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# DISSERTATION

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„Impact of flavoring food compounds on mechanisms regulating gastric acid secretion“

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*Für meine Familie*

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## List of abbreviations

| | |
|---|---|
| AC | Adenylate cyclase |
| ATP | Adenosine triphosphate |
| CaM | Calmodulin |
| cAMP | Cyclic adenosine monophosphate |
| CCK | Cholecystokinin |
| CCK-B | Cholecystokinin receptor |
| CHRM3 | Cholinergic M3 receptor |
| CNS | Central nervous system |
| DAG | Diacylglycerol |
| ECL | Enterochromaffin-like |
| ED | Eriodictyol |
| Gα | G protein subunit α |
| Gβγ | G protein subunit βγ |
| GERD | Gastro-esophageal-reflux disease |
| GLP-1 | Glucagon-like peptide 1 |
| GNAT2 | Transducin |
| GNAT3 | Gustducin |
| GPCRs | G protein-coupled receptors |
| HCl | Hydrochloric acid |
| HED | Homoeriodictyol |
| HRH2 | Histamine H2 receptor |
| $IP_3$ | Insositol triphosphate |
| MLF | Malolactic fermentation |
| PDE | Phosphodiesterase |
| PGG | Pentagalloylglucose |
| $PIP_2$ | Phosphatidylinositol bisphosphate |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| PLC*β*2 | Phohpholipase *β*2 |
| SNP | Single nucleotide polymorphism |
| SSTR2 | Somatostatin R2 receptor |
| TAS2R | Taste receptor, type 2 |

# I. Introduction

Gastric acid secretion is necessary for digestion of proteins, absorption of iron and calcium and activation of the intrinsic factor [1]. Furthermore, it prevents bacterial overgrowth and degrades food allergens [1]. Besides these essential functions, increased gastric acid secretion can cause discomfort accompanied by symptoms like epigastric pain, reflux or heartburn. In contrast, decreased acid secretion can cause epigastric fullness or nausea [2,3]. Dysregulation of gastric acid secretion is associated with chronic diseases such as gastro-esophageal-reflux disease (GERD) [4], gastritis or ulcer [5], which can, in the long run, cause carcinogenesis in the stomach and lower esophagus [2,6]

The first target in treating GERD and peptic ulcer is usually neutralization or reduction of gastric acid. Neutralization can easily be accomplished by administration of an alkaline solution. The core strategy to reduce gastric acid secretion still is the medication with proton pump inhibitors. However, these medications can cause several side effects. For newer therapeutics targeting sphincter pressure, the risk factors need to be evaluated [7].

Besides medication, people suffering from symptoms of GERD or peptic ulcer are advised not to drink coffee or alcoholic beverages such as wine and beer. This indicates that certain foods may affect mechanisms of gastric acid secretion and that it might be possible to influence gastric acid secretion by food intake.

The present thesis aimed at investigating the impact of multiple wine and beer samples including selected flavoring compounds thereof on mechanisms regulating gastric acid secretion. Furthermore, the underlying mechanisms of how flavoring compounds can stimulate gastric acid secretion in the stomach should be clarified by using the well-studied pro-secretory bitter compound caffeine. Identification of molecules that can activate molecular targets and in turn further regulative mechanisms of gastric acid secretion can support the development of new therapeutic strategies in treating gastric diseases. Furthermore, this knowledge can be used to develop stomach friendly food products.

## 1.1 Regulation of gastric acid secretion

The gastric mucosa produces 2-3 L of gastric juice, which mainly consists of hydrochloric acid (HCl), intrinsic factor, pepsinogenes, mucin and bicarbonate [8]. While mucin and bicarbonate are produced and secreted continuously, the secretion of HCl and pepsinogenes is regulated in dependence of food digestion by neural, hormonal, and paracrine pathways as well as by mechanical and chemical stimuli [9]. Pepsinogen is the zymogen of the active digestion enzyme pepsin. It can only be activated in an acidic environment, which, in turn, is created by HCl secretion. Pepsin is essential for the digestion of food proteins, degrading them into peptides [9].

The stomach consists of two major areas; the oxyntic gland area, which spreads out over approximately 80 % of the gastric surface, and the antral gland area, representing 20 % of surface area [10]. In addition, the stomach can also be subdivided into fundus, corpus and antrum. While fundus and corpus represent the oxyntic gland area, the antrum represents the antral gland area (Figure 1). The presence of parietal cells characterizes the oxyntic mucosa. These cells produce gastric acid. It has been estimated that around 1 x $10^9$ parietal cells are present in the human stomach [9,11]. Oxyntic glands also consist of pepsinogen and leptin secreting chief cells, mucus producing neck cells, and several enteroendocrine cells including histamine secreting enterochromaffin-like (ECL) cells, somatostatin-secreting D cells, and ghrelin-secreting Gr or A-like cells [12].

Gastric acid secretion associated with food consumption can be divided into three stages; cephalic phase, gastric phase and intestinal phase of digestion [2]. The classification is based on the controlling site: brain, stomach or duodenum, respectively. These phases can occur simultaneously. Beside these phases, a fourth phase can be distinguished: the basal state, which occurs between meals, the so-called interdigestive phase. In this phase only 10 – 15 % of maximal acid output occurs. After vagotomy, the transection of vagus nerve and dissection of antrum and removal of G cells basal secretion stops [13].

The cephalic phase starts with the pure thinking of food, and is further stimulated by smelling and tasting it. These processes are controlled in the central nervous system (CNS) which may lead to a parasympatical stimulation of gastric acid secretion by the vagus nerve. It has been shown that vagotomy stops the cephalic phase [13]. Maximum acid output in this phase reaches 45% of total acid secretion [13]. Richardson and colleagues [14] showed that cephalic stimulation induced by modified

sham feeding, where volunteers had to chew and expectorate an appetizing meal, accounts for one third of total acid secretion. Acid secretion was determined by *in vivo* intragastric titration in 9 volunteers. Food distention was induced by 600 mL liquid test meal and acted as another stimulus [14].

Emotions can also influence gastric acid secretion: anger, pain and sorrow can inhibit gastric acid secretion, while emotions like stress and aggression can promote gastric acid secretion as well as hypoglycemic situations [13].

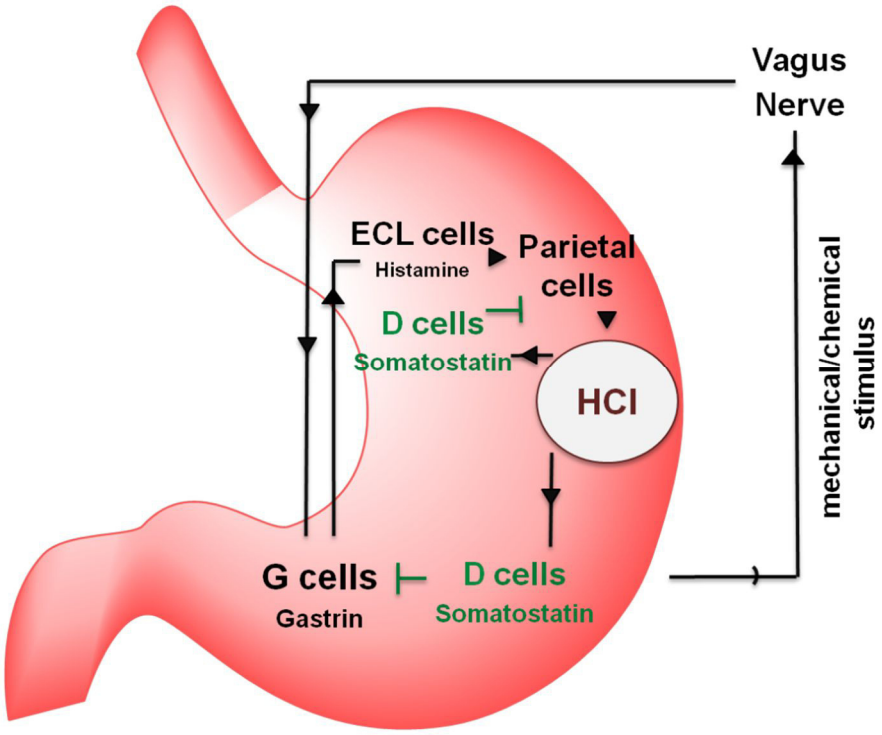



**Figure 1. Schematic overview illustrating physiological regulation of gastric acid secretion by vagus nerve, gastrin, histamine and somatostatin. Vagus nerve stimulates release of gastrin from G cells, and in turn, stimulates histamine release of ECL cells. Histamine paracrine activates parietal cells, which produce HCl. Somatostatin released from D cells finally inhibit gastric acid secretion (adapted from Konturek 2008 [10]).**

The gastric phase of gastric acid secretion is induced by mechanical and chemical receptor afferents as well as efferents of *Nervus vagus*.

Acetylcholine released by nerve fibers induce gastric acid secretion directly by activation of parietal cells, or indirectly via activation of G- cells or ECL cells [9]. Antral G-cells release gastrin into the blood stream. Gastrin binds to the CCK-B receptor, also known as CCK-2 or gastrin receptor, a G protein-coupled receptor also expressed on

ECL cells to release histamine. Histamine is the principal and final common mediator of gastric acid secretion [15]. Within the paracrine pathway, histamine binds to the histamine H2 receptor (encoded by the gene *HRH2*) on parietal cells to stimulate gastric acid secretion.

The gastric phase contributes 50 % to the total gastric acid secretion. When the gastric pH is below pH 3 somatostatin is released from the D-cells of the gastric oxyntic and pyloric mucosa. Somatostatin inhibits gastric acid secretion in a paracrine fashion directly by inhibiting parietal cells or indirectly by inhibiting histamine secretion from ECL cells or gastrin secretion from G cells. The biological action of somatostatin is mediated via G protein-coupled receptors, especially via the somatostatin receptor 2 (encoded by the gene *SSTR2*) [9].

The intestinal phase is defined as the phase when the chyme reaches the duodenum. This can have a stimulating effect, but only up to 5 % of total acid output, or a more pronounced inhibiting effect. Distention of the duodenal wall, a pH below 4 or passage of digestive products, such as fat, induces secretin release in duodenal S-cells [13,16]. Secretin inhibits gastric acid secretion, however, the exact mechanism is not fully understood. So far, it has been shown that secretin stimulates somatostatin production in D cells. Furthermore, it has been suggested that secretin inhibits gastric acid secretion via afferent signals, which leads to neuronal modulation of gastric acid secretion. However, another study showed that the inhibitory effects of secretin are independent of vagotomy [16].

### 1.1.1 Parietal cells

Parietal cells secrete HCl in a concentration of approximately 160 mmol/l or pH 0.8 [1]. For the production of gastric acid, three types of ions are involved. Most importantly, the protons ($H^+$) are pumped into the gastric lumen by a proton pump, the $H^+$-$K^+$-ATPase, to acidify the gastric lumen. In exchange, potassium ions are pumped from the lumen into the parietal cell. The apical chloride channels transport chloride into the lumen for HCl formation. Disruption of one of these ion transport mechanisms inhibits gastric acid secretion [16]. The $H^+$-$K^+$-ATPase exchanges one intracellular hydrogen

ion for one extracellular potassium ion at the expense of energy delivered from adenosine triphosphate (ATP). ATP is provided by mitochondria, which occupy up to 40 % of the cell volume [16]. In the resting phase, the $H^+$-$K^+$-ATPase is stored in tubulovesicles throughout the parietal cell. Stimulation of the parietal cell results in a membrane transformation. The tubulovesicles fuse with the apical pole, which is characterized by multiple microvilli-lined membrane invaginations, the secretory canaliculi. Thus, the cell surface is maximized and the insertion of $H^+$-$K^+$-ATPase into the membrane is allowed [9,15-17].

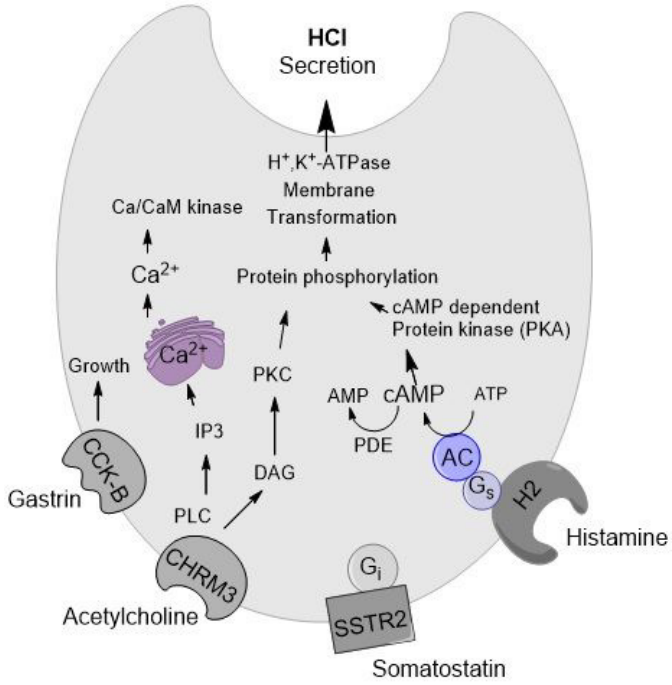



**Figure 2. Model illustrating signaling pathways of a parietal cell resulting in membrane transformation and HCl production (adapted from Forte 2010 [15]).**

Figure 2 illustrates the signaling pathways in parietal cells. For activation of the parietal cell, it is necessary that either acetylcholine or histamine binds to the respective receptor, which leads to the activation of two different signaling pathways. Histamine binding to the histaminic H2 receptor leads to the activation of adenylate cyclase (AC) which catalyzes ATP conversion to cAMP and further activates cAMP dependent protein kinase (PKA) [15]. The phosphodiesterase (PDE) breaks down cAMP to AMP. Binding of acetylcholine to the cholinergic (M3) receptor activates the phospholipase C, which cleaves membrane bound phosphatidylinositol bisphosphate ($PIP_2$) to yield insositol triphosphate ($IP_3$) and diacylglycerol (DAG). $IP_3$ activates release of $Ca^{2+}$ from

release of $Ca^{2+}$ from membrane stores. Protein phosphorylation by PKA, PKC and calmodulin (CaM) kinases activates recruitment of $H^+$-$K^+$-ATPase into the apical membrane, which is the basis for active HCl secretion [15]. Binding of somatostatin to the SSTR2 receptor inhibits HCl secretion directly. Although CCK-B receptors were found on parietal cells, gastrin does not activate HCl secretion directly. Activation of this receptor contributes to the regulation of cell maintenance and growth [15].

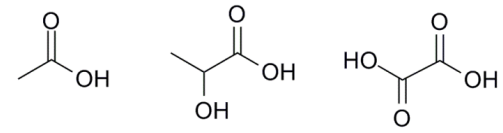
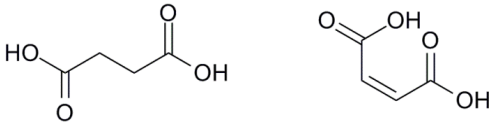
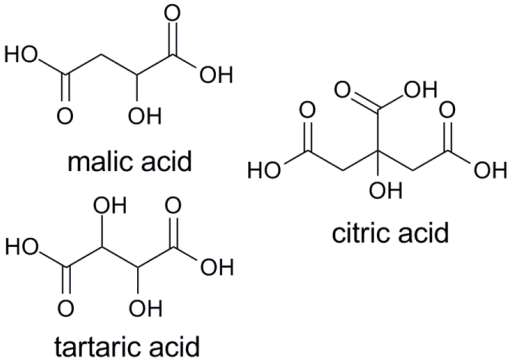
## 1.2 Impact of alcoholic beverages and constituents thereof on gastric acid secretion

Wine and beer are the most consumed alcoholic beverages in Europe according to the WHO Global status report on alcohol and health 2014. People who suffer from gastric discomfort are advised to avoid alcoholic beverages [18]. Therefore, several studies have examined the influence of different alcoholic beverages on gastric acid secretion using intragastric titration in humans [19-22] as well as in animals, such as rats [23], dogs [24], or in isolated glands from rabbits [25].

The group of Singer and Teyssen [20,22] measured gastric acid output in six volunteers using an intragastric titration method and revealed that fermented beverages like beer and wine stimulated gastric output, while distilled alcoholic beverages like whisky or campari containing a higher ethanol concentration showed very little or no effect. Furthermore, within these experiments, blood samples were drawn and plasma gastrin concentrations were determined. Plasma gastrin concentrations were also increased after administration of fermented beverages, indicating that gastric acid output was gastrin mediated. Matsuno and colleagues [25] revealed in isolated rabbit glands that red wine also induces gastric acid secretion via a second gastrin-unrelated pathway [25]. Consumption of ethanol itself stimulated gastric acid output only in concentrations between 1.4 and 4 % (v/v) but not in higher concentrations [22]. However, ethanol administration showed no effect on plasma gastrin concentrations, indicating that ethanol stimulates gastric acid secretion via a different pathway. These findings are in accordance with the study of Lenz and colleagues [26], who measured gastric acid output in eight healthy volunteers after administration of 5 % ethanol or white wine. Furthermore, Teyssen and colleagues [20,23] showed after distillation of beer, sherry and white wine that only the nonvolatile parts, acted prosecretory. These results revealed that other compounds

ethanol present in fermented alcoholic beverages contribute to the prosecretory effect. Based on this hypothesis, Teyssen and colleagues [21] fractionated yeast-fermented glucose and revealed that maleic acid and succinic acid are strong stimulants of gastric acid secretion, while acetic acid, lactic acid and oxalic acid showed little or no effect in six volunteers. Furthermore, the authors hypothesized that the length of the carbon chain, as well as the two carboxylic groups, are the main determinants of a molecule's effect on stomach acid secretion (Table 1). These structural characteristics are also found in other organic acids in wine, including tartaric acid, citric acid and malic acid, which are the predominant acids in wine and important for the taste of the beverage [27].

**Table 1.** Molecular structure of organic acids as putative stimulants of gastric acid secretion (adapted from Liszt et al. [28])

| | |
|---|---|
| **Low effect** on gastric acid secretion (12) |  acetic acid, lactic acid, oxalic acid |
| **Strong effect** on gastric acid secretion (12) |  succinic acid, maleic acid |
| **Unknown effect** on gastric acid secretion |  malic acid, citric acid, tartaric acid |

Concentration of malic acid can be reduced during the winemaking process via malolactic fermentation (MLF), a secondary fermentation step, after the completion of alcoholic fermentation. MLF is defined as the conversion of L-malic acid to L-lactic acid with the production of $CO_2$ [29]. This reaction is used to reduce wine acidity, decreasing the acidic taste of wine, improving the microbial stability and modifying the organoleptic character of wine to some extent [27]. The majority of red wines undergo MLF, while it

is less common in white wines. Therefore, total acidity is higher in white wines as well as the malic acid content.

Two previous studies compared the effect of red wine and white wine on gastric acid secretion, with inconsistent results. Tsukimi and colleagues [24] demonstrated a significantly stronger stimulation of red wine as compared to white wine after administration to dogs with vagally denervated Heidenhain pouches. In accordance to that finding, Peterson and colleagues [19] detected that after administration of 300 mL red or white wine in eight healthy subjects, the plasma gastrin levels were higher after red wine administration compared to administration of white wine. However, there was no difference in gastric acid output after administration of red or white wine, may due to the fact that the maximum gastric acid output was reached by both wines.

Even though the major characteristic of red wine is its content of phenolic compounds, which contribute to its color and flavor, especially the bitter and astringent taste of red wine, the influence of these compounds on gastric acid secretion has not been investigated so far. There is one study which measured the effect of phenolic compounds of paeoniae radix on proton secretion: Ono and colleagues [30] treated isolated parietal cells of guinea pigs with pentagalloylglucose (PGG) and gallic acid and measured [$C^{14}$] aminopyrine accumulation as index of acid production. PGG was determined to act antisecretorily in histamine and dibutyryl-cAMP stimulated isolated parietal cells, whereas gallic acid, a compound also present in wine, had no effect. Therefore, the contribution of phenolic compounds in red wine still remains unclear and has to be clarified.

Beer, also a strong stimulator of gastric acid secretion [20,31], contains a large variety of substances that derive from ingredients other than fermented glucose. Especially the hop-derived bitter acids in beer contribute to its specific bitter taste. However, their impact on gastric acid secretion is largely unexplored, although bitter taste is often associated with effects on the digestive system. There is one study which showed that hop extracts increase gastric juice volume but not gastric acid secretion in rats [32]. However, this study did not consider the formation of reaction products during the brewing process and storage, which represent the majority of hop-derived compounds in finished beer [33-35]. In contrast, stimulation of pancreatic enzyme secretion induced by beer, hops, barley extract or fractions thereof has been described previously [36-38]. This indicates that there might be a relevance of various beer constituents on digestion, and, therefore, on stomach physiology.

Little is known about the mechanisms of action by which wine and beer stimulate gastric acid secretion, and which compounds, besides organic acids, may contribute to this effect. Therefore, the effect of wine organic acids, phenolic compounds in wine and the beer bitter compounds on gastric acid secretion and regulating mechanisms thereof needed to be investigated.

## 1.3 Impact of caffeine on gastric acid secretion

The prominent bitter compound in coffee, caffeine, is the most consumed behaviorally active substance worldwide [39] and has been shown to stimulate gastric acid secretion [40-44].

Rudolph and colleagues [45] surveyed the caffeine consumption in 700 subjects aged between 14 – 39 years in Austria. The average total caffeine intake was 357 ± 400 mg per day with a median intake of 259 mg per day. Caffeine intake mostly derived from coffee (60.8%), energy drinks (11.9%) and colas (9.5%). Nevertheless, other sources for caffeine intake are tea, cocoa beverages and chocolate products [39,46].

Rubach and colleagues [40,47] tested the effect of several quantitatively predominant coffee compounds on gastric acid secretion. For this purpose they established the measurement of the intracellular pH in the human gastric tumor cell line (HGT-1 cells) using a pH sensitive fluorescence dye as indicator for proton secretion. Using this cell model, Rubach et al. [40,47,48] identified the pro- and anti-secretory activity of coffee samples and coffee components. HGT-1 cells have the characteristics of parietal cells, the cell type which actively produces gastric acid. Caffeine was shown to be a potent stimulator of proton secretion in parietal cells. In addition, caffeine showed the strongest stimulation of proton secretion compared to other tested coffee compounds chlorogenic acid, pyrogallol, catechol, βN-alkanoylhydroxytryptamides and N-methylpyridinium.

