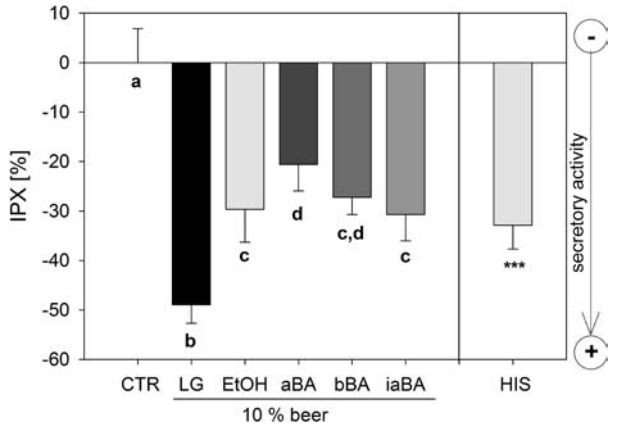

Figure 4. Effect of bitter acids on proton secretion of HGT-1 cells. Treatment of cells with a 1:10 dilution (10%) of the α-bitter acids (aBA), β-bitter acids (bBA), and iso-α-bitter acids (iaBA) compared to lager (LG) and 5.2% ethanol (EtOH) for 10 min. Positive control was histamine (HIS) 1 mM, ***$p \leq 0.001$. Data represent mean ± SEM of $n = 4$. Significant differences ($p \leq 0.05$) are indicated by the letters a to d.

lager beer. All tested compounds, measured in a 1:10 dilution, showed a significantly greater effect than nontreated control cells ($p \leq 0.001$). The IPX of the α-acids was −20.6%, of the β-acids −27.2% and of the iso-α-acids −30.7%. Ethanol had an effect significantly stronger than that of the α-acids ($p \leq 0.05$), but its effect was not different from those of the β-acids and the iso-α-acids. However, the α-acids affected gastric acid secretion significantly weaker than that of the β-acids ($p \leq 0.05$) and the iso-α-acids ($p \leq 0.01$).

The bitter acids showed a strong effect on the proton secretion, a key mechanism of gastric acid secretion. In fact, treatment of the cells with β-acids led to a comparable IPX, reached with a treatment of the highest concentration tested for citric

acid even though the concentrations of β-acids is only one thousandth of the concentration of citric acid. The differences in impact among the bitter acids cannot be attributed to the differences in test concentrations, because β-acids were applied at a concentration over 40 times lower than that of α-acids (0.081 mg/L and 3.57 mg/L) yet showed a significantly greater effect. A correlation can rather be drawn between the compound's contribution to the perceived bitter taste and their acid output. Here, the iso-α-acids comprise the greatest contribution followed by the β-acids, which account for a long-lasting bitterness, whereas the α-acids only make a small contribution to the bitter taste of beer.[12] Further tests would be required to elucidate the effects of downstream reaction products formed in the brewing process that were not evaluated here.[10,11,29,35]

Effects of Lager Beer, Ethanol, and Bitter Acids on the Expression of Genes Relevant for Gastric Acid Secretion. In order to gain insight into the pathways of gastric acid secretion affected by beer and beer components, expression of secretory-relevant genes was measured by real-time qPCR. Therefore, we tested lager, alcohol-free beer, 5.2% ethanol, α-acids, β-acids, and iso-α-acids in lager beer-representative concentrations for their influence on the expression levels of four target genes involved in gastric acid secretion. The expression of three prosecretory genes (*ATP4A, HRH2*, and *CHRM3*) and one antisecretory gene (*SSTR2*) was compared after treatment with different compounds at a dilution of 1:10 over 30 min.

The effect of lager beer on all four tested genes (Figure 5) occurred after 10 to 15 min, with a maximum relative expression level of the acetylcholine receptor (*CHRM3*) after 15 min (1.64 ± 0.70, $p \leq 0.05$) compared to nontreated cells (= 1). Additionally, the other two prosecretory genes *ATP4A*, encoding the H^+/K^+-ATPase and *HRH2*, encoding the histamine receptor were up-regulated to a maximum of 1.30 ± 0.34 after 15 min ($p \leq 0.05$) and 1.43 ± 0.13 after 10 min ($p \leq 0.05$), respectively. These effects were counter-regulated after 20 to 30 min treatment. In contrast, the antisecretory gene *SSTR2*, encoding the somatostatin receptor, was not regulated significantly by the lager beer. These results underscore the findings of the functional assays that lager beer is a stimulant of acid secretion. Additionally, a relatively weak up-regulation (<1.5) of the prosecretory genes led to a strong decrease of the intracellular pH, indicating a strong proton secretion, by the lager beer, showing that a significant up- or down-regulation below 2.0 can affect the proton output, indicating relevance for gastric acid secretion. However, the lager beer ingredients might act through different mechanisms of action. Therefore, it was necessary to further investigate the influence of ethanol and the alcohol-free beer on the target genes (Figure 5A).

Ethanol showed the strongest effect of all tested solutions on the expression of the *CHRM3* after 5 min (2.30 ± 1.77, $p \leq 0.05$). However, the effect got strongly counter-regulated after 10 min of treatment. In addition, also the *ATP4A* was up-regulated rapidly (5 min) by the tested EtOH concentration. In contrast to these fast responses, the *HRH2* became up-regulated only after 25 min by EtOH. Therefore, the effect of EtOH does not fully fit the findings for the lager beer. EtOH influences the gene expression of the relevant genes tested, but the effect seen for the lager beer is also influenced by other compounds found in the beer and cannot be explained by the effect of ethanol alone.

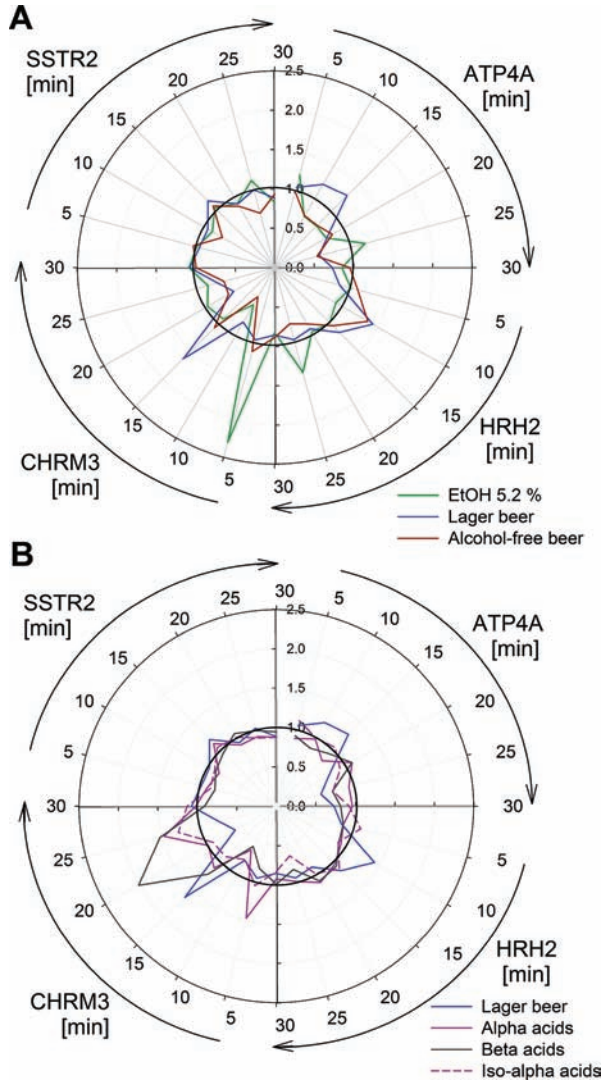

Figure 5. Time-dependent effect of a 1:10 dilution of (A) lager, alcohol-free beer, and 5.2% ethanol, and (B) the bitter acid extract, α-acids, β-acids, and iso-α-acids on gene expression of *ATP4A*, *HRH2*, *CHRM3*, and *SSTR2* after 5, 10, 15, 20, 25, and 30 min of incubation. Data represent the mean of $n = 3$.