Furthermore, several human intervention studies explored the effect of caffeine on gastric acid secretion by administration of caffeine directly into the stomach [41,42], or given intravenously [43] or intramuscularly [44].

Conventionally, gastric acid measurements in humans are performed by aspiration of gastric contents through a nasogastric tube, which is commonly instilled during the entire measurements. Gastric content can be manually aspirated using a syringe.

Acidity of the gastric juice is determined by titration to a pH 7 using an alkaline solution and chemical indicators or pH meters [49]. Thus, the oral cavity was bypassed in most studies [41-44,50].

Roth and Ivy [44] showed that caffeine did not stimulate gastric acid secretion in dogs, whereas in cats, a stimulating effect was shown. Furthermore, they [44] demonstrated in 10 human subjects that administration of 250 mg caffeine via a nasogastric tube stimulated gastric acid secretion more pronounced than intramuscular administration. The highest caffeine responses were shown after 40 to 50 min or at 70 min, respectively. Thus, it can be concluded that the stimulating effect of caffeine does not solely depend on the local effect of the drug on the stomach mucosa.

In a following study, Roth and Ivy [50] investigated the effect of 0.05 to 0.2 mg histamine, 250 mg caffeine or a combination thereof in 10 human subjects in consecutive gastric acid measurements as described above. During the first 20 min, no stimulation of gastric acid secretion by caffeine was detected, whereas histamine showed an immediate response. The gastric secretory response to histamine and caffeine was greatly enhanced and prolonged after caffeine administration and histamine administration earlier.

In the study of Musick and colleagues [41], a time dependent effect of caffeine was demonstrated after administration of 486 mg caffeine diluted in 200 mL water to 10 healthy and 25 ulcer diseased or 20 previously diseased patients. During the first 20 minutes after caffeine administration by Rehfuss tube directly into the stomach, no stimulation of gastric acid secretion was detected. However, after 30 min post-dose, an increased gastric acid output was measured. In addition, gastric acid secretion was significantly more pronounced in duodenal ulcer patients than in the healthy controls.

Two studies investigating the effect of orally administered caffeine were conducted [51,52]. Litman and colleagues [51] orally administered 500 mg caffeine diluted in 200 mL water to 48 patients with duodenal ulcer and 82 control patients. After 90 minutes, gastric content was evacuated and gastric acidity was analyzed. However, a basal control or water control was not included in the study. Nevertheless, approximately 30 % of the control patients showed a free acid concentration at or near the zero level and 39 % of the control patients showed only a low free acid concentration between 1 – 14 meq/L (clinical units). In contrast, free acid concentrations of 25 meq/L or higher were found in 47 out of 48 duodenal ulcer patients. This indicates, that caffeine does not

induce gastric acid secretion when administered orally in the majority of healthy subjects, but does so in ulcer patients.

Nieuwenhoven and colleagues [52] investigated the influence of a sports drink during exercise with or without the addition of 150 mg/L caffeine compared to a water control on gastrointestinal function, including intragastric pH measurements in the fundus of the stomach via a solid state pH electrode. Ten well-trained volunteers underwent a 60 minutes pre-exercise resting phase, followed by 90 min cycling period during which the test drinks were administered, and a final 210 min post-exercise resting phase. Gastric pH and reflux during the pre-exercise, the cycling, and the post-exercise episode after administration of the three different drinks did not differ significantly However, 62.2 % of the volunteers drinking the sports drink and 99.8 % of the volunteers drinking water had a gastric pH below 4, while only 14.9 percent of volunteers drinking the sports drink with caffeine had a gastric pH below 4 during the cycling stage. The median pH in the cycling stage during consumption of the three test drinks, sport drink without caffeine, water and sportsdrink with caffeine were 3.6, 3.1 and 4.3, respectively. These results indicate that total consumption of ~120 mg caffeine rather inhibits gastric acid secretion than stimulating it.

To summarize, caffeine administered bypassing oral cavity stimulates gastric acid secretion time dependently [41,44,50] and more pronounced in ulcer patients than in non-ulcer patients [41,51]. Intramuscular administration of caffeine lead to a delayed and less pronounced stimulation of gastric acid secretion [44]. However, when caffeine was administered orally, the majority of non-ulcer patients showed no or very low free acid concentrations [51]. Furthermore, oral administration of caffeine during exercise had a rather inhibiting than stimulating effect on gastric acid secretion [52]. These findings indicate that oral administration elucidates different effects than intragastric administration. Therefore, the effects of an oral vs. an intra-gastric administration of caffeine on gastric acid secretion including the underlying mechanisms still need to be clarified.

### 1.3.1. Cellular targets of caffeine postulated to regulate gastric acid secretion

Cohen and colleagues [43] tested, via intravenous administration, whether caffeine and pentagastrin can act synergistically in four healthy human volunteers. It was assumed that gastrin acts by stimulating the adenyl cyclase system, promoting the

formation of cAMP from ATP. Since caffeine is known to inhibit the phosphodiesterase (PDE), which promotes the degradation of cAMP to AMP, the authors hypothesized that gastrin and caffeine might act synergistically on gastric acid secretion. The authors showed that intravenous administration of 15 mg caffeine per kg body weight per hour stimulated gastric acid secretion to approximately 30 % of the maximum pentagastrin response and that there was no synergistic effect between pentagastrin and caffeine.
Previous studies showed that caffeine can stimulate gastric acid secretion via intragastric [41,42], intramuscular [44] and intravenous [43] administration. However, the cellular targets in the stomach need to be clarified. Since Rubach and colleagues [40] demonstrated a pro-secretory activity of caffeine in a parietal cell model, it can be assumed that caffeine acts on the parietal cell directly, not excluding other targets as well.
One molecular mechanism for the gastric acid-stimulating effect of caffeine could be an inactivation of PDE activity [39].Since PDE breaks down cAMP, caffeine may increase cAMP levels in the parietal cell. In accordance with this hypothesis, Rubach and colleagues [40] demonstrated that treatment of the human parietal cell model HGT-1 with 3 mM caffeine increased the intracellular cAMP levels. However, about 40 times higher caffeine concentrations for caffeine induced inhibition of PDE compared to its antagonistic effect on adenosine receptors are needed [39]. The antagonistic effect of caffeine on adenosine receptors [39] has been demonstrated to either, lead to an inhibition (adenosine receptor A2) [53] or indirectly to a stimulation (adenosine receptor A1) [54] of gastric acid secretion. Caffeine has been demonstrated to be a competitive antagonist to adenosine receptors A2 and A1 [39]. Adenosine receptor 1 has been shown to be involved in the inhibition of a histamine stimulated acid secretion [54]. In contrast, a stimulation of adenosine A2 receptor via adenosine led to an increase of gastric acid secretion and cyclic AMP levels in canine parietal cells [53]. Thus, caffeine might block the stimulating effect induced by adenosine receptor A2 on gastric acid secretion. An inhibition of adenosine receptor A1, could, thus, lead indirectly to an enhanced gastric acid production.
Caffeine was also shown to activate the bitter taste receptors TAS2Rs 7, 10, 14, 43 and 46 [55]. Pirastu and colleagues [56] identified a significant correlation between TAS2R43 single nucleotide polymorphism (SNP) variants and coffee liking by investigating 4066 human subjects from different parts of Europe and Central Asia for 88 SNPs covering the 25 TAS2R genes. Among compounds which are known to

activate TAS2R43, only caffeine is present in coffee [55]. Thus, it has been assumed that differences in caffeine perception could be responsible for differences in coffee liking. The perceived bitterness of caffeine can be reduced by addition of bitter masking compounds such as homoeriodictyol (HED) and eriodictyol (ED), which are extracted from the plant *yerba santa* [57,58]. A bitter reduction of 43 % by HED has been demonstrated in a human sensory panel consisting of 10 sensorically trained subjects by Ley and colleagues [57,58]. Therefore, HED might be used to study the influence of caffeine evoked bitter perception on gastric acid secretion.

## 1.4 Localisation and functionality of bitter taste receptors

As described before, the taste of food contributes to the initial phase, the cephalic phase, of gastric acid secretion which is controlled by the central nervous system [13]. The primary functions of the gustatory system are recognition of essential food nutrients and activation of the digestive system, as well as protection against poisonous or otherwise harmful compounds in food [59]. Taste perception in the oral cavity takes place in the taste buds, which are capable of detecting the five known taste qualities bitter, sweet, salty, sour, umami and probably free fatty acids [60]. The taste receptor cells located in the taste buds can be classified into four types; Type I, II, III and IV [61]. The two major classes are Type II and III. Type II cells express G protein-coupled receptors (GPCRs) and downstream effectors. Type II cells are directly stimulated by sweet, bitter, and umami taste compounds [61]. In contrast to Type II cells, Type III cells possess features of synapses, display typical neuronal features and do not express taste GPCRs. However, they respond directly to sour and salty taste stimulation. Therefore, Type II cells are termed as receptor cells and Type III cells as presynaptic taste cells [61]. Cell–cell interactions in taste buds are involved in signal processing.

Activation of taste cells via ligand binding leads to depolarization of taste cells and in turn, to neurotransmitter release. Taste buds are innervated by the cranial nerves VII (facial), IX (glossopharyngeal) and X (vagus). Upon recognition of a ligand, taste receptor cells initiate a signal transmitted by synaptic connection with sensory neurons, which ultimately transmit a signal to the brain stem and the thalamus [59,62] .

Taste receptors for sweet, umami and bitter belong to the family of the G-protein coupled receptors. The bitter taste receptor family is encoded by the TAS2R (taste receptor, type 2) gene family, of which mammalian species exhibit 15 – 36 genes, in humans 25 subtypes are expressed [63]. Some of the bitter receptors are activated by various, chemically diverse bitter compounds (TAS2R10, TAS2R14) while others are specialized to specific molecules (TAS2R3, TAS2R50) [55,64].

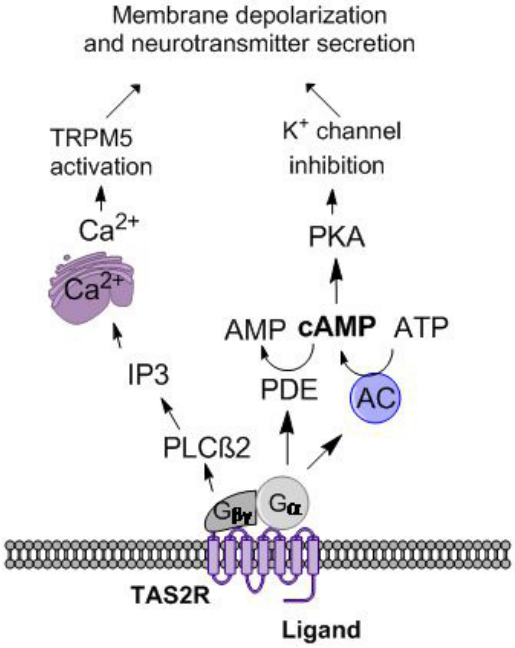



**Figure 3. Activation of TAS2Rs leads to the dissociation of heterotrimeric G proteins, which are known to activate the two major signal transduction pathways cAMP and PLCß2 (adapted from Cygankiewicz 2013 [59]).**

Binding of ligands to TAS2 receptors leads to the dissociation of heterotrimeric G proteins and activation of two major signal transduction pathways. The *α*-subunit of G proteins (Gα) probably can cause both, an increase in cAMP levels by activation of the adenylyl cylclase (AC), and a decrease in cAMP levels by phosphodiesterase (PDE) activation. Protein kinase A (PKA) is activated by elevated cAMP levels and phosphorylates potassium channels, causing its closing and subsequent membrane depolarization. In addition, it also leads to an increased neurotransmitter release via voltage-gated calcium ions. The *βγ* subunits of G proteins initiate the second pathway by activation of phospholipase *β*2 (PLC*β*2). PLC*β*2 hydrolyzes phosphoinositolphosphate (PIP2) to diacylglycerine (DAG) and inositoltriphosphate (IP3). Binding of IP3 to its receptor leads to a release of calcium ions from intracellular

compartments. This, in turn activates TRPM5 channels, associated with an influx of sodium ions and membrane depolarization [59].

Besides in the mouth, taste receptors have also been found in non-gustatory tissues including airway epithelia [65], intestinal cells [66,67] of humans and rodents, and in gastric epithelia of rats and mice [68,69]. In gastric epithelia of mice, brush cells were identified to express TAS1R3 [70], which can be activated by sweet and umami taste, and signaling proteins which are characteristic for taste cells, such as TRPM5 and gustducin (GNAT3), a G protein which is coupled to taste receptors [71,72]. In accordance to these findings, Janssen and colleagues [73] revealed α-gustducin positive cells in brush cells of mice, in the region of the limiting ridge, the boundary between fundus and corpus of the stomach, and in some endocrine cells. In contrast, α-transducin (GNAT2) positive cells were only found in endocrine cells. Wu and colleagues [68] showed in the gastric fundus of mice that the base of fundic glands is rich in transducin (GNAT2), while gustducin was not detected in this area of the glands. Those peripheral taste receptors are assumed to be activated by the same agonists that activate the taste receptors in the oral cavity. This suggests that taste receptors have additional functions apart from taste perception. For example, a role in digestive mechanisms has been proposed [74]. Janssen et al. [73] showed in mice that bitter taste receptors regulate the secretion of ghrelin with functional effects on food intake and gastric emptying. In another study, Le Nevé et al. [67] demonstrated that a bitter compound of the plant *Hoodia gordonii* elicits cholecystokinin (CCK) secretion from the human enteroendocrine cell line HuTu-80 via activation of two bitter taste receptors (TAS2R7 and TAS2R14). Both, ghrelin and CCK play a role in regulating gastric acid secretion [75] indicating a regulation of functional processes through chemosensing in the intestine. Kim and colleagues [66] demonstrated that activation of gut-expressed bitter taste receptors stimulates Glucagon-like peptide-1 (GLP-1) secretion in a PLC-dependent manner in diabetic mice.

To summarize, previous studies showed a functional role of chemosensors in the gastro-intestinal-tract, for example regulation of food intake, satiety and digestion. Besides, also an influence on gastric acid secretion seems possible, although not shown yet.

# II. Objectives

People, who are suffering from symptoms of GERD or peptic ulcer are disadvised from drinking wine, beer or coffee, indicating that these beverages or compounds thereof may interfere with the regulation of gastric acid secretion.
The pro- and anti-secretory activity of coffee and coffee compounds was studied in-depth in the research group of V. Somoza. However, the impact of an activation of bitter taste receptors by caffeine on gastric acid secretion has not been investigated so far. Beside caffeine from coffee, also flavoring compounds from beer and wine may have an impact on gastric acid secretion, although mechanistic studies are scarce.

The present thesis aimed at investigating the impact of several flavoring food compounds from wine, beer and coffee, with special focus on bitter substances like caffeine, on mechanisms regulating gastric acid secretion. Therefore, a combined approach of *in vivo* measurements determining the gastric pH in humans [49,76,77] and *in vitro* analyses in the well-established cell model HGT-1 cells [40,47,48,78-80] to study the underlying mechanisms of action was applied.

First, the effect of different wine and beer samples on gastric acid secretion were examined and revealed that organic acids and bitter compounds in wine and beer representative concentrations of selected wine and beer samples are potent stimulators of gastric acid secretion.
The second part of the thesis focused on the hypothesis, that bitter compounds regulate gastric acid secretion via activation of bitter taste receptors in the stomach. In a first step, the expression of bitter taste receptors in human parietal cells and HGT-1 cells was proven for the first time. Subsequent mechanistic studies demonstrated an association of taste receptor activation and proton secretion in HGT-1 cells using siRNA knockdown assays. Finally, a proof of-concept study was conducted to validate the hypothesis, that bitter taste receptors are involved in the regulation of gastric acid secretion.

# III. Results

## (1) "Identification of organic acids in wine that stimulate mechanisms of gastric acid secretion"

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This study investigated the effect of one red wine, and one white wine sample, and organic acids thereof on gastric acid secretion *in vivo* and *in vitro*.

Red wine stimulated gastric acid secretion more effectively than white wine, with succinic acid and malic acid as the key organic acids that contribute to gastric acid secretion. Red wine interfered with the expression of genes relevant for gastric acid secretion more pronounced than white wine. Ethanol reduced the pro-secretory activity of tartaric acid in HGT-1 cells.

I participated in the experimental design and analyzed the effect of the wines, the organic acids and ethanol on proton secretion and cell viability in HGT-1 cells. In addition, I performed the qPCR experiments of genes relevant for gastric acid secretion. I determined the concentration of organic acids in the wine samples and the buffer capacity of the test solutions in the human intervention trial. Furthermore, I planned and performed the *in vivo* experiments. I did the statistical analysis and prepared the manuscript.

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# Identification of Organic Acids in Wine That Stimulate Mechanisms of Gastric Acid Secretion

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**ABSTRACT:** Wine may cause stomach irritation due to its stimulatory effect on gastric acid secretion, although the mechanisms by which wine or components thereof activate pathways of gastric acid secretion are poorly understood. Gastric pH was measured with a noninvasive intragastric probe, demonstrating that administration of 125 mL of white or red wine to healthy volunteers stimulated gastric acid secretion more potently than the administration of equivalent amounts of ethanol. Between both beverages, red wine showed a clear trend for being more active in stimulating gastric acid secretion than white wine ($p$ = 0.054). Quantification of the intracellular proton concentration in human gastric tumor cells (HGT-1), a well-established indicator of proton secretion and, in turn, stomach acid formation in vivo, confirmed the stronger effect of red wine as compared to white wine. RT-qPCR experiments on cells exposed to red wine also revealed a more pronounced effect than white wine on the fold change expression of genes associated with gastric acid secretion. Of the quantitatively abundant organic acids in wine, malic acid and succinic acid most actively stimulated proton secretion in vitro. However, addition of ethanol to individual organic acids attenuated the secretory effect of tartaric acid, but not that of the other organic acids. It was concluded that malic acid for white wine and succinic acid for red wine are key organic acids that contribute to gastric acid stimulation.

**KEYWORDS:** *red wine, white wine, ethanol, organic acids, gastric acid secretion*

## INTRODUCTION

Wine consumption is known to increase gastric acid secretion[1−3] and to induce reflux in patients with gastroesophageal reflux disease (GERD)[4] as well as in healthy subjects.[4,5] Chronic gastric acid secretion may cause gastric irritation such as ulcer disease,[6] heartburn, and GERD,[7] which may lead to adenocarcinomas in the lower esophagus.[8] Subjects with these diseases are often advised to refrain from drinking alcoholic beverages such as wine. Approximately 15% of the world's population suffers from GERD.[9] In an unselected population-based study in Japan, 82,894 subjects between the ages of 30 and 89 completed a questionnaire asking for symptoms of heartburn. The prevalence of heartburn, a typical GERD symptom, was high in about 20% of the subjects.[10] In another population-based study, conducted in Germany, an even higher GERD prevalence of 34% was reported.[11]

In the past few decades, several studies have investigated the influence of alcoholic beverages on gastric acid secretion using intragastric titration in humans[1−3,12] as well as in animals, such as rats[13] or dogs,[14] or in isolated gastric glands from rabbits.[15] To our knowledge, there have been only two studies comparing the effect of red wine and white wine on gastric acid secretion. In a human intervention trial, Peterson and colleagues administered 300 mL of either red or white wine to healthy subjects and did not observe any difference in gastric acid secretion.[2] In contrast, Tsukimi et al.[14] demonstrated a significantly stronger stimulating effect for red wine as compared to white wine after administering amounts ranging from 25 to 100 mL to dogs with vagally denervated Heidenhain pouches.

Apart from wine, other alcoholic beverages have been studied for their effects on gastric acid secretion. One of the major findings was that fermented alcoholic beverages are strong stimulants of gastric acid secretion, whereas spirits with a higher ethanol concentration showed very little or no effect.[1,3] These results indicate that the acid stimulatory effect of alcoholic beverages derives not just from ethanol, indicating the presence of other stimulating components.[1−3,13] Following this hypothesis, Teyssen and colleagues investigated fractions of fermented glucose and identified maleic acid and succinic acid as strong stimulants of gastric acid secretion. Other organic acids detected in the fermentation mixture, such as acetic acid, oxalic acid, and lactic acid, showed no influence. Hence, the authors hypothesized that the length of the carbon chain and the two carboxylic groups are the main determinants of a molecule's effect on stomach acid secretion[12] (Table 1). These structural characteristics are also found in other organic acids of wine, including tartaric acid, malic acid, and citric acid (Table 1). None of these organic acids has been investigated for its effects on mechanisms of stomach acid secretion in wine representative concentrations, although tartaric acid and malic acid are the predominant organic acids in wine and significantly contribute to its pH.[16]

Gastric acid secretion takes place in the parietal cells of the stomach and is regulated by a number of cell surface receptors as well as functional and signaling proteins. Activation of cell surface receptors of parietal cells leads to signal transductions in which hormones and second messengers activate the key element in the complex process of gastric acid secretion, the $H^+$,$K^+$-ATPase (coded by the gene *ATP4A*). Activation of the $H^+$,$K^+$-ATPase leads to transport of hydrogen ions into the gastric lumen in exchange for potassium ions. The histamine

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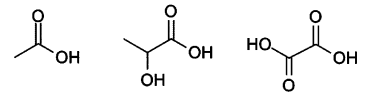
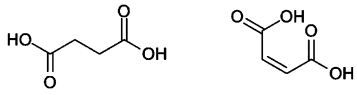
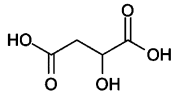
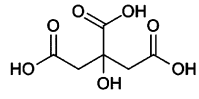
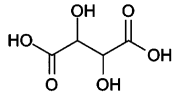
**Table 1. Molecular Structures of Organic Acids as Putative Stimulants of Gastric Acid Secretion**

| | |
|---|---|
| **Low effect** on gastric acid secretion (12) |  acetic acid, lactic acid, oxalic acid |
| **Strong effect** on gastric acid secretion (12) |  succinic acid;  maleic acid |
| **Unknown effect** on gastric acid secretion |  malic acid;  citric acid;  tartaric acid |

H2 receptor (coded by the gene *HRH2*) and the acetylcholine M3 receptor (coded by the gene *CHRM3*) have been determined to initiate these signal transduction pathways that regulate the expression of the respective prosecretory genes further downstream. The only cell surface receptor known to inhibit secretion is the somatostatin receptor (coded by the gene *SSTR2*). These cell surface receptors and their respective ligands, histamine, acetylcholine, and somatostatin, play a crucial role in the regulation of gastric acid secretion[17,18] and are expressed in the human gastric tumor cell line HGT-1, which has been established in our group for the identification of stomach acid regulating compounds in coffee and beer.[19−24]

The aim of the present study was to identify the impact of white and red wine organic acids and ethanol on mechanisms of gastric acid secretion. We measured the intragastric pH in six volunteers after consumption of white wine, red wine, or ethanol using a noninvasive intragastric pH-probe. Mechanisms of stomach acid secretion were studied by analyzing the intracellular proton concentration as a measure of proton secretory activity in HGT-1 cells by means of a pH-sensitive dye and by determining the expression of the *ATP4A*, *CHRM3*, *HRH2*, and *SSTR2* genes by RT-qPCR. Finally, we compared the effects of wines with those of the organic acids tartaric acid, malic acid, citric acid, succinic acid, and lactic acid and of ethanol in wine representative concentrations by analyzing the intracellular proton concentration in HGT-1 cells.