To ensure this hypothesis, the alcohol-free beer was tested. Like the lager beer, alcohol-free beer also showed a stimulating effect on the expression of *ATP4A* after 15 min of treatment (1.70 ± 0.68, $p \leq 0.05$). In contrast to the lager beer, the alcohol-free beer significantly down-regulated the *SSTR2* after 10 min (0.76 ± 0.29, $p \leq 0.01$) and 25 min (0.71 ± 0.19 $p \leq 0.05$). Interestingly, the expression of *CHRM3* was significantly down-regulated by the alcohol-free beer after 10 min. Comparing the results for lager, alcohol-free beer, and ethanol, clear differences can be seen in the effects on the *SSTR2* and the *CHRM3* in particular. This leads to the conclusion that EtOH contributes markedly to the effect of the lager beer on the regulation of the four tested genes, but other beer ingredients also have an impact on the regulation of these genes.

After the identification of the bitter acids as key players in the effect of beer on proton secretion in the functional assay, we wanted to analyze the mechanism by which these substances might contribute to the effect of beer. Thus, the bitter acids were tested for their influence on the four genes described

above in concentrations representative in lager beer (Figure 5B). The bitter acids up-regulated the *CHRM3* between 5 and 25 min of treatment, to a maximum extent by the β-acids (2.01 ± 0.81, $p \leq 0.01$) after 20 min. Furthermore, all types of bitter acids decreased the expression levels of the antisecretory gene *SSTR2* with a maximum effect of 0.81 ± 0.11 ($p \leq 0.001$) after 10 min. The ranking of the bitter acids by their effect on the gene regulation of gastric acid secretion-relevant genes is from the least to the most effective substance class: α-acids, iso-α-acids, and β-acids. Again, the α-acids, which contribute only little to the bitter taste of beer,[12] showed the lowest effects.

The results show that lager beer bears its effect on gastric acid secretion by increasing the expression of prosecretory genes *ATP4A, HRH2,* and *CHRM3,* whereas alcohol-free beer only stimulates expression of *ATP4A* and decreases expression of antisecretory gene *SSTR2*. These findings indicate that this difference can largely be explained by the absence of ethanol in alcohol-free beer, because ethanol mainly promotes the expression of *HRH2* and *CHRM3*. The decrease in expression of *SSTR2* can be observed throughout the effects of α-acids, β-acids, and iso-α-acids. The single bitter acid fractions mainly stimulated the gene expression of *CHRM3*.

In a rat pylorus-ligated model, Kurasawa et al.[10] showed that hops have a similar influence on gastric juice volume as carbachol, a drug that is a structure analogue of acetylcholine and therefore binds to and activates the acetylcholine receptor. Acetylcholine is a neurotransmitter that makes the parietal cell more sensitive to stimulation by the enteric nervous system through a higher expression of the *CHRM3*. However, the effect of the bitter acids on gastric acid secretion could also be mediated by other signaling pathways. The dependency of the bitterness on the effect leads to the assumption that the bitter receptor signaling could be involved. The bitter taste receptor TR2 has been identified in enterchromaffin (EC) cells, which play a crucial role in the endocrine system of the gastrointestinal tract, and it has been shown that caffeine, as a bitter compound, significantly increased serotonin release from the EC cells.[36] Taste receptors might also be expressed in other cell types in the gastrointestinal tract, and the bitter acids might therefore directly affect signaling pathways via binding to the bitter receptors.

In conclusion, beer has been shown to be a strong stimulant of gastric acid secretion, independent of the type of beer, comparing the prosecretory potential of five beers in vitro. The ethanol content contributes to the effect, although other prosecretory substances are present in beer. Of these, the organic acids were analyzed, and it could be shown that not only maleic acid and succinic acid are responsible for the effect, as previous studies proclaimed,[9] but also malic acid and citric acid, which could be quantified in the beer samples analyzed. For the first time, the hop-derived α-, β-, and iso-α-acids could be identified as a class of substances heavily linked to gastric acid secretion, and these findings suggest that their impact is correlated with the contribution to bitter taste of beer. The data obtained are the scientific basis toward the manufacturing of stomach-friendly beer by tailoring the bitter acid composition of beer by the choice of hops, the time of addition, and the temperature during wort boiling.

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Notes

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ABBREVIATIONS USED

ATP4A, H^+/K^+-ATPase α-subunit; HRH2, histamine receptor H2; CHRM3, acetylcholine receptor M3; SSTR2, somatostatin receptor 2; PPIA, peptidylproyl isomerase A; KRHB, Krebs–HEPES buffer; IPX, intracellular proton index; EtOH, ethanol.

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