## MATERIALS AND METHODS

**Chemicals.** Cell culture materials such as Dulbecco's Modified Eagle Medium (DMEM), trypsin, glutamine, penicillin/streptomycin, and histamine as well as L-(+)-tartaric acid, succinic acid, and DL-lactic acid were obtained from Sigma-Aldrich. Fetal bovine serum was purchased from Invitrogen, Karlsruhe, Germany.

Citric acid and L-malic acid were included in the enzyme kits from R-Biopharm (Roche, Darmstadt, Germany) and used for their quantitative analysis. 1,5-Carboxy-seminaphtorhodafluor acetoxymethyl ester (SNARF-1-AM) and nigericin were obtained from Invitrogen. For RNA isolation, we used the RNeasy Mini Kit obtained by Qiagen, Hilden, Germany, and the SV Total RNA Isolation System obtained from Promega, Madison, WI, USA. High Capacity RNA to cDNA Master Mix was purchased from Applied Biosystems, Munich, Germany.

**Samples.** A total of five red wine samples of the variety "Blauer Zweigelt Klassik" and five white wine samples of the variety "Grüner Veltliner", both produced in 2009 by Wegenstein, Niederösterreich (Lower Austria), Austria, were purchased from a local store (Table 2). Edible ethanol (96%) was obtained from a local pharmacy and diluted to a concentration of 12% v/v with double-distilled water. In the cell culture experiments, samples were diluted 1:100 or 1:10 in DMEM.

**Determination of Wine Buffer Capacity.** The buffering capacity of 125 mL of each wine with (buffer capacity 1) and without (buffer capacity 2) 5 mL of saturated $NaHCO_3$ was determined by titration with 1 N HCl from initial pH to pH 1.5 using a pH-meter pH 211 (HANNA Instruments, BW, Germany).

**Photometric and Enzymatic Quantification of Organic Acids in Wine.** Tartaric acid was quantified through its reaction with vanadate and photometrically determined at a wavelength of 530 nm, as described by Matissek et al.[25] Citric acid and L-malic acid were determined using enzymatic kits from R-Biopharm (Roche). Here, citric acid quantification is based on the conversion of citrate into oxaloacetate and acetate in the presence of citrate lyase. Oxaloacetate and acetate are reduced in the presence of L-malate dehydrogenase and L-lactate dehydrogenase by reducing nicotinamide adenine dinucleotide (NADH). The decrease of NADH is photometrically determined at a wavelength of 340 nm and is stochiometric to the amount of citrate. The L-malic acid enzyme kit is based on the oxidization of L-malic acid to oxaloacetate by NADH in the presence of L-malate

**Table 2. Primers Used for Gene Expression Analysis of *ATP4A*, *HRH2*, *CHRM3*, and *SSTR2* with *PPIA* as Housekeeping Gene[19,21,23]**

| direction | gene | sequence (5′−3′) | product length (bp) |
|---|---|---|---|
| forward | *PPIA* | CCA CCA GAT CAT TCC TTC TGT AGC | |
| reverse | *PPIA* | CTG CAA TCC AGC TAG GCA TGG | 144 |
| forward | *ATP4A* | CGG CCA GGA GTG GAC ATT CG | |
| reverse | *ATP4A* | ACA CGA TGG CGA TCA CCA GG | 176 |
| forward | *CHRM3* | AGC AGC AGT GAC AGT TGG AAC | |
| reverse | *CHRM3* | CTT GAG CAC GAT GGA GTA GAT GG | 117 |
| forward | *HRH2* | TGG GAG CAG AGA AGA AGC AAC C | |
| reverse | *HRH2* | GAT GAG GAT GAG GAC CGC AAG G | 154 |
| forward | *SSTR2* | TCC TCC GCT ATG CCA AGA TGA AG | |
| reverse | *SSTR2* | AGA TGC TGG TGA ACT GAT TGA TGC | 189 |

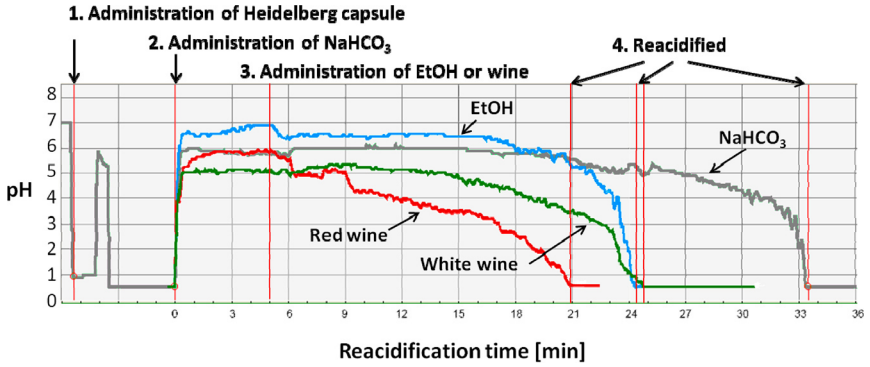



**Figure 1.** Gastrogram of four Heidelberg capsule measurements from one test subject. At 0 min, the pH was brought up to about 6 by administration of 5 mL of a saturated $NaHCO_3$ solution. After 5 min, either nothing (gray line) or 125 mL of ethanol (blue line), white wine (green line), or red wine (red line) was administered.

dehydrogenase. The amount of NADH formed is stochiometric to the L-malic acid content.

For succinic acid and DL-lactic acid quantification we used enzymatic test kits from Megazyme International (Wicklow, Ireland). For succinic acid quantification, the decrease of NADH indicates the conversion of succinate into succinyl-CoA measured photometrically at a wavelength of 340 nm. For DL-lactic acid quantification, also the increase of NADH is measured, which indicates the oxidation of DL-lactic acid to pyruvate. Absorptions were measured using an Infinite 200 PRO Plate Reader (Tecan, Männedorf, Switzerland). Analyses were done according to the protocols of the distributor.

**Subjects.** Six healthy, female volunteers between 25 and 30 years of age with a body mass index between 19.6 and 32.3 $kg/m^2$ were studied. None of them had been diagnosed for gastrointestinal disease, and none took any medication or antibiotics for 2 months prior to the tests. Habitual alcohol consumption was <20 g of pure alcohol per day. Each volunteer was fully informed about the test, gave written consent, and was treated following the ethical principles of the declaration of Helsinki. The trial subjects had to fast from food and liquid for 10 h prior to the intervention. During the experiment, the subjects remained in a supine left-sided position.

**Analyses of Gastric pH in Healthy Subjects.** The intragastric pH was analyzed by means of a Heidelberg Detection System (Heidelberg Medical Inc., Mineral Bluff, GA, USA). This test system has been approved by the U.S. Food and Drug Administration for measuring the intragastric pH. The system consists of a pH-sensitive capsule (called a Heidelberg capsule) that has to be swallowed and contains a miniature radio transmitter. A transceiver placed on the abdomen of the volunteer receives the signal and sends it to the recorder connected to a computer.

Prior to administration, Heidelberg capsules were activated for 5 min in a 0.9% sterile filtered NaCl solution (filter pore size = 0.22 $\mu$m) and calibrated using two calibration points, pH 1 and 7. Afterward, the subjects swallowed the capsule. When the intragastric pH was constant at pH <1 for at least 3 min, the capsule was considered to be in the stomach. Afterward, each trial started with the administration of 5 mL of a saturated sodium bicarbonate solution ($NaHCO_3$). This alkaline challenge triggers a rise in stomach pH and subsequently leads to the secretion of stomach acid by the parietal cells (Figure 1).

Each volunteer completed at least four interventions. In the first intervention, the volunteer was administered 5 mL of $NaHCO_3$ solely. To test the effect of the samples, the subject received, first, the alkaline solution, and, second, 5 min later, 125 mL of either white wine, red wine, or ethanol (12% v/v).

Reacidification time was analyzed with the Heidelberg Detection System software (Figure 1). The area under the curve (AUC) and the slope of the reacidification plot over time were analyzed using the software ImageJ 1.43 (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The slope of the curve was calculated from the point of the start of acid secretion to the end point at which the initial baseline pH was reached and is given as pH/min. Data are presented as AUC per minute of reacidification time normalized to the buffer capacity.

**Cell Culture.** The human gastric tumor cell line HGT-1 was obtained from Dr. C. Laboisse (Laboratory of Pathological Anatomy, Nantes, France). The cells were cultured under standard conditions at 37 °C, 95% humidity, and 5% $CO_2$. DMEM with 4 g/L glucose was used as culture medium and supplemented with 10% fetal bovine serum, 2% L-glutamine, and 1% penicillin/streptomycin.

**Cytotoxicity Test.** Cellular viability was tested by trypan blue staining using a hemocytometer (Brand, Wertheim, Germany). A total of about 560,000 HGT-1 cells was seeded per well in a 24-well plate (Greiner Bio-One, Kremsmünster, Austria) and allowed to settle for 24 h at 37 °C and 5% $CO_2$. Cells were washed once with Krebs–HEPES–buffer (KRHB; 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 KOH) and treated with dilutions from 1:500 to 1:5 of red wine, white wine, or 12% v/v ethanol during incubations at 37 °C, 95% humidity, and 5% $CO_2$ for up to 45 min, depending on the subsequent analysis. Then, the cells were washed twice with KRHB, harvested with trypsin, and stained with trypan blue. The number of living cells as well as blue-colored dead cells was counted with a hemocytometer. The viability of treated cells was calculated and compared to the viability of nontreated cells (=100%). Three biological with two technical replicates per sample were measured.

**Determination of the Intracellular pH in HGT-1 Cells.** The intracellular pH ($pH_i$) was measured as an indicator for proton secretion with the pH-sensitive fluorescence dye SNARF-1-AM. A total of 100,000 viable cells per well was spread in a white 96-well plate and allowed to settle for 24 h at 37 °C, 95% humidity, and 5% $CO_2$. Cells were washed once with KRHB and incubated at previous conditions for 30 min with the fluorescence dye SNARF-1-AM at a concentration of 3 $\mu$M.[19,20,23,24] Afterward, cells were washed twice with KRHB and treated with 100 $\mu$L of the diluted sample in DMEM for 10 min. Cells treated with 1 mM histamine were used as positive control. Nontreated cells were used as control and compared to cells treated with different concentrations of the wine samples or ethanol. The organic acids tartaric acid, citric acid, malic acid, succinic acid, lactic acid, and a combination thereof as recombinate were tested in a 1:100 dilution of their respective concentration in white and red wines. In this high dilution of 1:100, any pH effects originating from tested compounds can be excluded. Furthermore, we tested the influence of ethanol on the effect induced by the organic acids by adding ethanol in the respective concentration of wine to the organic acids, the recombinate, and red and white wines to which ethanol was added to reach a 2-fold higher concentration compared to the original product.

Treatment was followed by a washing step with KRHB. Afterward, 100 $\mu$L of KRHB was added and the 96-well plate was placed into an Infinite 200 PRO Plate Reader. Fluorescence was analyzed at an excitation of 488 nm and emission wavelengths of 580 and 640 nm. The ratio of the fluorescence intensities from those two emission


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Table 3. Buffer Capacity, pH, Ethanol Content, and Organic Acid Content of White Wine and Red Wine[a]

| | white wine | red wine | ethanol |
|---|---|---|---|
| pH | 3.5 ± 0.0 | 3.6 ± 0.1 | 6.5 ± 0.7 |
| buffer capacity 1 (mmol HCl) | 11.1 ± 0.1 | 11.8 ± 0.4 | 5.5 ± 1.4 |
| buffer capacity 2 (mmol HCl) | 15.9 ± 0.8 | 16.0 ± 0.3 | 10.2 ± 0.2 |
| ethanol (% v/v) | 11.5 | 13 | 12 |
| malic acid (g/L) | 2.42 ± 0.03 | 0.021 ± 0.004 *** | |
| tartaric acid (g/L) | 1.86 ± 0.12 | 1.75 ± 0.19 | |
| lactic acid (g/L) | 0.44 ± 0.01 | 1.69 ± 0.07 *** | |
| citric acid (g/L) | 0.30 ± 0.03 | 0.19 ± 0.05 ** | |
| succinic acid (g/L) | 0.27 ± 0.01 | 0.57 ± 0.05 *** | |

[a]Buffer capacity is given as consumption of HCl determined by titration of 125 mL of the samples with (buffer capacity 1) and without (buffer capacity 2) 5 mL of saturated $NaHCO_3$ to pH 1.5 of white wine, red wine, and ethanol. Data are given as the mean ± SD from triplicate analyses (statistics: two-tailed *t* test; significant differences vs concentrations of white wine are indicated by ** = $p < 0.01$ and *** = $p < 0.001$).

wavelengths allows an accurate determination of pH when plotted on a calibration curve.[19−24]

A calibration curve was generated for each experiment by staining the cells in potassium buffer solutions of varying pH values, ranging from 7.2 to 8.2 adjusted with NaOH using a pH-meter pH 211 (HANNA Instruments), in the presence of 2 μM nigericin to equilibrate intracellular pH ($pH_i$) and extracellular pH ($pH_{ex}$). The potassium buffer calibration solutions for the intracellular pH measurement consisted of 20 mM NaCl, 110 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 18 mM D-glucose, and 20 mM HEPES. The $pH_i$ calibration was fit to a linear regression. Intracellular $H^+$ concentration was calculated from the $pH_i$. The intracellular proton index (IPX) was calculated by log 2 transformation of the intracellular proton concentration ratio between treated cells and control cells.[19−21,23] The effect of ethanol addition to the organic acids on IPX values is shown as percent difference.

Because the ethanol concentration of white wine was 11.5%, whereas red wine contained 13%, we used a 12% ethanol solution as control solution in the Heidelberg capsule experiments. Prior to the Heidelberg experiment, these three ethanol concentrations (11.5, 12, and 13% v/v) were tested in a 1:100 dilution in HGT-1 cells and did not show any different effects on the IPX (data not shown).

**RNA Isolation and cDNA Synthesis.** In six-well plates, 100,000 HGT-1 cells/well were seeded and grown until confluence. Then, cells were treated with a 1:100 dilution of white wine, red wine, or ethanol and a 1:10 dilution of white wine or ethanol for 5, 10, 15, 20, 25, 30, and 45 min. Afterward, cells were harvested for RNA isolation using the RNeasy Mini Kit and the SV Total RNA Isolation System. Quantity and quality of RNA were checked spectrophotometrically at 260 nm and by calculation of the ratio of 260 and 280 nm using the nanoquant plate for the Infinite 200 PRO Plate Reader. All samples used were in a ratio between 1.8 and 2.2. High-capacity RNA to cDNA Master Mix (Applied Biosystems, Munich, Germany) was used for cDNA synthesis following the manufacturer's protocol.

**Gene Expression Assays.** Primers for the $H^+$,$K^+$-ATPase α-subunit (*ATP4A*), the histamine H2 receptor (*HRH2*), the somatostatin receptor (*SSTR2*), and the acetylcholine receptor M3 (*CHRM3*) (Table 2) were designed and validated previously.[19,21] Peptidylprolyl isomerase A (*PPIA*) was used as housekeeping gene. Real-time PCR assays were performed on a StepOne plus (Applied Biosystems) using the Fast SYBR green master mix (Applied Biosystems). Cycling conditions were set as follows: 20 s/95 °C (activation), 3 s/95 °C (denaturation), 30 s/60 °C (annealing), 15 s/72 °C (elongation with fluorescence measurement). Cycling conditions for *HRH2* were set to 20 s/95 °C, 3 s/95 °C, 30 s/62 °C, and 15 s/77 °C, respectively.

**Statistical Analysis.** Statistical analysis was performed using Excel 2007 (Microsoft, Seattle, WA, USA) and Sigma Plot software 11.0 (Systat Software, Erkrath, Germany). Outliers were excluded by Nalimov outlier analysis. Significant differences in the human intervention trial between samples were tested by a one-way ANOVA with Holm−Sidak post hoc analysis and a one-sided paired Student's *t* test. The cytotoxicity of the samples on HGT-1 cells compared to nontreated cells was determined with the two-tailed Student's *t* test and considered to be significant at $p < 0.05$. Significant differences in the data set of the proton secretory analysis were determined by a one-way ANOVA with Holm−Sidak post hoc analysis and the two-tailed Student's *t* test. For analyzing time-dependent effects on gene expression, we performed the two-way ANOVA with Holm−Sidak post hoc analysis. At least three biological replicates and two technical replicates were analyzed for each cell culture experiment. Data under Results as well as in diagrams are given as the mean ± SEM, unless indicated otherwise.

## RESULTS

**Effect of White Wine, Red Wine, and Ethanol on Gastric Acid Secretion in Healthy Volunteers Determined in Vivo by Gastric pH Measurement.** To determine the influence of white wine and red wine on gastric acid secretion in comparison to a 12% v/v ethanol solution, we measured the stomach pH of six fasted volunteers by means of a noninvasive pH-sensitive intragastric probe. First, the subject's gastric pH was challenged by a 5 mL solution of saturated $NaHCO_3$, resulting in a stable pH of 5−7 for at least 5 min postload and a mean reacidification time of 35.4 ± 6.3 min. Intervention with the 12% v/v ethanol solution resulted in a clear trend toward a shorter reacidification time of 23.9 ± 2.6 min compared to 25.3 ± 3.5 min ($p < 0.70$) and 27.2 ± 3.3 min ($p < 0.57$) for red wine and white wine, respectively (data not shown). Next to the reacidification time, the slope of the reacidification curve is a valuable measure of a compound's effect on gastric acid secretion: the greater the slope, the faster the pH is falling. Administration of the 12% v/v ethanol solution (−0.76 ± 0.09 pH/min) caused a stronger decline compared to red wine (−0.41 ± 0.05 pH/min, *p* vs ethanol = 0.016), white wine (−0.48 ± 0.06 pH/min, *p* vs ethanol = 0.021), and the saturated $NaHCO_3$ solution (−0.54 ± 0.05 pH/min, *p* vs ethanol = 0.201; data not shown). Because the lower buffering capacity of ethanol compared to wine (Table 3) might affect these results, we normalized the AUC to reacidification time and buffer capacity. Reacidification parameters of the saturated $NaHCO_3$ solution administered alone were defined as control and were set to 100%. All treatments were compared to this control. Thus, a lower percent value refers to a stronger acid secretion. Administration of red wine and white wine as well as 12% v/v ethanol significantly ($p < 0.001$) increased gastric acid secretion compared to the saturated $NaHCO_3$ solution alone. Red wine (12.8 ± 1.5%) showed a clear trend for the strongest stimulation of gastric acid secretion compared to white wine

(14.5 ± 1.6%, $p$ vs red wine = 0.054) and ethanol (22.5 ± 0.7%, $p$ vs red wine < 0.001; data not shown). For illustration, Figure 2 shows a typical gastrogram from one study subject.

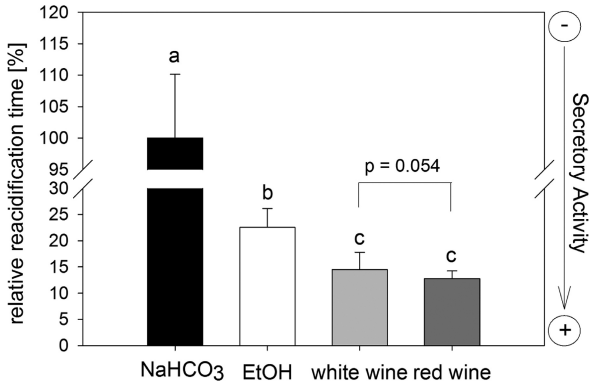


**Figure 2.** In vivo determination of gastric pH using Heidelberg-pH-probe. Displayed data refer to AUC/min normalized to buffer capacity results from administration of $NaHCO_3$, ethanol (EtOH) 12% v/v, white wine, and red wine; 5 mL of $NaHCO_3$ alone was set to 100%, and data are displayed as the mean ± SEM, $n$ = 6 (statistics: one-way ANOVA with Holm−Sidak post hoc test; letters indicate significant differences between groups; $p$ < 0.001 and a one-tailed $t$ test between red wine and white wine).

**Cytotoxicity of White Wine, Red Wine, and Ethanol in HGT-1 Cells.** We conducted a trypan blue toxicity test for testing which concentrations of wine and ethanol can be used in cell culture experiments without exhibiting cytotoxic effects. Results are shown in Table 4. Red wine was toxic in a 1:10 dilution but not in a 1:100 dilution, whereas white wine and ethanol in a dilution of 1:10 showed no toxicity (Table 4). Therefore, all cell culture experiments were carried out in dilutions of 1:10 and 1:100 using ethanol and white wine, whereas red wine was tested only in a 1:100 dilution.

**Table 4. Cell Viability in Percent versus Nontreated Cells (Control): 100 ± 2%[a]**

| dilution abs | 12% v/v ethanol | white wine | red wine |
|---|---|---|---|
| 1:500 | nd | nd | 99 ± 3 |
| 1:250 | nd | nd | 98 ± 1 |
| 1:100 | nd | 99 ± 1 | 95 ± 3 |
| 1:10 | 99 ± 2 | 95 ± 4 | 37 ± 8 *** |
| 1:5 | 98 ± 2 | 80 ± 12 * | 4 ± 1 *** |

[a]Data are given as the mean ± SD, $n$ = 3, tr = 2. nd, not determined (statistics: two-tailed $t$ test vs control; significant differences vs nontreated control cells are indicated by * = $p$ < 0.05 and *** = $p$ < 0.001).

**Effect of White Wine, Red Wine, and Ethanol on Intracellular Proton Concentrations in HGT-1 Cells.** To study the influence of wine and ethanol on mechanisms of proton secretion in parietal HGT-1 cells, we measured the intracellular pH using the pH-sensitive dye SNARF-AM and analyzed the data as the IPX. The lower the proton concentration in the cell, the lower the IPX and the stronger is the proton secretion[19,20,22−24] HGT-1 cells treated with histamine (1 mM), a physiological stimulant of gastric acid secretion,[17] resulted in a significant decrease of the IPX (−0.21 ± 0.03; $p$ < 0.001) compared to nontreated cells (Figure 3). Ethanol and white wine in 1:10 dilutions significantly decreased

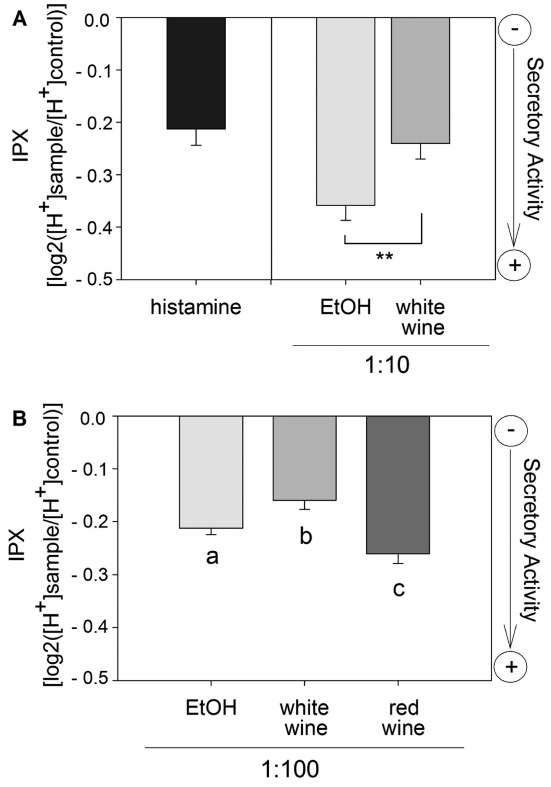


**Figure 3.** Intracellular proton index (IPX) of HGT-1 cells treated for 10 min with histamine (1 mM): (A) 1:10 or (B) 1:100 dilution of ethanol (EtOH, 12% v/v), white wine, or red wine. Data are displayed as the mean ± SEM, $n$ > 4; tr = 3−6 (statistics: (A) two-tailed $t$ test, ** = $p$ < 0.01; (B) one-way ANOVA with the Holm−Sidak post hoc test; letters indicate significant differences between groups, $p$ < 0.05).

the IPX compared to nontreated cells ($p$ < 0.001) (Figure 3A). The IPX values after the cell's treatment with dilutions of 1:10 of white wine and ethanol were −0.24 ± 0.03 and −0.36 ± 0.03, respectively. Comparison of the effects of 1:100 dilutions of red wine (Figure 3B), white wine, and 12% v/v ethanol demonstrated a significantly stronger decrease of the intracellular pH, as indicator of a higher proton secretion, for red wine (IPX = −0.26 ± 0.02) compared to white wine (IPX = −0.16 ± 0.02, $p$ < 0.001) and ethanol (IPX = −0.21 ± 0.01, $p$ = 0.02). However, 12% v/v ethanol in dilutions of both 1:10 and 1:100 stimulated proton secretion, as indicated by a lower IPX in HGT-1 cells, more potently than white wine (1:100, $p$ < 0.05; 1:10, $p$ < 0.01).

**Influence of White Wine, Red Wine, and Ethanol on Gene Expression of *ATP4A*, *HRH2*, *CHRM3*, and *SSTR2*.** A time course experiment was performed to investigate the influence of a 1:100 dilution of white wine, red wine, and ethanol and, additionally, a 1:10 dilution of white wine and ethanol on the expression of genes involved in the regulation of gastric acid secretion.[19,21−23] Gene expression of *ATP4A*, *HRH2*, *CHRM3*, and *SSTR2* was measured by qPCR. Gene expression of *PPIA* served as the control. Results are given as relative gene expression; treated cells were compared to nontreated cells (control = 1) (Figure 4).

The ratios of gene expression for the target genes, compared to the housekeeping gene *PPIA*, were determined. Treatment with red wine in a 1:100 dilution for 10 min (*ATP4A*, 1.40 ± 0.35; *HRH2*, 1.62 ± 0.36; *CHRM3*, 1.88 ± 0.36; *SSTR2*, 1.85 ± 0.33) and 15 min (*ATP4A*, 1.46 ± 0.21; *HRH2*, 1.91 ± 0.21;

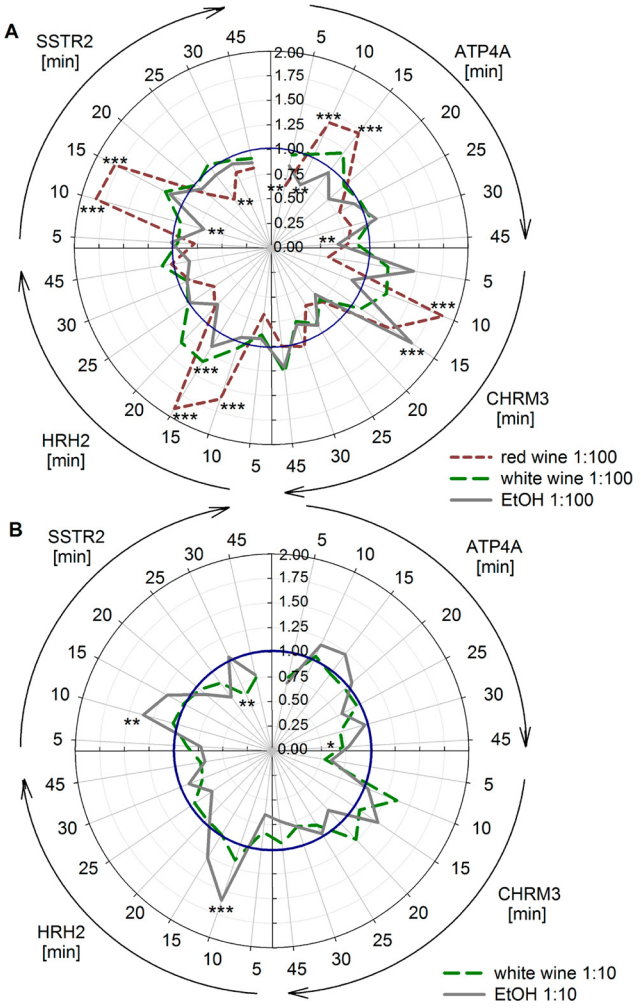


**Figure 4.** Time-dependent indices of gene expression for the *ATP4A*, *CHRM3*, *HRH2*, and *SSTR2* in HGT-1 cells after treatment with (A) 1% ethanol (12% v/v), white wine, and red wine or (B) 10% ethanol (EtOH, 12% v/v) and white wine compared to nontreated cells. Data are displayed as mean values, $n = 3–4$, tr = 3 (statistics: two-way ANOVA with the Holm−Sidak post hoc test; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

*CHRM3*, 1.46 ± 0.17; *SSTR2*, 1.80 ± 0.16) resulted in the most pronounced change in gene expression of all four genes when compared to the cells' treatment with white wine and ethanol. Whereas treatment of the cells with the 1:100 dilution of ethanol increased the *CHRM3* expression after 15 min (1.72 ± 0.40, $p < 0.001$), administration of the higher concentration of 1:10 ethanol increased *HRH2* (1.60 ± 0.35, $p < 0.001$) and *SSTR2* (1.36 ± 0.21, $p = 0.002$) expression already after 10 min of exposure. Treatment of the HGT-1 cells with the 1:100 dilution of white wine significantly increased the expression of *HRH2* (1.35 ± 0.22, $p < 0.001$). Additionally, the 1:10 dilution of white wine decreased the expression of *SSTR2* (0.62 ± 0.04, $p = 0.005$) and *ATP4A* (0.72 ± 0.04, $p = 0.012$) after 30 and 45 min.

**Quantification of Organic Acids in Wine.** Organic acids in the wine samples were quantified to apply wine representative concentrations in the experiments. The composition of organic acids in the two wines varied considerably (Table 3). The concentration of malic acid was much higher in white wine (2.42 ± 0.03 g/L) compared to red wine (0.021 ± 0.004 g/L). In contrast, the concentration of lactic acid was higher in red wine (1.69 ± 0.07 g/L) compared to white wine (0.44 ± 0.01 g/L). The concentration of succinic acid in red wine (0.57 ± 0.05 g/L), which has been identified as a strong stimulant of gastric acid secretion,[12] was double that of white wine (0.27 ± 0.01 g/L).

**Effect of Organic Acids in Wine Representative Concentrations on Intracellular Proton Concentrations in HGT-1 Cells.** We tested tartaric acid, citric acid, malic acid, succinic acid, and lactic acid individually and as a recombinate in concentrations representing a 1:100 dilution of white and red wines (Figure 5). All organic acids in the respective concentration of white wine and red wine significantly ($p < 0.001$) stimulated gastric acid secretion compared to nontreated cells.

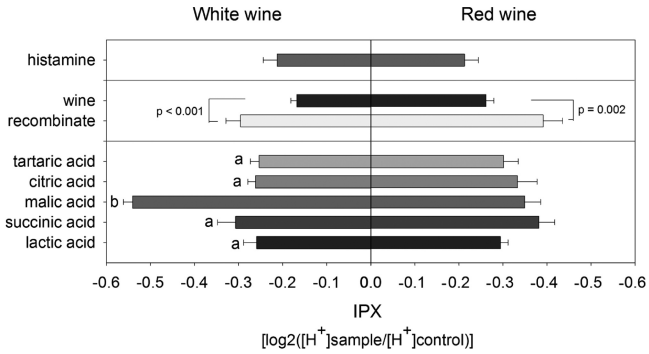


**Figure 5.** Intracellular proton index (IPX) of HGT-1 cells treated for 10 min with organic acids in 1:100 dilutions of white and red wine representative concentrations in the absence of ethanol. White wine representative concentrations: 18.3 mg/L tartaric acid, 3.0 mg/L citric acid, 24.2 mg/L malic acid, 2.7 mg/L succinic acid, 4.4 mg/L lactic acid, and a recombinate of those acids in these concentrations. Red wine representative concentrations: 17.5 mg/L tartaric acid, 1.9 mg/L citric acid, 0.21 mg/L malic acid, 5.7 mg/L succinic acid, 16.9 mg/L lactic acid, and a recombinate of those acids in these concentrations. The control was nontreated cells. Data are displayed as the mean ± SEM, $n \geq 3$; tr = 3–6 (statistics: wine vs recombinate, two-tailed *t* test; organic acids vs each other, one-way ANOVA with the Holm−Sidak post hoc test; letters indicate significant differences between groups, $p < 0.05$).

In white wine, malic acid was the most abundant organic acid with a concentration of 24.2 mg/L and showed the strongest stimulation of proton secretion of the tested organic acids as indicated by an IPX value of −0.54 ± 0.02. The white wine recombinate of organic acids (IPX = −0.30 ± 0.03) showed a significantly ($p < 0.001$, two-tailed *t* test) stronger effect on proton secretion, as indicated by a lower IPX, compared to white wine (IPX = −0.16 ± 0.02).

In red wine representative concentrations, the effects of singly applied organic acids were not significantly different from each other. However, succinic acid was very potent, resulting in the lowest IPX value of −0.38 ± 0.04. Although this result was statistically not different from the IPX values obtained for the other organic acids, there was a clear trend for a higher proton secretory potential of succinic acid applied in a concentration of 5.7 mg/L compared to lactic acid ($p = 0.12$) and tartaric acid ($p = 0.18$), which were applied in higher concentrations of 16.9 and 17.5 mg/L, respectively. The organic acid recombinate (IPX = −0.36 ± 0.04) stimulated proton secretion more strongly than red wine (IPX = −0.26 ± 0.02; two-tailed *t* test, $p < 0.01$).

**Effect of the Addition of Ethanol to Wine and to Organic Acids in Wine Representative Concentrations on Intracellular Proton Concentrations in HGT-1 Cells.** To study whether ethanol, as a major compound in wine, interacts with the individual organic acids and with the organic acid recombinate to modulate proton secretion in HGT-1 cells, we added ethanol in wine representative concentrations (12% 1:100 diluted) (Figure 6). When ethanol was applied concomitantly to the individual organic acids and to the recombinate, only the effect of tartaric acid in white wine representative concentrations (18.6 mg/L) was reduced significantly by −36.6 ± 8.80% ($p < 0.01$) and by 30.4 ± 4.40% ($p < 0.05$) in red wine representative concentrations (17.5 mg/L). Addition of ethanol to organic acid recombinates of both wines did not significantly changed the IPX value. However, doubling the ethanol concentration in red wine or white wine attenuated the proton secretory effect of red wine by −31.9 ± 7.86% ($p < 0.001$), whereas the effect of white wine (0.84 ± 11.64%) remained unchanged compared to the effect demonstrated for the original sample.

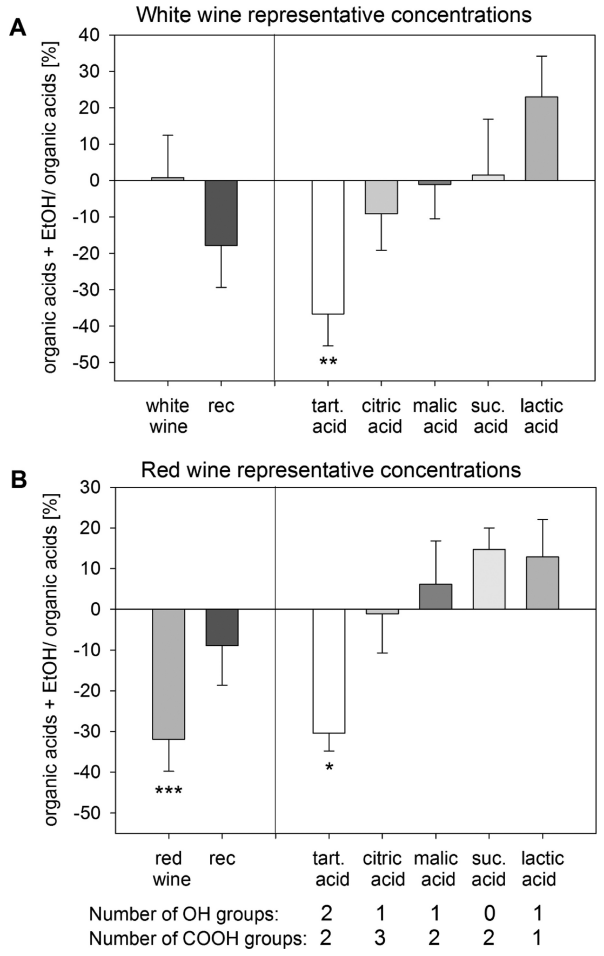


**Figure 6.** Intracellular proton index (IPX) of HGT-1 cells treated for 10 min with organic acids in concentrations of white wine (A) or red wine (B) with ethanol, shown as difference in percentage to organic acids without ethanol. White wine representative concentrations: 18.3 mg/L tartaric acid (tart. acid), 3.0 mg/L citric acid, 24.2 mg/L malic acid, 2.7 mg/L succinic acid (suc. acid), 4.4 mg/L lactic acid, and a recombinate (rec) of those acids in these concentrations. Red wine representative concentrations: 17.5 mg/L tartaric acid (tart. acid), 1.9 mg/L citric acid, 0.21 mg/L malic acid, 5.7 mg/L succinic acid (suc. acid), 16.9 mg/L lactic acid, and a recombinate (rec) of those acids in these concentrations. Data are displayed as the mean ± SEM, $n \geq 3$; tr = 3−6 (statistics: A and B, two-tailed $t$ test, effect of organic acids without ethanol vs effect of organic acids with ethanol; *** = $p < 0.001$; ** = $p < 0.01$ * = $p < 0.05$).

## DISCUSSION

The purpose of this study was to identify whether organic acids and ethanol in white wine and red wine contribute to their effects on gastric acid secretion. Gastric acid secretion in healthy subjects after administration of white wine, red wine, or ethanol was studied by means of pH-sensitive Heidelberg capsules. Molecular mechanisms of gastric acid secretion in the presence of white or red wine, ethanol, or organic acids were also studied in human gastric tumor cells (HGT-1), a cell line that has been established in our group for the identification of coffee and beer compounds that regulate mechanisms of stomach acid secretion.[19−24]

Organic acids have been shown to stimulate mechanisms of gastric acid secretion in humans.[12,22] The different processing technologies of white and red wine result in a characteristic, wine-specific composition of organic acids. In red wine, a second fermentation, the malolactic fermentation, is commonly used to reduce the amount of the sourer tasting malic acid by converting it into lactic acid, which has a less sour taste.[26] In white wine, by contrast, a sourer taste is preferred and the malolactic fermentation is typically not applied. Therefore, we investigated the contribution of organic acids to the stimulatory potential of white and red wine on stomach acid secretion and mechanisms thereof.

First, we conducted a human intervention study to investigate whether red and white wines have different effects on stomach acid secretion. Here, we show that administration of 125 mL of either red wine or white wine stimulated gastric acid secretion. These effects were even stronger than the effect of an equivalent amount of ethanol. The finding that red and white wine stimulate gastric acid secretion is in agreement with results from previous studies in which 300−500 mL of white[1,3] or red wine[2] instilled intragastrically by a tube also increased gastric acid secretion in healthy subjects. However, results reported for ethanol administered in beverage representative amounts are conflicting.[1,2,27,28] Lenz et al.[28] and Singer et al.[1] demonstrated that ethanol only in low concentrations ranging from 1.4 to 10% (equivalent to a total amount of 5.5−19 g ethanol) stimulated gastric acid secretion, but not when administered in higher concentrations.[1,28] Peterson et al.[2] did not find a significant effect on gastric acid secretion in healthy subjects after administration of a total amount of 28 g of ethanol, given in concentrations from 5 to 36%. In agreement with these data, we demonstrated an increase of gastric acid secretion after administration of 12% v/v (or a total amount of 12 g) ethanol to healthy subjects. We suggest that ethanol exhibits hormetic effects, being able to stimulate gastric acid secretion in lower but not in higher concentrations.

Another finding of our human intervention was a clear trend for the red wine ($p = 0.054$) being more potent than the white wine in stimulating gastric acid secretion. Furthermore, in our parietal cell model, we measured a significantly stronger effect for red wine on the IPX, as an indicator of proton secretion in HGT-1 cells, compared to white wine. To further elucidate the differential effects of red wine and white wine on mechanisms of proton secretion, we conducted gene expression analyses in HGT-1 cells after treatment with white wine, red wine, or

ethanol. We performed a time course experiment to analyze the expression of the prosecretory genes *ATP4A*, *HRH2*, and *CHRM3* and the antisecretory gene *SSTR2*. Here, we demonstrated for the first time that red wine strongly increased the expression of all tested genes in HGT-1 cells after 10 and 15 min of exposure, but also inhibited the expression of the antisecretory receptor *SSTR2* after 25 min of treatment. In contrast, white wine solely stimulated the expression of *HRH2*. This suggests that red wine acts more effectively and through different mechanisms of proton secretion in the parietal cell compared to white wine. Tsukimi et al.[14] also reported a stronger stomach acid secretion after administration of red wine to six dogs with vagally denervated Heidenhain pouches compared to white wine.

Next, we wanted to know whether the different effects of white and red wine could be attributed to their individual contents of organic acids. Therefore, we quantified the most common organic acids in wine: succinic acid, tartaric acid, citric acid, malic acid, and lactic acid. All concentrations quantified were in accordance with previously published data.[16,29] The red and white wine samples contained similar concentrations of tartaric acid (Table 3). Due to the malolactic fermentation typically applied to red wine,[26] the most abundant organic acid in red wine was lactic acid, whereas malic acid was quantitatively dominating in white wine. Additionally, the concentration of succinic acid was twice as high in red wine as in white wine. For answering the question which of the organic acids contributes the most to the effect of red wine and white wine, we tested the organic acids for their effects on the IPX in HGT-1 cells in wine representative concentrations. In a red wine representative concentration of 5.7 mg/L, there was a clear trend for succinic acid to decrease the intracellular proton concentration, as an indicator of proton secretion, more potently compared to lactic acid ($p = 0.12$) and tartaric acid ($p = 0.18$), which were applied in higher concentrations of 16.9 and 17.5 mg/L, respectively (Figure 5). In white wine representative concentrations, the most abundant organic acid, malic acid, exhibited the strongest stimulation of proton secretion. Teyssen et al.[12] also showed a stimulatory effect of organic acids on gastric acid secretion. In their study, the effects of acetic acid, oxalic acid, lactic acid, maleic acid, and succinic acid, produced by glucose fermentation, were tested, and only maleic acid and succinic acid showed a significant stimulation of gastric acid secretion in six healthy volunteers. Thus, a structure-dependent effect was hypothesized by Teyssen et al.,[12] suggesting that the length of the carbon chain and the presence of two carboxylic groups are necessary for a compound to stimulate gastric acid secretion (Table 1). We here identified malic acid and succinic acid as the most potent acids in wine, which not only supports the findings by Teyssen et al.[12] but also is in agreement with our own previous results, showing that malic acid and succinic acid contribute to the stomach acid secretory potential of beer.[22] However, we also observed a strong effect by lactic acid on mechanisms of proton secretion and can, therefore, not confirm the hypothesis that two carboxylic groups are necessary for a compounds' stimulatory effect on gastric acid secretion.

Furthermore, we tested the effects of biomimetic organic acid recombinates compared to white and red wine in the respective wine representative concentrations. As a result, both recombinates stimulated mechanisms of proton secretion more potently than red wine or white wine. We then questioned whether a wine component reacts with, for example, the hydroxyl or carboxyl groups of the organic acids as structural elements hypothesized to be responsible for the ability to stimulate mechanisms of gastric acid secretion. Because ethanol is one of the predominant compounds in wine and has been demonstrated to stimulate stomach acid secretion in our Heidelberg experiment with healthy subjects in a less pronounced manner than wine, and is known to esterify organic acids, we tested whether the addition of ethanol to the individual organic acids and their recombinate could have an effect on the intracellular proton index. Here, we could show that the addition of ethanol to tartaric acid resulted in a significantly attenuated proton secretion compared to the effect of the tartaric acid alone and compared to the other organic acids tested. Because tartaric acid bears the highest number of hydroxyl groups among these organic acids, we hypothesize that esterification in the presence of ethanol may lessen the effect of tartaric acid on proton secretion. However, addition of ethanol to the recombinate of organic acids in white and red wine representative concentrations did not lead to a significant attenuation of the recombinate's stimulatory effect. This result was also observed for malic acid and succinic acid, the two most active organic acids in white and red wine. Interestingly, when ethanol was added to red wine, the stimulatory effect of red wine was also reduced, whereas addition of ethanol to white wine did not change proton secretion. However, this may be a result of white wine's already reduced ability to stimulate acid secretion. However, we cannot exclude that ethanol interacts with red wine components other than organic acids to lessen its effects on proton secretion.

For white wine, the results from the Heidelberg experiment in healthy subjects, as well as those obtained from the cell culture experiments in HGT-1 cells, indicate a less pronounced effect on mechanisms of stomach acid secretion compared to red wine. Because the addition of ethanol to white wine did not affect its proton secretory potential, other compounds in white wine must be responsible for its less stimulating effect on stomach acid secretion compared to red wine.

In conclusion, we could show that red wine enhances gastric acid secretion by regulation of the prosecretory genes coding for $H^+,K^+$-ATPase, histamine H2 receptor, and acetylcholine M3 receptor and the antisecretory somatostatin receptor more potently than white wine. Furthermore, we found that organic acids, especially malic acid and succinic acid, are potent gastric acid stimulants in wine. Ethanol is also a potent stimulant, but we also show that ethanol can lower the stimulatory potential of tartaric acid and red wine. Identification of wine components responsible for the less pronounced effect of white wine compared to red wine on stomach acid secretion has to be addressed in future studies.

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS USED

GERD, gastroesophageal reflux disease; NADH, nicotinamide adenine dinucleotide; SNARF-1-AM, 1,5-carboxyseminaphtorhodafluor-acetoxymethyl ester; IPX, intracellular proton index; HGT-1, human gastric tumor cell line 1; *ATP4A*, $H^+$,$K^+$-ATPase $\alpha$-subunit; *HRH2*, histamine H2 receptor; *SSTR2*, somatostatin receptor; *CHRM3*, acetylcholine receptor M3; *PPIA*, peptidylprolyl isomerase A; *n*, biological replicate; tr, technical replicate.

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## (2) "Identification of catechin, syringic acid and procyanidin B2 in wine as stimulants of gastric acid secretion"

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The aim of this study was to identify whether the different effects of red wine vs. white wine on mechanisms of gastric acid secretion can be explained by the different concentrations of malic and lactic acid and/or phenolic compounds.
Red wine samples stimulated proton secretion more pronounced than white wine samples. It has been shown that malic acid and lactic acid stimulate proton secretion concentration dependently and regulate genes relevant for gastric acid secretion. Furthermore, it has been revealed that phenolic compounds, such as catechin, syringic acid and procyanidin B2 present in red wine contribute to the stimulation of proton secretion in HGT-1 cells, and when added to white wine enhancing its effect on proton secretion.

I participated in the study design and measured the proton secretion in HGT-1 cells. Furthermore, I performed the MTT assays in HGT-1 cells to exclude cytotoxic effects of the samples. I prepared the samples and analyzed the gene expression. I did the statistical analysis, as well as data interpretation. Finally, I prepared the manuscript.

# Identification of Catechin, Syringic acid and Procyanidin B2 in Wine as Stimulants of Gastric Acid Secretion

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**Keywords:** gastric acid secretion, red wine, white wine, organic acids, phenolic compounds

**Abstract:**

Organic acids of wine, in addition to ethanol, have already been identified as stimulants of gastric acid secretion. Here, we characterize the influence of other wine compounds, particularly phenolic compounds, on proton secretion. Forty wine parameters were determined in four red wines and six white wines, including the content in acids and phenolic compounds. The secretory activity of all 10 wines was determined in a gastric cell culture model (HGT-1 cells) by means of a pH sensitive fluorescent dye. Red wines stimulated proton secretion more than white wines. Lactic acid and the phenolic compounds syringic acid, catechin, and procyanidin B2 stimulated proton secretion, and correlated with the pro-secretory effect of the wines. Addition of the phenolic compounds to the least active white wine sample enhanced the proton secretory effect of the modified wine. These results indicate that not only malic and lactic acid, but also bitter and astringent tasting phenolic compounds in wine contribute to its stimulatory effect on gastric acid secretion.

## Introduction

Gastric acid secretion is stimulated by dietary intake of foods, especially proteins, and many beverages, such as coffee[1, 2], and fermented beverages such as beer and wine.[3-7] Parietal cells in the stomach secrete about two liters of gastric acid per day in the form of hydrochloric acid (HCl). HCl functions to kill bacteria, to aid digestion by solubilizing food, and by establishing an optimal pH (between 1.8 - 3.5) for the activity of digestive enzymes.[8] Although gastric epithelia are intrinsically resistant to the damaging effects of HCl, the eptihelia of the esophagus is not resistant. Therefore, reflux of gastric acid into the esophagus cause pain, the so called heartburn, and in the long run lesions of the epithelia.[9] Wine consumption can result in gastro-esophageal-reflux[10, 11] and about 20 percent of the population showing at least weekly symptoms of gastro-esophageal-reflux.[12]

Gastric acid secretion by parietal cells takes place in the corpus and fundus areas of the stomach. The activity of these cells is regulated by a number of cell surface receptors as well as functional and signalling proteins. The key protein regulating gastric acid secretion is the $H^+$,$K^+$-ATPase (encoded by the gene *ATP4A*). This protein can be activated through histamine H2 cell surface receptors (encoded by the gene *HRH2*) and the acetylcholine M3 receptor (encoded by the gene *CHRM3*), which transmit signals through hormones and second messengers. Activation of $H^+$,$K^+$-ATPases results in the transport of hydrogen ions into the gastric lumen in exchange for potassium ions. Activation of the somatostatin receptor (encoded by the gene *SSTR2*) inhibits proton secretion. These three cell surface receptors and their respective ligands, histamine, acetylcholine and somatostatin, are important in the regulation of gastric acid secretion[8, 13]. All of these receptors as well as the $H^+$,$K^+$-ATPase are functionally expressed in the human gastric tumor cell line HGT-1.[3, 14, 15] This cell line has been established in our group for the identification of stomach acid

regulating compounds in coffee, wine and beer [3, 7, 14, 16-19], with the reliability of the results confirmed with *in vitro* results from human intervention trials.[1, 3]

In one of our previous studies, in which we identified organic acids in wine as potent stimulants of gastric acid secretion, we also demonstrated that a sample of red wine stimulated gastric acid secretion of healthy volunteers more potently than a sample of white wine.[3] Although only two types of wines were tested, our results were in accordance with findings by Tsukimi and colleagues[20], who demonstrated a significantly stronger effect for red wine as compared to white wine after administration to dogs with vagally denervated Heidenhain pouches.[20] Also Peterson and colleagues[6] showed that administration of 300 mL of red wine resulted in higher serum gastrin concentrations compared to administration of the same amount of white wine. Gastrin stimulates gastric acid secretion by binding to cholecystokinin B receptors, thereby stimulating the release of histamine in enterochromaffin-like cells, which induces the secretion of protons into the gastric lumen through the $K^+$,$H^+$-ATPase.

Although these results clearly demonstrate differential effects of red versus white wine on gastric acid secretion, the key active compounds responsible for this difference have not yet been identified. Quantitatively, red and white wines primarily differ in their concentrations of malic and lactic acid[3], and of phenolic compounds. Ethanol, as major gastric acid stimulating constituent in wine, can be neglected since (i) the ethanol content in red and white wine is similar, and (ii) previous studies by Singer *et al.*[2] and Teyssen *et al.*[3] demonstrated that only fermented beverages such as beer and wine stimulate gastric acid output, while distilled alcoholic beverages with a higher ethanol concentrations show very little or no effect.[4, 5] Moreover, ethanol itself induces gastric acid secretion only in concentrations lower than those present in wine.[21] Based on these results, Teyssen and colleagues[22] fractionated fermented glucose, and identified the organic acids maleic acid and succinic acid as strong stimulants of gastric acid

secretion when administered to healthy subjects. Since the major organic acids in wine are tartaric, citric, malic, succinic and lactic acid, one of our previous studies aimed to identify whether these constituents contribute to the stimulating effect of wine on gastric acid secretion.[3] All tested organic acids stimulated proton secretion, with malic acid in wine representative concentrations showing the most pronounced effect. Hydroxyl and carboxyl groups were proposed to be the functionally active groups of the organic acids.[1,22] Since these structural characteristics are shared by phenolic compounds as well, we hypothesized that phenolic constituents, which are present at higher concentrations in red wine as compared to white wine, contribute to the more pronounced effect of red wine on gastric acid secretion.

To our knowledge, no studies have investigated the effect of the phenolic compounds of wine on gastric acid secretion. However, we found one study in which the effect of phenolic compounds on proton secretion was tested; Ono and colleagues[23] measured [$C^{14}$] aminopyrine accumulation as an index of acid production in isolated parietal cells of guinea pigs. The isolated cells were stimulated with histamine or dibutyryl-cAMP and compared to cells treated with histamine or dibutyryl-cAMP combined with pentagalloylglucose (PGG) extracted from paeoniae radix, or with gallic acid. The amount of [$C^{14}$] aminopyrine accumulation in the cells reflects the acid secretory state of the cells. PGG inhibited the histamine- or dibutyryl-cAMP-provoked effect in isolated parietal cells while gallic acid, a compound also present in wine, had no effect.

The aim of this study was to identify whether the different effects of red wine vs. white wine on mechanisms of gastric acid secretion can be explained by the different concentrations of malic and lactic acid and/or phenolic compounds. The secretory activity of four red wines and six white wines was tested using the well-established HGT-1 cell model.[3, 7, 16, 18] The results on cellular proton secretion were correlated with the quantitated content of organic acids and phenolic compounds. The most promising

compounds were tested individually, and in a recombinate of all, that was added to a white wine sample which showed the least pronounced effect on proton secretion.

## Materials and Methods

**Chemicals.** DL-lactic acid, L- malic acid, (+)- catechin, syringic acid, procyanidin B2, histamine, MTT-reagent, and chemicals for the cell culture experiments, trypsin, glutamine, penicillin/streptomycin, and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma-Aldrich. 1,5 Carboxy-seminaphtorhodafluor acetoxymethylester (SNARF-1-AM), nigericin and fetal bovine serum were obtained from Invitrogen (Vienna, Austria). All other chemicals were obtained from Roth (Karlsruhe, Germany).

**Samples.** Wine samples were provided by the Federal College and Research Institute for Viticulture and Pomology, Klosterneuburg, Austria. A total of 4 red wine samples of the varieties “Blauer Burgunder” 2010 (R1), “Rösler” 2009 (R2), “Cabernet Sauvignon-Merlot” 2009 (R3) produced by the Federal College and Research Institute for Viticulture and Pomology, Klosterneuburg, Austria and “Zweigelt Reserve” 2009 (R4) by Rosner, Lower Austria and a total of 6 white wine samples of the varieties “Grüner Veltliner” 2010 (W1) produced by Rosner, Lower Austria, “Gelber Muskateller” 2010 (W2), “Grüner Veltliner” 2010 (W3), “Chardonnay ice wine” 2009 (W4), “Chardonnay” 2010 (W5), “Riesling” 2010 (W6) produced by the Federal College and Research Institute for Viticulture and Pomology, Klosterneuburg, Austria were tested. For cell culture experiments, samples were diluted 1:100 in DMEM[1].

**Cell culture.** For cell culture experiments, the human gastric tumor cell line HGT-1, obtained from Dr. C. Laboisse (Laboratory of Pathological Anatomy, Nantes, Frances),

was used. They were cultured in DMEM with 4 g/L glucose, supplemented with 10 % fetal bovine serum, 2 % L-glutamine, and 1 % penicillin/streptomycin under standard conditions at 37 °C, 95% humidity, and 5% $CO_2$.

**Cell cytotoxicity.** Cytotoxic effects of wine samples were excluded by staining the cells with the yellow tetrazole MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent. A total of 100 000 cells per well were seeded in a 96-well plate, and allowed to settle for 24 h. Afterwards, the medium was discarded and cells were treated with wine samples in a 1:100 dilution, individual compounds in wine-representative concentrations, and white wine (W7) in combination with a recombinate of catechin, procyanidine B2 and syringic acid added to DMEM for 30 min under standard conditions. Test samples were removed and 100 µL MTT-working solution, consisting of 1 part 5 mg/mL MTT solution and 5 parts DMEM, were added. In this assay, viable cells reduce the yellow tetrazole MTT to a purple formazan. After 15 min, the MTT solution was removed and the formazan was diluted in dimethyl sulfoxide. Absorbance was measured at 550 nm and at reference wavelength of 690 nm using an Infinite 200 Pro Plate Reader. Cell viability was determined relative to medium-only treated control cells (untreated controls = 100%).

**Intracellular pH measurement in HGT-1 cells.** The intracellular pH was measured as a marker of proton secretion in HGT-1 cells by means of the pH-sensitive fluorescence dye SNARF-1-AM.[3, 7, 16, 18] HGT-1 cells were seeded in a 96-well plate at a density of 100 000 viable cells per well under standard conditions at 37 °C, 95% humidity, and 5% $CO_2$ and allowed to settle for 24 h. Afterwards, cells were washed once with Krebs-Ringer-bicarbonate-HEPES-Buffer (KRBH), and loaded with 3 µM SNARF-1-AM in KRHB for 30 min at standard conditions. The cells were washed twice

and treated with either the wine samples or the wine constituents in wine representative concentrations, or white wine (W7) in combination with a recombinate of catechin, procyanidine B2 and syringic acid in DMEM, all diluted 1:100, for 10 min at standard growth conditions. As positive control, 1 mM histamine was used. Afterwards, the test substances were removed, the cells washed twice with KRBH, and 100 µl KRBH per well was added prior to the measurement of fluorescence using an Infinite 200 Pro Plate Reader. Fluorescence was detected at 580 nm and 640 nm emission after excitation at 488 nm. The ratio between the two measured emission wavelengths was used to calculate the pH using a standard calibration curve. The calibration curve was generated by treating the cells with potassium buffer solutions of varying pH values, ranging from 7.2 to 8.2 in the presence of 2 µM nigericin to equilibrate intracellular and extracellular pH in the cells. The potassium buffer calibration solutions consisted of 20 mM NaCl, 110 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 18 mM D-Glucose and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The analyzed intracellular pH of the calibration solutions was fit to a linear regression. Using the intracellular pH, the intracellular $H^+$ concentration was calculated. The ratio between treated and medium-only/non-treated cells was calculated and $\log_2$ transformed to determine the intracellular proton index (IPX).[3, 7, 16, 18] The lower the IPX the stronger the proton secretion by the cell.

**Quantitation of wine constituents and parameters.**

*Analysis of general wine parameters.* Reducing substances, titratable acidity, volatile acidity, pH-value, free and total sulphurous acid were analysed by means of standard methods as described by Eder and Brandes.[24] For reducing substances, the traditional iodometrical titration with Fehling reagent was applied. Titratable acids were

determined potentiometrically with 0.1 N NaOH up to an endpoint of pH 7.0. The pH-measurement was performed in the undiluted sample using a pH-sensitive electrode. For the determination of volatile acids, the acid content of water steam distillate was measured by titration with 0.01 N NaOH and phenolphthalein as indicator (pH 8.1). The effect of disturbing sulphurous acid was subtracted by extra iodometric titration. Free und total sulphurous acid were determined acidimetrically following the procedure described by Paul.[25]

*Organic acids***.** Quantitation of the major organic acids (tartaric, malic, lactic, citric, oxalic acid) was achieved by ionic chromatography as described by Prasnikar et al.[26]. Succinic acid was quantified using an enzymatic kit from Megazyme International (Wicklow, Ireland) according to the protocol of the manufacturer.

*Sugars, Glycerol and acetic acid.* Fructose, glucose, glycerol, acetic acid were analyzed enzymatically[27] using the Konelab 20 automatic system as described by Stojanovic et al.[28]

*Aromatic phenols.* The concentrations of aromatic phenols (vanillin, syringaldehyde, coniferylaldehyde sinapaldehyde and scopoletin) were determined after solid phase enrichment on LiChrolut EN 200 mg, 3 ml column (Merck) as described by Matejicek et al.[29]. The SPE column was conditioned by subsequent washing with 3 ml dichlormethane, 3 ml methanole and 3 ml ethanol (13 % (v/v) in water). Then 5 ml of the sample with internal standard (4-methoxybenzaldehyde) were slowly applied onto the column. After washing the column with 2-3 volumes of ethanol (13 % (v/v) in water), the column was dried by drawing nitrogen for 30 min. Finally, the phenols were eluted by twice adding 1 ml dichloromethane. The eluate was dried on a rotavapor (240 mbar, 40°C) and re-dissolved in 1 ml HPLC solvent (0.5 % formic acid, 10 % methanol). Before injection into HPLC system, the eluate was purified by membrane filtration (Multoclear, 13 PVDF, 0.45 µm). HPLC was performed on a Rapid Resolution system

(RR 1200 Agilent) equipped with a binary pump (SL, Agilent) and a diode array detector (DAD, Agilent) as well as a fluorescence detector (1260 Infinity FLD, Agilent). Separation was achieved on reversed-phase column Zorbax SB-C-18 (150 x 2.1 mm, 1.8 µm) with gradient elution of a) 0.5 % formic acid and b) methanol within 87 min. Flowrate was set at 0.25 ml/min, column oven temperature at 40°C. Detection wavelengths were 260, 280, 290, 313, and 350 nm. Scopoletine was also detected using the FLD (excitation: 345nm emission: 460nm). A volume of 5 µl of the filtered SPE eluate was injected for analysis of the phenolic acids (vanillic acid, syringic acid, ellagic acid and gallic acid).

*Phenolic substances.* The other phenolic substances (caftaric acid, cis and trans-coutaric acid, fertaric acid, para-cumaric acid, ferulic acid, caffeic acid, catechine, epicatechine, tyrosol, and procyanidine B1 and B2) were analyzed with a modified HPLC method published by Vrhovsek et al (1997). The HPLC system consisted of a Rapid Resolution 1200 system with binary pump, DAD detector (Agilent) and a Poroshell 120 SB-C18 column (150 x 2.1, 2.7 µm; Agilent). A gradient elution with solvent a) formic acid (0.5%), and b) methanol and a flow rate of 0.2 ml/min was performed. Injection volume was 5 µl and detection was at 280 and 320 nm.

*Anthocyanins.* Total monomeric anthocyanins were seperated and detected by HPLC-UV, and the content was calculated as malvidol-3-glucoside as a reference compound, as described by Eder et al.[30]. For the determination of the total phenol content, a photometric method using the Folin Ciocalteus reagent was used as described by Linskens and Jackson[31].

**Gene Expression.** A total of 100,000 HGT-1 cells per well were seeded in six-well plates and grown until confluence. Then the cells were treated with 10 mg/L DL-lactic acid and 10 mg/L L-malic acid for 5, 10, 15, 20, 25, and 30 min and harvested for RNA

isolation using the RNeasy Mini Kit and SV Total RNA Isolation System (Promega, Madison, USA). RNA quantity and quality were checked spectrophotometrically at 260 nm and 280 nm by calculation of the ratio of those wavelengths using the nanoquant plate for the Infinite 200 PRO Plate Reader. All cell samples had a ratio between 1.8 – 2.2. cDNA synthesis was carried out with the High capacity RNA to cDNA Master Mix (Applied Biosystems, Munich, Germany) according to the manufacturer's protocol. Peptidylprolyl isomerase A (PPIA) was used as internal control. Primers for the $H^+$,$K^+$-ATPase alpha-subunit (*ATP4A*), the histamine H2 receptor (*HRH2*), the somatostatin receptor (*SSTR2*) and the acetylcholine receptor M3 (*CHRM3*) and *PPIA* were designed and validated previously [3, 7, 14, 17] and were carried out as previously described[3].

**Statistical analysis.**

The concentration of organic acids and phenolic compounds in wine was correlated to the IPX of the ten wine samples using the correlation analysis after Spearman with SPSS 19.0.0 (IBM Statistics). Data below the limit of detection were replaced by the LOD/$\sqrt{2}$.Statistical analysis was performed using Excel 2007 (Microsoft), SigmaPlot software 11.0 (Systat Software). Outliers were excluded by Nalimov outlier analysis. Cytotoxic effects of the samples on HGT-1 cells compared to non-treated cells were determined with the two-tailed Student's *t*-test, and considered to be significant at a $p < 0.05$. Significant differences in the data set of the intracellular pH measurements were determined by a one-way ANOVA with Holm-Sidak *post hoc* analysis or the two-tailed Student's *t*-test as indicated in the figure legends. The two-way ANOVA with Holm-Sidak *post hoc* analysis was applied for analyzing time dependent effects on gene expression. At least three biological replicates and 2 technical replicates were

analyzed for each cell culture experiment. Data in the results section as well as in diagrams is given as mean ± SEM, except indicated otherwise.

## Results

### Cell vitality of HGT-1 cells after treatment with wine samples and wine additions.

We previously showed that red wine in concentrations higher than a 1:100 dilution caused cytotoxic effects in HGT-1 cells[3]. To exclude cytotoxic effects of the wine samples used in this study in HGT-1 cells, we conducted a MTT-assay using the same dilution (**Table 1)**. The MTT assay is an indicator for the metabolic status of the cell. Data is represented as percent relative to non-treated cells. Lower values for sample-treated cells indicate a reduction of the metabolic status, which is an early indicator of cytotoxic effects. Neither the wine samples, nor any of the individual wine constituents tested, nor the combination of the white wine Riesling (W7) with the recombinate showed cytotoxic effects on HGT-1 cells.

### Effect of wine samples on proton secretion in HGT-1 cells.

For studying the influence of the four red wines and six white wines on proton secretion in HGT-1 cells, cells were treated with 1:100 dilutions of each wine sample and effects were compared to nontreated cells (control). Each wine sample itself stimulated proton secretion ( $p < 0.001$, one way ANOVA with Holm – Sidak *post hoc* test vs. control ) (**Table 2)**. The red wine varieties “Blauer Burgunder” and “Roesler” showed the highest secretory activity ( $p < 0.001$), with an IPX of -0.39 ± 0.02 and -0.37 ± 0.02, respectively. The white wine variety “Riesling” showed the least pronounced stimulation of the tested wines with an IPX of -0.10 ± 0.02. IPX. The average of the IPX values of the red wine samples was lower ($p < 0.016$ ) than that of the red wine samples (**Figure 1**), clearly

demonstrating the more pronounced stimulating effect on proton secretion for red wines.

**Characterization of wines.**

All wines were produced in Lower Austria from the 2009 and 2010 vintages. The characteristics of the 4 red wines and 6 white wines are reported in **Table 2** and **Table 3**. Alcoholic strength was in the range of 13 – 14.1 % for red and 11.8 -13.1 % for white wines. Titratable acidity was higher in white wines ranging from 5.9 – 10.5 g/L compared to red wines ranging from 4.9 – 5.8 g/L. Organic acid content mainly differed in the concentration of lactic and malic acid. While lactic acid was only quantified in red wine samples in concentrations from 2–3.5 g/L, malic acid was only quantified in white wine samples in concentrations from 3.3–4.6 g/L.

Mean concentrations of total phenolics were higher in red wines ranging from 1.11 – 1.84 g/L compared to white wines 0.05–0.09 g/L. Although several phenolic compounds were not detected in the majority of the white wine samples, caftaric acid as well as tyrosol were also quantified in white wines to a nearly similar amount as in red wine samples. Coniferylaldehyde and sinapaldehyde were not detected, neither in white nor in red wine. The phenolic compound with the highest amounts in wine samples was catechin with concentrations ranging from 29.5–85.5 mg/L in red wines and 1.8 – 4.8 mg/L in white wines.

**Correlation between IPX and wine parameters.**

To determine which of the wine constituents quantified correlate with the stimulatory effect of wine samples on proton secretion in HGT-1 cells, the IPX data of the ten wine samples were correlated with the amount of organic acids and phenolic compounds. **Table 4** shows the results of the correlation analysis. Negative correlations indicating

a pro-secretory effect were determined for lactic acid, procyanidin B1 and B2, ellagic acid, syringic acid, syringaldehyde, vanillic acid and vanillin. According to the hypothesis that a higher amount of hydroxyl and carboxyl groups induce gastric acid secretion, we further investigated procyanidine B2 (10 hydroxyl groups) and syringic acid (1 hydroxyl and 1 carboxylgroup). Since catechin (5 hydroxyl groups) showed the highest amount in the red wine samples of the measured phenolic compounds and is the parent structure of procyanidine B2 and several other phenolic compounds in wine, it has been included for further analysis. In addition, lactic and malic acid were investigated since both are either only present in white or in red wine.

**Effect of DL-lactic acid and L-malic acid on intracellular proton concentration.**

L-malic and DL-lactic acid were tested in a concentration range quantified in the ten wine samples, namely 20 – 40 mg/L and 30 – 60 mg/L, respectively (**Figure 2**). Both organic acids stimulated proton secretion ($p < 0.001$) in HGT-1 cells in a concentration dependent manner compared to non-treated cells (lactic acid; -0.30 ± 0.01 to -0.52 ± 0.02, malic acid; IPX ranging from -0.26 ± 0.03 to -0.44 ± 0.03).

**Gene expression of HGT-1 cells after treatment with lactic acid and malic acid.**

In order to gain a mechanistic insight into whether DL-lactic acid or L-malic acid affect the regulation of secretory-relevant genes, HGT-1 cells were treated with 10 mg/mL DL-lactic or L-malic acid over a time period of 30 min and gene expression of *ATP4A*, *HRH2*, *SSTR2* and the *CHRM3* was determined by qPCR.

Gene expression of the target genes are presented as ratios normalized to the endogenous control *PPIA* (control = 1) (**Figure 3**). Treatment of HGT-1 cells with lactic acid or malic acid down regulated expression of *ATP4A* and *HRH2* at 10 min (*ATP4A* 0.57 ± 0.06 and 0.55 ± 0.06, *HRH2* 0.66 ± 0.09 and 0.54 ± 0.08, respectively). None

of the tested organic acids affected the expression levels of *CHRM3*. The main difference in effects between DL-lactic acid and L-malic acid were detected on expression levels of *SSTR2.* L-malic acid reduced expression of *SSTR2* at 10 min (0.64 ± 0.08, $p < 0.01$). In contrast, DL-lactic acid increased mRNA expression of *SSTR2* at 25 min (1.29 ± 0.08, $p < 0.05$), an effect that was significantly different from that of L-malic acid at 25 min (0.95 ± 0.08, $p < 0.01$).

**Effect of catechin, procyanidin B2 and syringic acid on intracellular proton concentration**

Catechin, syringic acid and procyandidin B2 were tested in a wine representative concentration range. Catechin in the range of 0.1 – 1 mg/L stimulated proton secretion (IPX -0.27 ± 0.03) as well as procyanidin B2 in the concentration range of (0.01 – 0.25 mg/L) and syringic acid in the concentration range of 0.1 – 2.5 mg/L to the highest extend, with IPX values of -0.17 ± 0.03 and -0.25 ± 0.05, respectively (**Figure 4**).

**Enhancing the stimulating effect of white wine on proton secretion via addition of a phenolic recombinate**

To verify that catechin, syringic acid and procyanidin B2 can stimulate proton secretion in a complex wine matrix, a recombination of these substances in two concentrations were added to the white wine of the variety Riesling (W6). This wine showed the lowest pro-secretory activity (**Table 2**). Treatment of the cells with the W6 sample in combination with three phenolic compounds in concentrations representative for red wine lead to IPX values of – 0.14 ± 0.02 ($p > 0.05$), whereas treatment with a twofold addition of the recombinate (IPX – 0.16 ± 0.02) increased the proton secretion significantly ($p < 0.05$) compared to the cells treated with the white wine solely (IPX – 0.10 ± 0.02 ).

## Discussion

In two previous studies, red wine was demonstrated to stimulate gastric acid secretion to a greater extent than white wine. However, since one of these studies was carried out in dogs with vagally denervated Haidenhain pouches[20], and in the other study, only one red wine was tested vs. one white wine in six healthy subjects[1], we hypothesized here (i) that the more pronounced effect of red wine is reproducible for a greater variety of wines, (ii) that major quantitative differences in red and white wine contribute to this effect. Forty wine parameters were analyzed in four red wine samples and in six white wine samples. The major quantitative differences were analyzed for two classes of compounds: the organic acids malic acid and lactic acid, which were only quantified in white and red wine samples, respectively, and phenolic compounds. Correlation analysis between the quantitative data and the proton secretory activity of the given constituents in parietal HGT-1 cells, a well-established cell culture model representing mechanisms of gastric acid secretion in human[3, 7, 16, 18], revealed significant associations for the two organic acids as well as the phenolic compounds procyanidin B1 and B2, ellagic acid, syringic acid, syringaldehyde, vanillic acid, vanillin, and scopoletin when tested in wine-representative concentrations. Since the red wine samples were, again, demonstrated to stimulate proton secretion more pronounced compared to the white wines, the contribution of malic acid, lactic acid and selected phenolic compounds was studied.

In one of our previous studies, malic acid and lactic acid were identified to increase proton secretion in HGT-1 cells, independent of the ethanol content of the wine sample.[1] However, this finding was left on a descriptive level since no mechanistic data were provided. In this study, we were able to demonstrate that both organic acids regulate the mRNA expression of gene involved in proton secretion to a similar extend when tested in wine-representative concentrations, which is in line with their effect on proton

secretion. However, the proton secretory activity of malic and lactic acid cannot chiefly account for the different effect of red versus white wine, since malic acid was only quantified in white wine, whereas lactic acid, in a similar concentration range, was quantified in red wine solely. This can be explained by the fact that the majority of red wines undergo malolactic fermentation, while this technology is less common used for the production of white wines.[32] In malolactic fermentation, a secondary fermentation after completion of the alcoholic fermentation, L-malic acid is transformed to the less sour L-lactic acid.[33] This reaction is used to reduce the acidic taste, to modify the organoleptic character, and to improve the microbial stability of wine.[32] Although the malolactic fermentation makes the red wine taste smoother, our findings suggest that this technology does not reduce its secretory potential.

Phenolic compounds were also hypothesized to contribute to the gastric acid secretory potential of red wine. This hypothesis was not only based on the quantitative differences of phenolic compounds in red versus white wine and their correlation with the effect on proton secretion. Previous results from Teyssen et al.[22], and our own group[1], suggest that the length of the carbon chain, the presence of carboxylic groups and hydroxyl groups are structural determinants for compound to stimulate gastric acid secretion. Therefore, procyanidin B2 (10 hydroxyl groups, complex carbon molecule structure, bitter taste), syringic acid (one hydroxyl and one carboxyl group, bitter taste), as well as catechin as parent structure (10 hydroxyl groups, bitter taste and astringent compound) were selected. For all three phenolic compounds, a contribution to bitter and astringent taste of wine has been reported.[34] To our knowledge, this is the first study which tested and demonstrated a stimulating effect of phenolic compounds on gastric acid secretion in wine-representative concentrations. Furthermore, when these compounds were added to the least active white wine (Riesling W6) in concentrations two-fold higher than those quantified in red wine, a significant increase in proton

secretion was determined. This result clearly demonstrates that catechin, syringic acid as well as procyanidin B2 elicit a pro-secretory activity even when added to a complex solution such as white wine. Moreover, we suggest that the higher amount of phenolic compounds in red wine contributes to its higher stimulatory effect on gastric acid secretion compared to white wine.

In conclusion, we demonstrated that red wines stimulated mechanisms of gastric acid secretion more than white wines. Furthermore, we showed for the first time that the organic acids DL-lactic acid and L-malic acid regulate gene expression relevant for gastric acid secretion. In addition, the phenolic constituents catechin, procyanidine B2 and syringic acid were demonstrated to stimulate proton secretion in gastric parietal cells and are suggested to contribute to the more pronounced effect of red wine on gastric acid secretion compared to white wine.

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**Figure captions:**

**Figure 1.** Intracellular proton index (IPX) means after treatment of HGT-1 cells with red wines or white wines (1:100 dilution). Means from *n*=5-6, technical replicates = 3-6; (statistic: 2-sided *t*-test)

**Figure 2.** Intracellular proton index (IPX) of HGT-1 cells treated for 10 min with (A) DL- Lactic acid and (B) L – Malic acid in different wine representative concentrations. The control (C) was nontreated cells and the positive control was 1 mM histamine (HIS). Data are displayed as mean ± SEM, *n* = 3, *tr* = 4 – 6, (statistics: one-way ANOVA with the Holm-Sidak post hoc test; letters indicate significant differences between groups, $p < 0.05$)

**Figure 3.** Time-dependent indices of gene expression for the *ATP4A*, *CHRM3*, *HRH2*, and *SSTR2* in HGT-1 cells after treatment with 10 mg/L DL-lactic acid (DL-LA) and 10 mg/L L-malic acid (L-MA) compared to non treated cells. Data are displayed as mean values, *n* = 2-3, *tr* = 3, (statistics: two-way ANOVA with Holm-Sidak *post hoc* test; ** = $p < 0.01$, *** = $p < 0.001$)

**Figure 4.** Intracellular proton index (IPX) of HGT-1 cells after treatment with (**A**) procyanidin B2, (**B**) catechin, (**C**) syringic acid in wine represantitive concentrations. The control (C) was nontreated cells and the positive control was 1 mM histamine (HIS). Data are displayed as mean ± SEM, *n* = 4, *tr* = 4 6, (statistics: one-way ANOVA with the Holm-Sidak *post hoc* test; letters indicate significant differences between groups, $p < 0.05$)

**Figure 5.** Intracellular proton index (IPX) of HGT-1 cells after treatment with white wine of the variety Riesling (2010) with or without recombinats of catechin, syringic acid, procyanidin B2 (REC) in red wine representative concentrations. The control (C) was nontreated cells and the positive control was 1 mM histamine (HIS). Data are displayed as mean ± SEM, $n$ = 5-11, $tr$ = 6, (statistics: one-way ANOVA with the Holm-Sidak *post hoc* test vs. Control; Student's $t$-test WW vs. WW + RECx2, #, $p < 0.05$; ***, $p < 0.001$)

**Table 1.** Cell vitality in percent versus nontreated cells (Control: 100 ± 8 %).

| | **Mean ± SD** |
|---|---|
| **Red wines** | |
| Blauer Burgunder 2010 | 100 ± 7 |
| Rösler 2009 | 100 ± 7 |
| Cabernet Sauvignon-Merlot 2009 | 100 ± 7 |
| Zweigelt Reserve 2009 (Rosner) | 97 ± 8 |
| **White wines** | |
| Grüner Veltliner 2010 (Rosner) | 109 ± 8 ** |
| Gelber Muskateller 2010 | 102 ± 7 |
| Grüner Veltliner 2010 | 120 ± 12 *** |
| Chardonnay Eiswein 2009 | 98 ± 9 |
| Chardonnay 2010 | 106 ± 9 |
| Riesling 2010 | 100 ± 15 |
| **Recombinates** | |
| Recombinate x2 | 98 ± 5 |
| Riesling 2010 + Recombinate | 106 ± 13 |
| Riesling 2010 + Recombinatex2 | 106 ± 12 |

Wines were tested in a 1:100 dilution. The recombinate consisted of 0.8 mg/L (+) – catechin, 0.025 mg/L syringic acid and 0.2 mg/L procyanidin B2. The concentrations were doubled in recombinate 2. Data are given as the mean mean ± SD, $n$ = 3 - 6, $tr$ = 3 – 6. (Statistics: one way ANOVA with the Holm – Sidak post *hoc* test vs. control; significant differences vs. non treated control cells are indicated by ** = $p < 0.01$ and *** = $p < 0.001$)

**Table 2.** Characterization of wine samples. n.d. = not detected

| | Red wines | | | | White wines | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Parameter** | **R1** | **R2** | **R3** | **R4** | **W1** | **W2** | **W3** | **W4** | **W5** | **W6** |
| **IPX mean** | -0.39 | -0.37 | -0.29 | -0.25 | -0.28 | -0.26 | -0.19 | -0.16 | -0.13 | -0.10 |
| **IPX SEM** | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.04 | 0.01 | 0.03 | 0.02 |
| **Density [20°C/20°C]** | 1.00 | 1.00 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 1.07 | 0.99 | 0.99 |
| **Alcohol [vol%]** | 13.0 | 13.4 | 13.7 | 14.1 | 12.0 | 12.7 | 12.8 | 11.8 | 13.1 | 12.4 |
| **pH** | 3.9 | 3.9 | 3.9 | 3.7 | 3.5 | 4.0 | 3.6 | 4.1 | 3.7 | 3.7 |
| **SO2 acid:** | | | | | | | | | | |
| **Free [mg/l]** | 38 | 23 | 28 | 19 | 23 | 32 | 17 | 19 | 23 | 26 |
| **Total [mg/l]** | 129 | 97 | 81 | 65 | 57 | 121 | 100 | 143 | 74 | 99 |
| **Glycerine [g/L]** | 8.3 | 8.3 | 8.5 | 9.3 | 5.7 | 5.6 | 6.0 | 20.2 | 6.2 | 5.9 |
| **red. Compounds [g/L]** | 1.2 | 0.6 | 1.4 | 5.8 | 4.7 | 0.4 | 2.7 | 153 | 1.3 | 2.9 |
| **Fructose [g/L]** | 0.5 | 0.4 | 0.4 | 2.6 | 2.3 | 0.5 | 2.0 | 96.9 | 0.8 | 2.6 |
| **Glucose [g/L]** | n.d. | n.d. | n.d. | 1.8 | 1.6 | n.d. | 0.2 | 53.1 | n.d. | n.d. |
| **Titr. acidity [g/L]** | 5.7 | 5.6 | 4.9 | 5.0 | 7.1 | 5.9 | 6.1 | 10.5 | 7.3 | 6.8 |
| **Volatile acid [g/L]** | 1.4 | 1.5 | 1.1 | 0.7 | 0.5 | 0.4 | 0.6 | 1.6 | 0.1 | 0.3 |
| **Tartaric acid [g/L]** | 1.4 | 1.3 | 1.2 | 2.0 | 2.7 | 0.3 | 1.6 | 0.6 | 1.0 | 1.0 |
| **Malic acid [g/L]** | n.d. | n.d. | n.d. | n.d. | 3.8 | 4.6 | 3.3 | 5.5 | 4.6 | 4.5 |
| **Lactic acid [g/L]** | 3.5 | 3.2 | 2.4 | 2.0 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **Citric acid [g/L]** | n.d. | n.d. | 0.1 | n.d. | 0.2 | n.d. | 0.2 | 2.7 | 0.1 | 0.1 |
| **Succinic acid [g/L]** | 0.6 | 0.7 | 0.8 | 0.6 | 0.3 | 0.4 | 0.3 | 0.4 | 0.4 | 0.3 |
| **Acetic acid [g/L]** | 1.2 | 1.2 | 1.0 | 0.7 | 0.6 | 0.4 | 0.6 | 1.3 | 0.4 | 0.4 |
| **Oxalic acid [mg/L]** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 28 | n.d. | n.d. |

R1 = Blauer Burgunder 2010, R2 = Rösler 2009, R3 = Cabernet Sauvignon-Merlot 2009, R4 = Zweigelt Reserve 2009 (Rosner), W1 = Grüner Veltliner 2010 (Rosner), W2 = Gelber Muskateller 2010, W3 = Grüner Veltliner 2010, W4 = Chardonnay Eiswein 2009, W4 = Chardonnay 2010, W5 = Riesling 2010

**Table 3.** Contents of phenolic compounds and amines in wine samples. Results are expressed as mg/L. n.d. = not detected

| | Red wines | | | | White wines | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Parameter** | **R1** | **R2** | **R3** | **R4** | **W1** | **W2** | **W3** | **W4** | **W5** | **W6** |
| **IPX mean** | -0.39 | -0.37 | -0.29 | -0.25 | -0.28 | -0.26 | -0.19 | -0.16 | -0.13 | -0.10 |
| **IPX SEM** | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.04 | 0.01 | 0.03 | 0.02 |
| **Phenols [g/L]** | 1.3 | 1.84 | 1.25 | 1.11 | 0.09 | 0.07 | 0.09 | 0.05 | 0.09 | 0.07 |
| **Anthocyanins** | 98 | 49 | 123 | 11 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **Catechin** | 85.5 | 73.1 | 37.6 | 29.5 | 2 | 2.2 | 3.8 | 1.8 | 4.8 | 2.4 |
| **Epicatechin** | 29.6 | 29.9 | 12.8 | 14.1 | n.d. | n.d. | 0.9 | n.d. | 1 | n.d. |
| **Gallic acid** | 30.2 | 24.7 | 37.4 | 66.2 | n.d. | 0.1 | 0.5 | 1.6 | 0.2 | 0.1 |
| **Proc. B1** | 33.7 | 19.8 | 31.6 | 14.7 | n.d. | 1.2 | 2.2 | n.d. | 3.9 | n.d. |
| **Proc. B2** | 22 | 18.1 | 11.9 | 6.5 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **Caffeic acid** | 18.2 | 15.9 | 5.3 | 12 | 1.7 | 1.7 | 2 | 3.5 | 1.9 | 2.1 |
| **Caftaric acid** | 11.7 | 8.9 | 50.2 | 29.6 | 9.4 | 7.6 | 25.5 | 1.7 | 26.1 | 35.7 |
| **Tyrosol** | 10.9 | 10.2 | 20 | 8.1 | 6.2 | 5.1 | 6.5 | 7.7 | 6.6 | 7.1 |
| **p-Cumaric acid** | 6.3 | 11.6 | 5.1 | 5.1 | 1 | 1.2 | 1.2 | 1.9 | 2.4 | 1.3 |
| **Ellagic acid** | 4.43 | 1.96 | 3.12 | 3.2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **Syringic acid** | 2.47 | 2.05 | 1.29 | 1.27 | 0.04 | n.d. | 0.02 | 0.05 | 0.02 | 0.03 |
| **Syringaldehyde** | 0.22 | 0.33 | 0.28 | 0.06 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **c-Coutaric acid** | 0.4 | 0.5 | 2.8 | 1.3 | 1.2 | 1.6 | 0.9 | 0.4 | 1.3 | 1.2 |
| **t-Coutaric acid** | 1.9 | 2.3 | 14.7 | 6.7 | 2 | 2.4 | 2.5 | 0.5 | 3.7 | 4 |
| **Vanillic acid** | 0.96 | 0.82 | 0.9 | 0.49 | n.d. | n.d. | 0.01 | 0.06 | 0.01 | 0.02 |
| **Vanillin** | 0.14 | 0.15 | 0.13 | 0.04 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **Fertaric acid** | 0.4 | 0.5 | 1.7 | 1.1 | 1.7 | 1.2 | 1.7 | 0.3 | 1.8 | 6.5 |
| **Ferulic acid** | 1.9 | 1.9 | 1 | 0.8 | 0.8 | 0.6 | 0.8 | 0.9 | 0.8 | 0.9 |
| **Scopoletin [µg/L]** | 63 | 146 | 35 | 21 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

R1 = Blauer Burgunder 2010, R2 = Rösler 2009, R3 = Cabernet Sauvignon-Merlot 2009, R4 = Zweigelt Reserve 2009 (Rosner), W1 = Grüner Veltliner 2010 (Rosner), W2 = Gelber Muskateller 2010, W3 = Grüner Veltliner 2010, W4 = Chardonnay ice wine 2009, W4 = Chardonnay 2010, W5 = Riesling 2010

**Table 4.** Significant results of the correlation analysis after Spearman between IPX values and phenolic wine compounds.

| | ***all wines*** | | |
|---|---|---|---|
| **IPX mean vs.** | Correlation coefficient | *P* 2-ways | n |
| Tartaric acid | -0.377 | 0.283 | 10 |
| **Malic acid** | **.696*** | **0.025** | **10** |
| **Lactic acid** | **-.792**** | **0.006** | **10** |
| Citric acid | 0.483 | 0.157 | 10 |
| Succinic acid | -0.491 | 0.150 | 10 |
| Oxalic acid | 0.290 | 0.416 | 10 |
| Acetic acid | -0.525 | 0.119 | 10 |
| SO2 total (acid) | 0.006 | 0.987 | 10 |
| | | | |
| Phenols [g/L] | -.763* | 0.010 | 10 |
| Anthocyanins | -.751* | 0.012 | 10 |
| | | | |
| **Catechin** | **-0.588** | **0.074** | **10** |
| Epicatechin | -0.600 | 0.067 | 10 |
| Gallic acid | -0.401 | 0.250 | 10 |
| Proc. B1 | -.669* | 0.034 | 10 |
| **Proc. B2** | **-.792**** | **0.006** | **10** |
| Caffeic acid | -0.474 | 0.166 | 10 |
| Caftaric acid | 0.188 | 0.603 | 10 |
| Tyrosol | -0.455 | 0.187 | 10 |
| p-Cumaric acid | -0.463 | 0.177 | 10 |
| Ellagic acid | -.683* | 0.030 | 10 |
| **Syringic acid** | **-.675*** | **0.032** | **10** |
| Syringaldehyde | -.751* | 0.012 | 10 |
| c-Coutaric acid | 0.116 | 0.749 | 10 |
| t-Coutaric acid | 0.261 | 0.467 | 10 |
| Vanillic acid | -.659* | 0.038 | 10 |
| Vanillin | -.778** | 0.008 | 10 |
| Fertaric acid | 0.509 | 0.133 | 10 |
| Ferulic acid | -0.466 | 0.175 | 10 |
| Scopoletin [µg/L] | -0.673 | 0.033 | 10 |

R1 = Blauer Burgunder 2010, R2 = Rösler 2009, R3 = Cabernet Sauvignon-Merlot 2009, R4 = Zweigelt Reserve 2009 (Rosner), W1 = Grüner Veltliner 2010 (Rosner), W2 = Gelber Muskateller 2010, W3 = Grüner Veltliner 2010, W4 = Chardonnay ice wine 2009, W4 = Chardonnay 2010, W5 = Riesling 2010

**Figure 1**

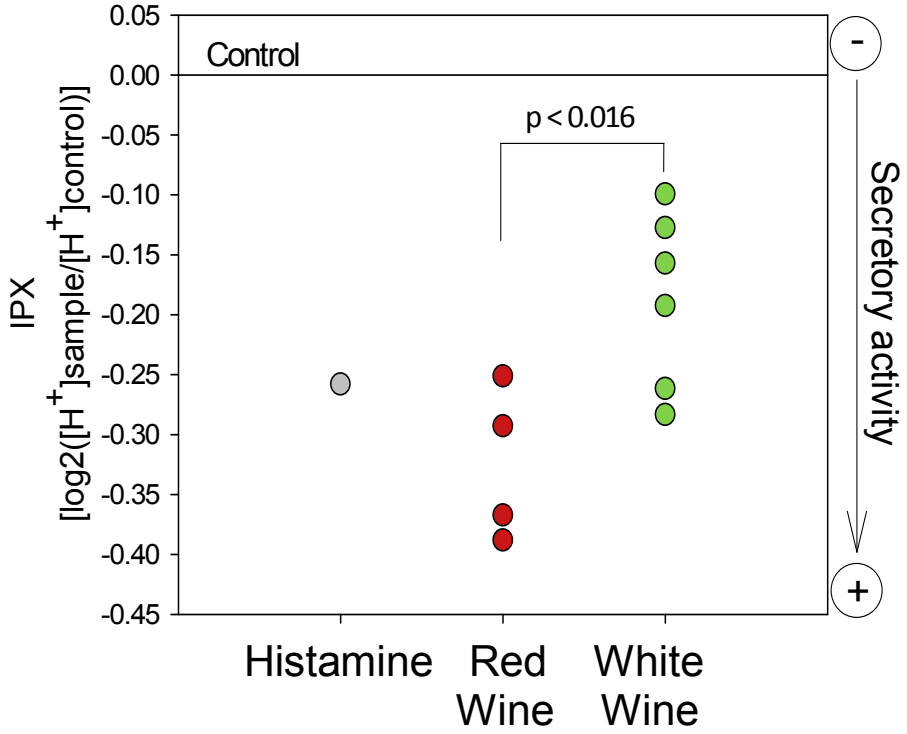

0.05
0.00
-0.05
-0.10
-0.15
-0.20
-0.25
-0.30
-0.35
-0.40
-0.45
Control
p < 0.016
IPX
[log2([H+]sample/[H+]control)]
-
+
Secretory activity
Histamine
Red Wine
White Wine

**Figure 2**

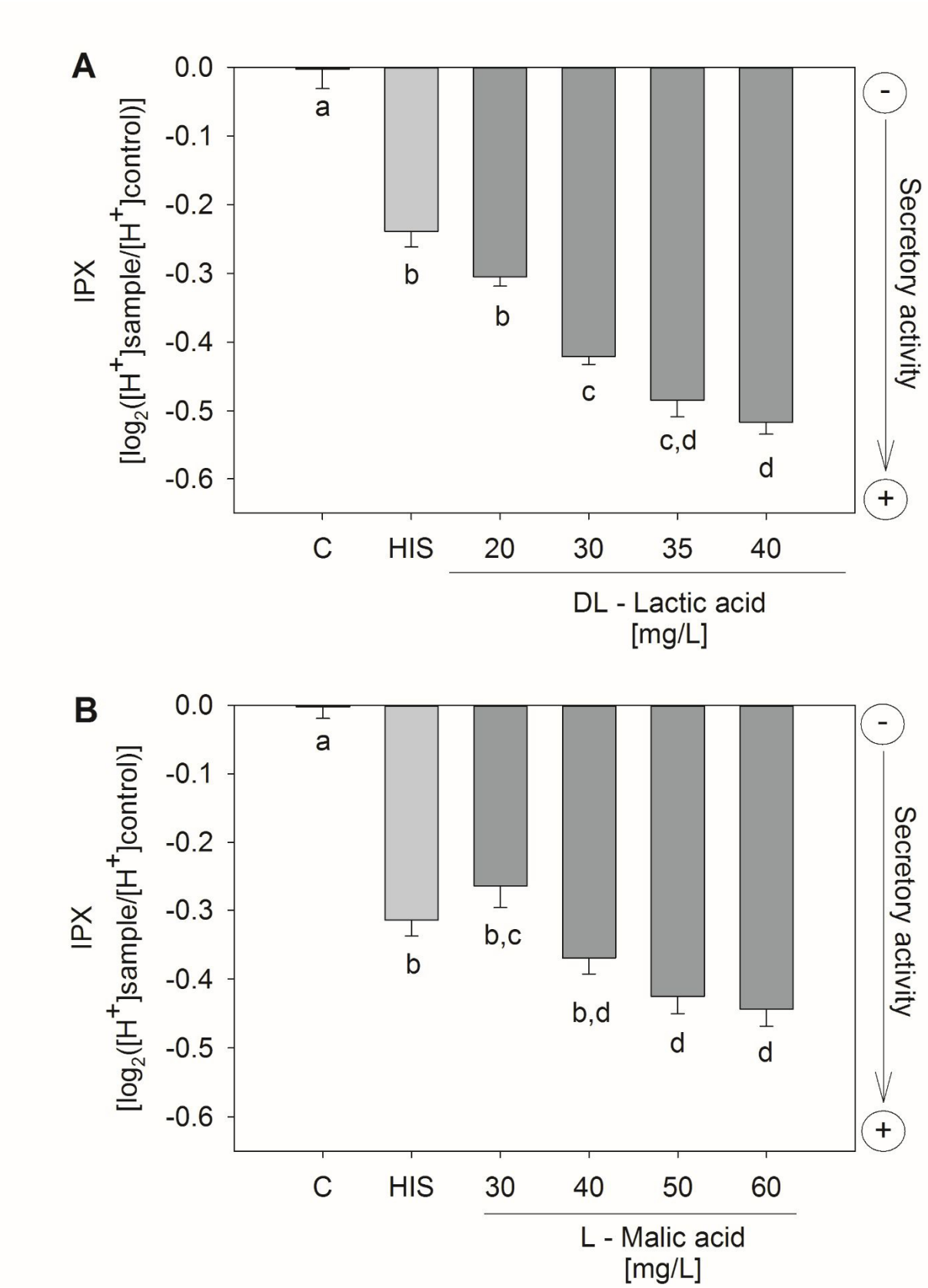

A
IPX
[log2([H+]sample/[H+]control)]
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
a
b
b
c
c,d
d
C
HIS
20
30
35
40
DL - Lactic acid
[mg/L]
-
+
Secretory activity
B
IPX
[log2([H+]sample/[H+]control)]
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
a
b
b,c
b,d
d
d
C
HIS
30
40
50
60
L - Malic acid
[mg/L]
-
+
Secretory activity

**Figure 3**

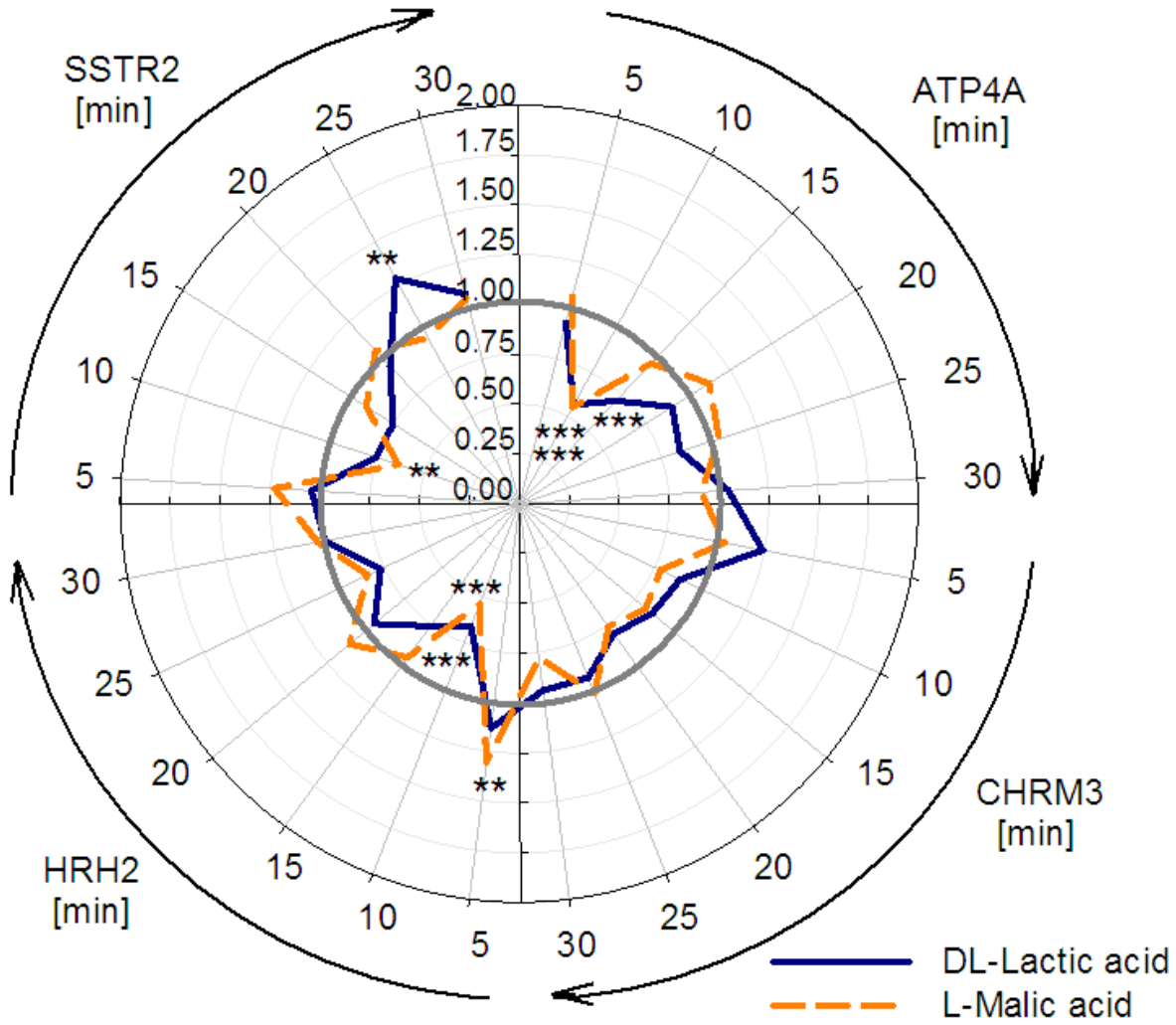

SSTR2
[min]
ATP4A
[min]
CHRM3
[min]
HRH2
[min]
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
DL-Lactic acid
L-Malic acid

**Figure 4**

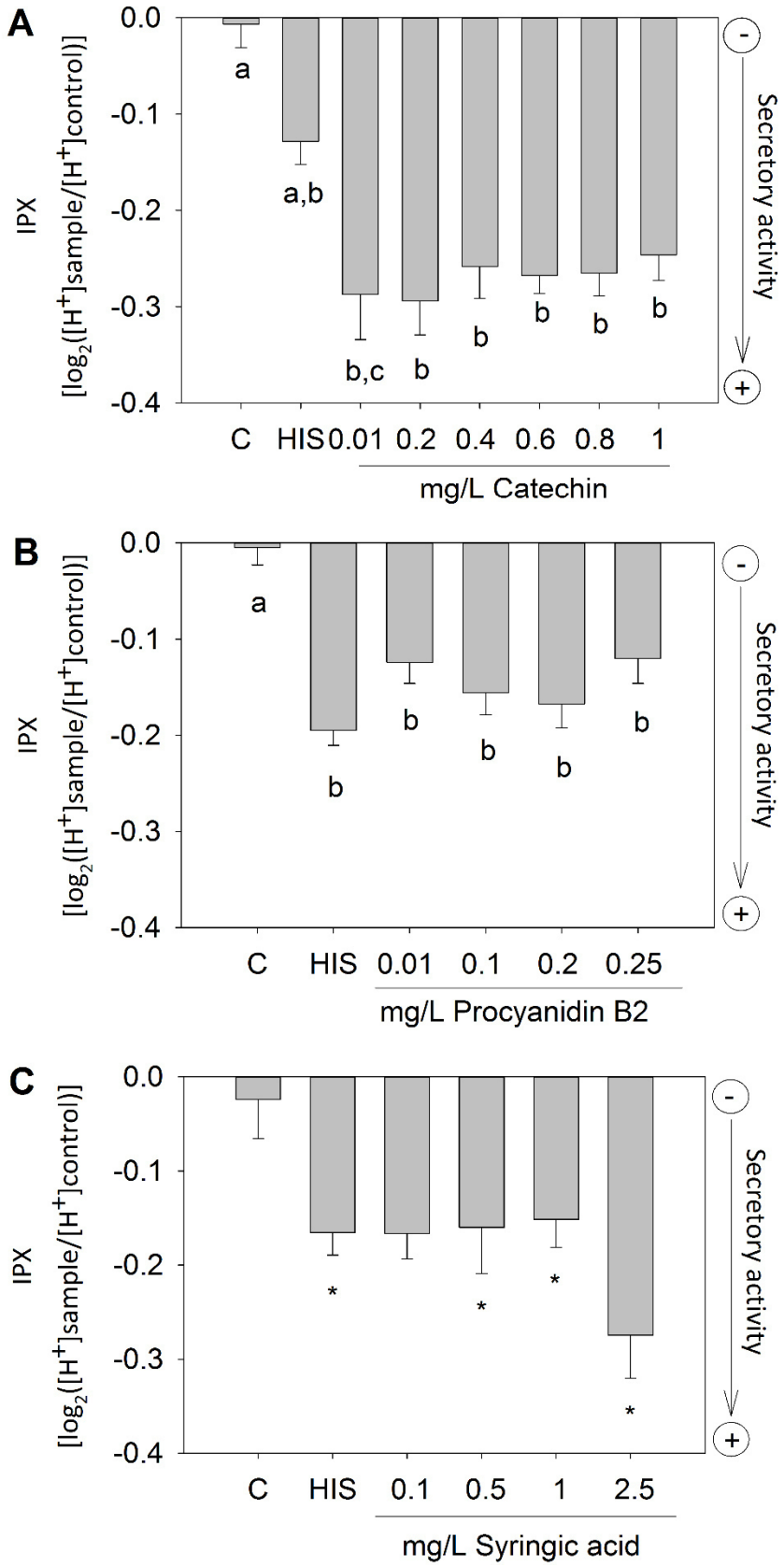
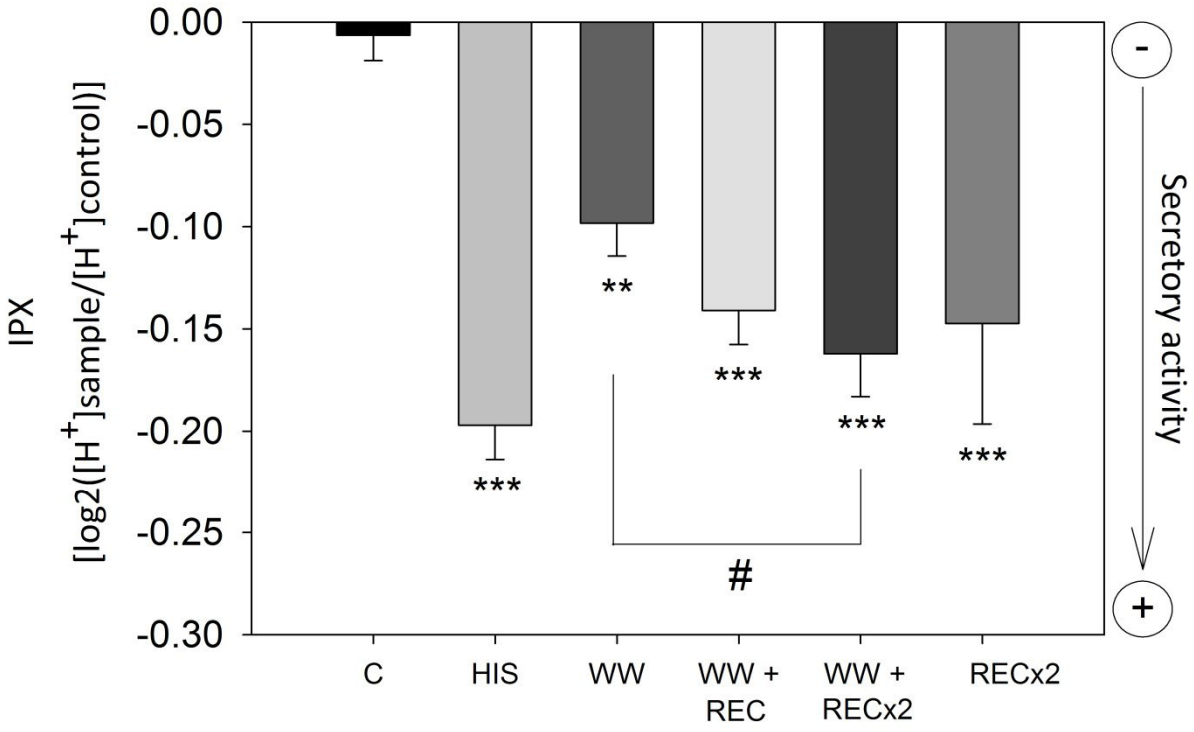

A
IPX
[log2([H+]sample/[H+]control)]
0.0
-0.1
-0.2
-0.3
-0.4
a
a,b
b,c
b
b
b
b
b
Secretory activity
-
+
C
HIS
0.01
0.2
0.4
0.6
0.8
1
mg/L Catechin
B
IPX
[log2([H+]sample/[H+]control)]
0.0
-0.1
-0.2
-0.3
-0.4
a
b
b
b
b
b
Secretory activity
-
+
C
HIS
0.01
0.1
0.2
0.25
mg/L Procyanidin B2
C
IPX
[log2([H+]sample/[H+]control)]
0.0
-0.1
-0.2
-0.3
-0.4
*
*
*
*
Secretory activity
-
+
C
HIS
0.1
0.5
1
2.5
mg/L Syringic acid

**Figure 5**




| | | | | |
|---|---|---|---|---|
| WW (1:100 diluted) | + | + | + | - |
| (+) - Catechin [mg /L] | - | 0.8 | 1.6 | 1.6 |
| Procyanidin B2 [mg/L] | - | 0.2 | 0.4 | 0.4 |
| Syringic acid [mg/L] | - | 0.025 | 0.05 | 0.05 |

## (3) „Identification of beer bitter acids regulating mechanisms of gastric acid secretion“

Jessica Walker,[†,§] Johannes Hell,[†,§] **Kathrin Ingrid Liszt**,[†] Michael Dresel,[‡] Marc Pignitter,[†] Thomas Hofmann,[‡] and Veronika Somoza[*,†]

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In this study, several beer types were tested for their ability to stimulate proton secretion from HGT-1 cells. The effect of non-alcoholic beer was less pronounced compared to the alcoholic varieties. Organic acids and the hop derived bitter acids, *α*-, *β*-, and iso-*α*-acids were shown to be active ingredients in beer regarding proton secretion in HGT-1 cells in beer representative concentrations indicating that HGT-1 cells are sensitive to the bitterness of these compounds.

I participated in the experimental design, in the measurements of proton secretion in HGT-1 cells and qPCR experiments. In addition, I was involved in the interpretation, as well as the discussion of the data and supported the preparation of the manuscript.

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Article

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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-$\alpha$-acid extract (30%) was prepared by preisomerization of a hop extract. Individual iso-$\alpha$-acids were isolated from an iso-$\alpha$-acid extract (30%; Hallertauer Hopfenveredelungsgesellschaft mbH, Mainburg, Germany), $\alpha$-acids and $\beta$-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 ($\alpha$-acids: 3.57 mg/L; $\beta$-acids: 0.081 mg/L; iso-$\alpha$-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.

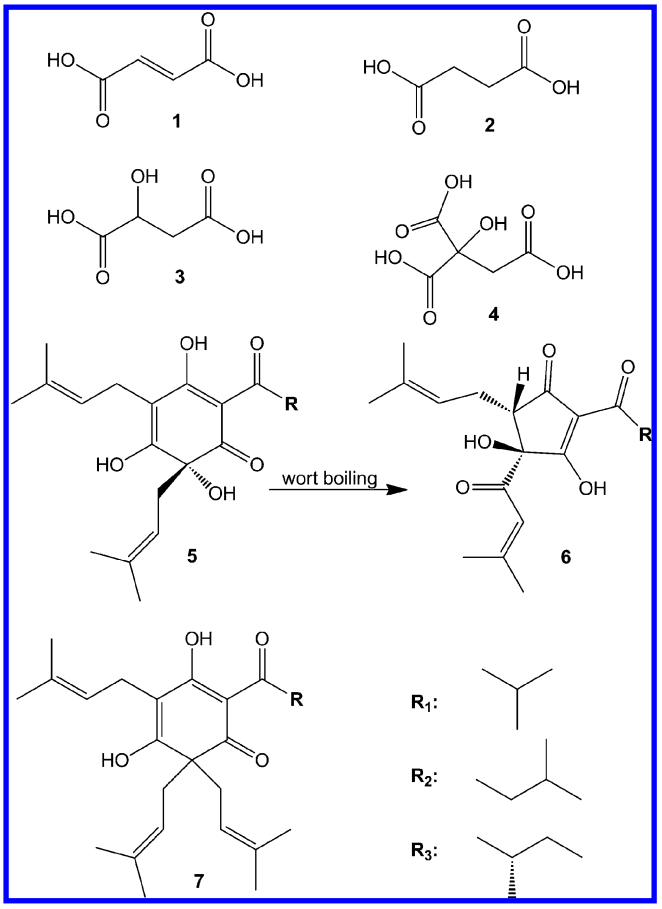


**Figure 1.** Beer constituents tested in this study: maleic acid (**1**), succinic acid (**2**), malic acid (**3**), citric acid (**4**), $\alpha$-bitter acids (**5**), iso-$\alpha$-bitter acids (**6**), and $\beta$-bitter acids (**7**). $\alpha$-Bitter acids: $R_1$, cohumulone-derivatives; $R_2$, humulone-derivatives; $R_3$, adhumulone-derivatives. $\beta$-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 $\mu$M 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 $\mu$M 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 $\mu$L 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 $\mu$L 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 $\mu$m 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 $\mu$m 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 $\mu$m nylon filter (Sigma-Aldrich), and 30 $\mu$L 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 $\mu$L) were degassed by ultrasonification and, after membrane filtration (0.45 $\mu$m, 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 $3.3 \times 10^{-3}$ s. Nitrogen was used as the collision gas ($4 \times 10^{-5}$ 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 $\mu$m 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

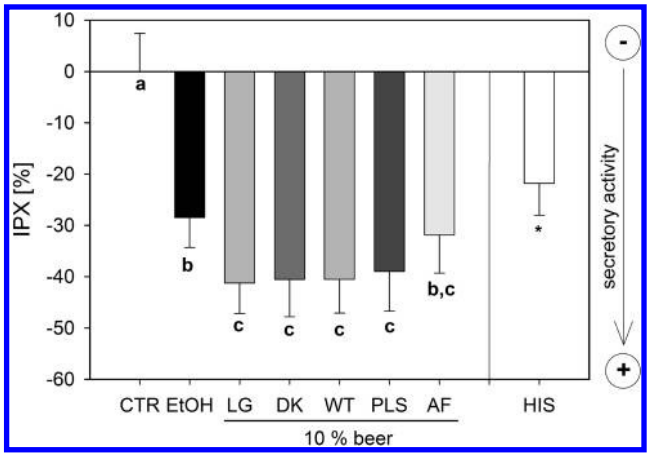


**Figure 2.** Effect of different types of beer on proton secretion of HGT-1 cells. Treatment with a 1:10 dilution of 5.2% ethanol (EtOH), lager (LG), dark beer (DK), wheat beer (WT), pilsener (PLS), and alcohol-free beer (AF) for 10 min. Positive control was histamine (HIS) 1 mM, $^{**}p \leq 0.01$. 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** | | | |
| $\alpha$-acids (mg/L) | 4.812 | 0.057 | 0.005 |
| $\beta$-acids (mg/L) | 0.142 | 0.012 | 0.005 |
| iso-$\alpha$-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 $\alpha$-, $\beta$-, and iso-$\alpha$-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 $\alpha$- and $\beta$-acids, which are the major phytochemicals in hops, are isomerized to give the corresponding iso-$\alpha$-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,

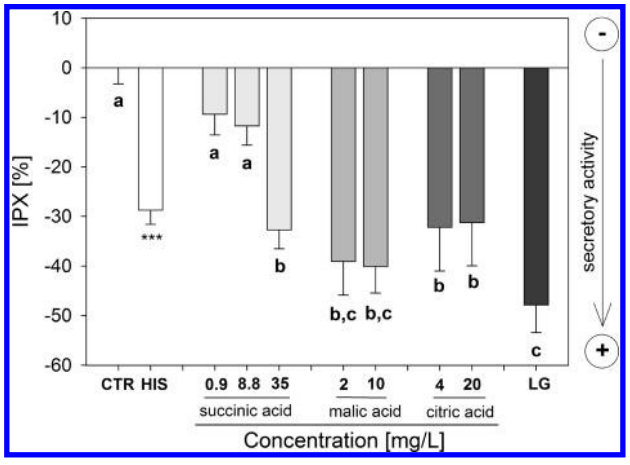


**Figure 3.** Effect of organic acids on proton secretion of HGT-1 cells. Treatment of cells with 1:10 to 1:400 dilutions of succinic acid (350.8 mg/L), malic acid (100 mg/L), and citric acid acid (200 mg/L) compared to a 1:10 dilution of lager beer for 10 min. Positive control was histamine (HIS) 1 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 $\alpha$-, $\beta$-, and iso-$\alpha$-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

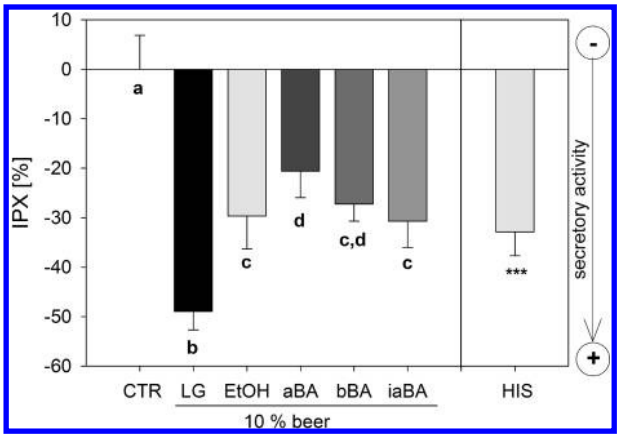


**Figure 4.** Effect of bitter acids on proton secretion of HGT-1 cells. Treatment of cells with a 1:10 dilution (10%) of the $\alpha$-bitter acids (aBA), $\beta$-bitter acids (bBA), and iso-$\alpha$-bitter acids (iaBA) compared to lager (LG) and 5.2% ethanol (EtOH) for 10 min. Positive control was histamine (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 $\alpha$-acids was −20.6%, of the $\beta$-acids −27.2% and of the iso-$\alpha$-acids −30.7%. Ethanol had an effect significantly stronger than that of the $\alpha$-acids ($p \leq 0.05$), but its effect was not different from those of the $\beta$-acids and the iso-$\alpha$-acids. However, the $\alpha$-acids affected gastric acid secretion significantly weaker than that of the $\beta$-acids ($p \leq 0.05$) and the iso-$\alpha$-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 $\beta$-acids led to a comparable IPX, reached with a treatment of the highest concentration tested for citric

acid even though the concentrations of $\beta$-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 $\beta$-acids were applied at a concentration over 40 times lower than that of $\alpha$-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-$\alpha$-acids comprise the greatest contribution followed by the $\beta$-acids, which account for a long-lasting bitterness, whereas the $\alpha$-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, $\alpha$-acids, $\beta$-acids, and iso-$\alpha$-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 ($1.64 \pm 0.70$, $p \leq 0.05$) 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 $1.30 \pm 0.34$ after 15 min ($p \leq 0.05$) and $1.43 \pm 0.13$ 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 ($2.30 \pm 1.77$, $p \leq 0.05$). 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.

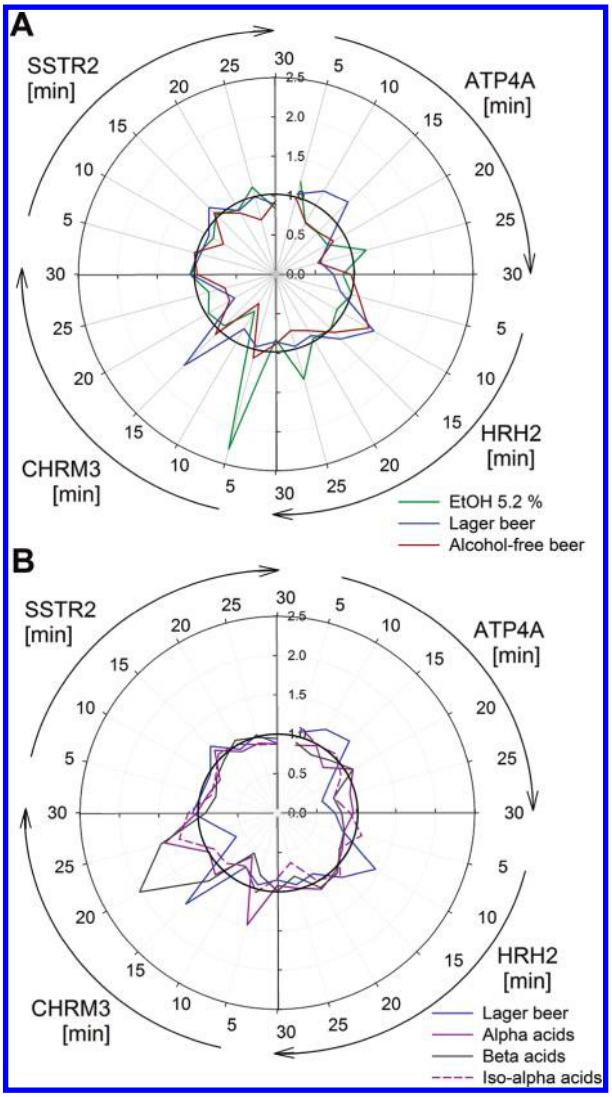


**Figure 5.** Time-dependent effect of a 1:10 dilution of (A) lager, alcohol-free beer, and 5.2% ethanol, and (B) the bitter acid extract, $\alpha$-acids, $\beta$-acids, and iso-$\alpha$-acids on gene expression of *ATP4A*, *HRH2*, *CHRM3*, and *SSTR2* after 5, 10, 15, 20, 25, and 30 min of incubation. 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 ($1.70 \pm 0.68$, $p \leq 0.05$). In contrast to the lager beer, the alcohol-free beer significantly down-regulated the *SSTR2* after 10 min ($0.76 \pm 0.29$, $p \leq 0.01$) and 25 min ($0.71 \pm 0.19$ $p \leq 0.05$). 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 $\beta$-acids ($2.01 \pm 0.81$, $p \leq 0.01$) after 20 min. Furthermore, all types of bitter acids decreased the expression levels of the antisecretory gene *SSTR2* with a maximum effect of $0.81 \pm 0.11$ ($p \leq 0.001$) 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: $\alpha$-acids, iso-$\alpha$-acids, and $\beta$-acids. Again, the $\alpha$-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 $\alpha$-acids, $\beta$-acids, and iso-$\alpha$-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 $\alpha$-, $\beta$-, and iso-$\alpha$-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

The authors declare no competing financial interest.

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## ABBREVIATIONS USED

ATP4A, $H^+/K^+$-ATPase $\alpha$-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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## (4) "Bitter taste receptors in the stomach regulate gastric acid secretion"

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This study investigated the role of oral and gastric bitter taste receptors in the regulation of gastric acid secretion. The expression of 22 out of 25 TAS2Rs (bitter taste receptors) in human was revealed in HGT-1 cells. In addition, the protein expression of TAS2R10 and the related G protein transudcin (GNAT2) in human gastric biopsies and in the HGT-1 cell line were demonstrated. Furthermore, stimulation of the proton secretion in HGT-1 cells by several bitter compounds, including caffeine and theobromine, was shown. Interestingly, the bitter-masking compounds homoeriodictyol and eriodictyol reduced the pro-secretory effect of the bitter substances caffeine and theobromine. Using siRNA targeted against *TAS2R10*, the involvement of *TAS2R10* in the pro-secretory effect of caffeine in HGT-1 cells was proven. A human intervention trial using the Heidelberg-capsule detection system demonstrated that caffeine reduces gastric acid secretion only when oral bitter taste receptors are activated. In

contrast, activation of gastric bitter taste receptors bypassing activation of oral bitter taste receptors stimulated gastric acid secretion time dependently.

I participated in planning the overall study design. Regarding the *in vitro* part of the manuscript, I performed gene expression analysis of the 25 bitter taste receptors and genes encoding the signaling proteins. In addition, I did the immunocytostaining analysis under supervision of Dr. Maik Behrens in HGT-1 cells. Furthermore, I was involved in the proton secretion experiments and performed the siRNA knockdown experiments. I planned and organized the human intervention study. Furthermore, I organized and performed the human sensory trial in cooperation with the Symrise AG. Finally, I did statistical analysis, data interpretation and prepared the manuscript.

**LETTER**

# Bitter taste receptors in the stomach regulate gastric acid secretion

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**The TAS2R family of receptors, when expressed in oral taste buds, is responsible for bitter taste perception[1], but has also been identified in non-gustatory tissues, including airway epithelia[2], intestinal cells[3,4] of humans and rodents, and in the gastric epithelia of rats and mice[5,6]. The TAS2R pathway in the gut is involved in the regulation of food intake, digestion, and satiation[3,4,7,8]. We show that TAS2Rs are expressed in human gastric epithelial cells of the corpus/fundus, and in human gastric tumour cells (HGT-1), a cell line representative of parietal cells. Furthermore, we show that bitter compounds stimulate proton secretion from HGT-1 cells, as well as in the stomach, with the involvement of *TAS2R10*. Activation of gastric TAS2Rs stimulate acid secretion, whereas activation of oral bitter taste receptors slows gastric acid secretion. Homoeriodictyol (HED)[9], known to mask caffeine bitterness, reduces the proton secretory effect of caffeine in HGT-1 cells as well as the caffeine-promoted gastric acid secretion in humans. Sensory analyses demonstrate reduced bitterness perception of caffeine in the presence of HED in correlation with its effects on gastric acid secretion. These findings support the hypothesis that bitter taste receptors in the stomach are involved in the regulation of gastric acid secretion and indicate that bitter tastants and bitter masking compounds could be used therapeutically to regulate gastric pH.**

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Gastric acid secretion, necessary for the digestion of food, absorption of iron, calcium and vitamin B12 and chemical barrier against pathogens[10], contributes to gastric discomfort and is the major target in the treatment of gastroesophageal reflux disease (GERD) or peptic ulcer disease[11]. Caffeine is generally thought to stimulate gastric acid secretion. This assumption has derived from studies where caffeine was administered directly into the stomach[12,13], or given intravenously[14] or intramuscularly[15]. While bitter substances are associated with toxicity, several are common and deliberate components of the human diet, e.g., caffeine, theobromine, iso-alpha acids and phenolic compounds. Of these, caffeine is particularly relevant because it is widely consumed, activates TAS2Rs 7, 10, 14, 43 and 46[16], and can be administered to volunteers in relatively high doses without toxicity concerns. Furthermore, caffeine is unique in that two tasteless flavanones from yerba santa, homoeriodictyol (HED) and its structural relative eriodictyol (ED), effectively mask its bitterness[9], allowing it to be modulated independently of its other physiological effects. To examine if TAS2Rs are involved in the regulation of gastric acid secretion, we first determined the mRNA expression of 25 human *TAS2Rs* in the HGT-1 cell line using RT-qPCR. The genes for the five *TAS2R*s known to be activated by caffeine, TAS2R 7, 10, 14, 43 and 46[16], as well as that of the other *TAS2R* genes, are expressed at similar or even higher levels than the M3 acetylcholine receptor *CHRM3* genes, one of the main regulators of gastric acid secretion (**Supplementary Table 1**). While *TAS2R5* and *TAS2R14* are the most highly expressed TAS2Rs, *TAS2R 8, 45, and 60* mRNAs were not found in HGT-1 cells. We also found that HGT-1 cells express mRNAs for TAS2R downstream signaling proteins PLCß2, transducin (GNAT2) and gustducin (GNAT3) (**Supplementary Table 1**). Like the parietal cell line HGT-1, the human gastric epithelium contains transcripts for the cognate receptors for caffeine *TAS2R* 7, 10, 14, 43 and 46[16] at levels similar to those of the M3 acetylcholine receptor

(**Supplementary Table 2**). Presence of the broadly tuned TAS2R10[17] in the gastric epithelium was confirmed by immunohistochemical staining of stomach surgical specimens from the antrum and fundus/corpus region. The specificity of the TAS2R10 antibody was verified in transiently transfected HEK239 cells (**Supplementary Figure 1**). Localization of TAS2R10 staining was confined to parietal cells and to gastric chief cells in the fundus/corpus showing strong cytoplasmic granular reactivity (**Fig. 1a, b**). Gastric antrum staining in glandular cells was faint, consisting of very weak cytoplasmic and focal intermediate membranous reaction (**Fig. 1e, f**). In contrast, mucus-producing foveolar cells in the fundus/corpus (**Fig. 1a, b**) and antrum (**Fig. 1e, f**) did not show expression of TAS2R10. Blocking experiments showed a clear staining reduction (**Fig. 1c, d, g, h**). GNAT2 was localized in parietal and chief cells in the fundus/corpus and in the membranes of foveolar cells (**Fig. 1i, j**). In the gastric antrum, TAS2R10 is present on the membranes of glandular cells, (**Fig. 1m, n**) but not in foveolar cells. TAS2R10 and GNAT2 expression was also detected in HGT-1 cells (**Fig. 1q**).

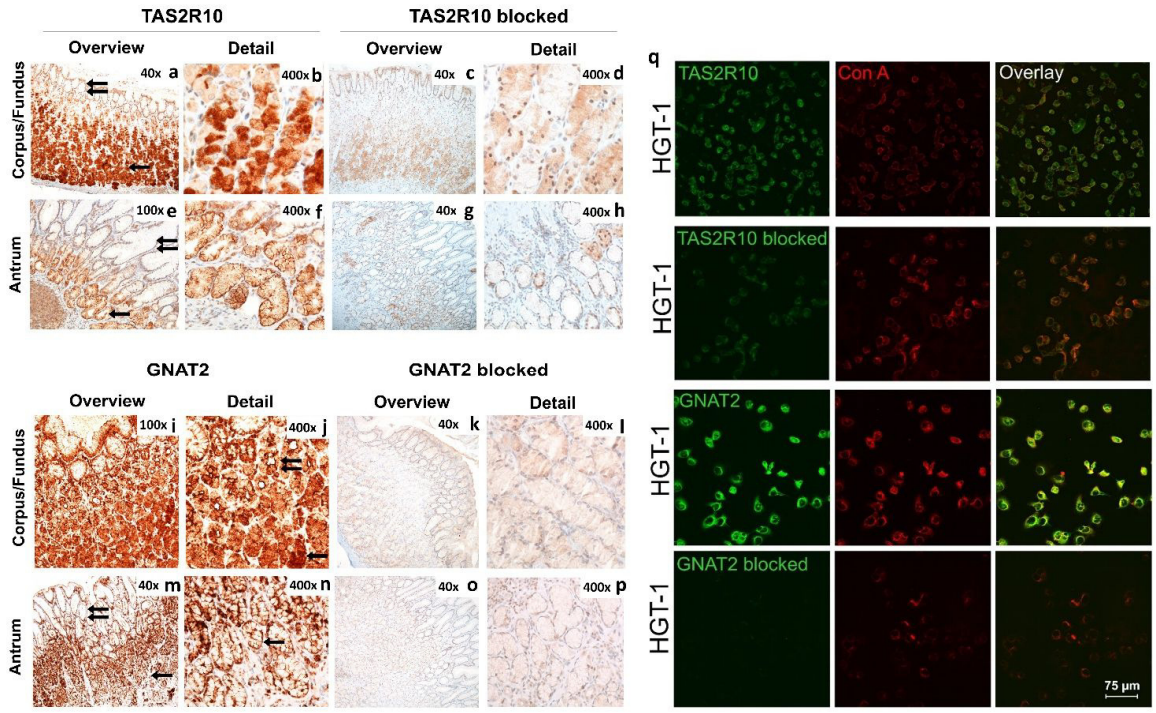


**Figure 1.** (**a–p**) **Immunochemical localization of TAS2R10 and GNAT2** in gastric tissue and (**q**) HGT-1 cells with and without preincubation with a blocking peptide. (**a**) In the gastric corpus/fundus, cytoplasmic reactivity of TAS2R10 in parietal and chief cells (one arrow) was detected while foveolar cells were negative (two arrows). Detail (**b**) shows parietal and chief cells. In the gastric antrum (**e, f**) very faint cytoplasmic and focal membranous reactivity of TAS2R10 in glandular cells was detected (one arrow). Foveolar cells are negative (two arrows). (**f**) Detail showing glandular cells. GNAT2 was localized in gastric fundus (**i,j**) parietal and chief cells (one arrow, **j**). Foveolar cells demonstrate membranous staining (two arrows, **j**). (**m,n**) in gastric antrum membranous reactivity of GNAT2 in glandular cells (one arrow, **m,n**) was detected while foveolar cells were negative (two arrows, **m**). (**c,d,g,h,k,l,o,p**) showing the corresponding negative controls. (**q**) Staining of HGT-1 cells with TAS2R10 and GNAT2 antisera (green) with and without specific blocking peptide and cell surface labeling with concanavalin A (Con A, red).

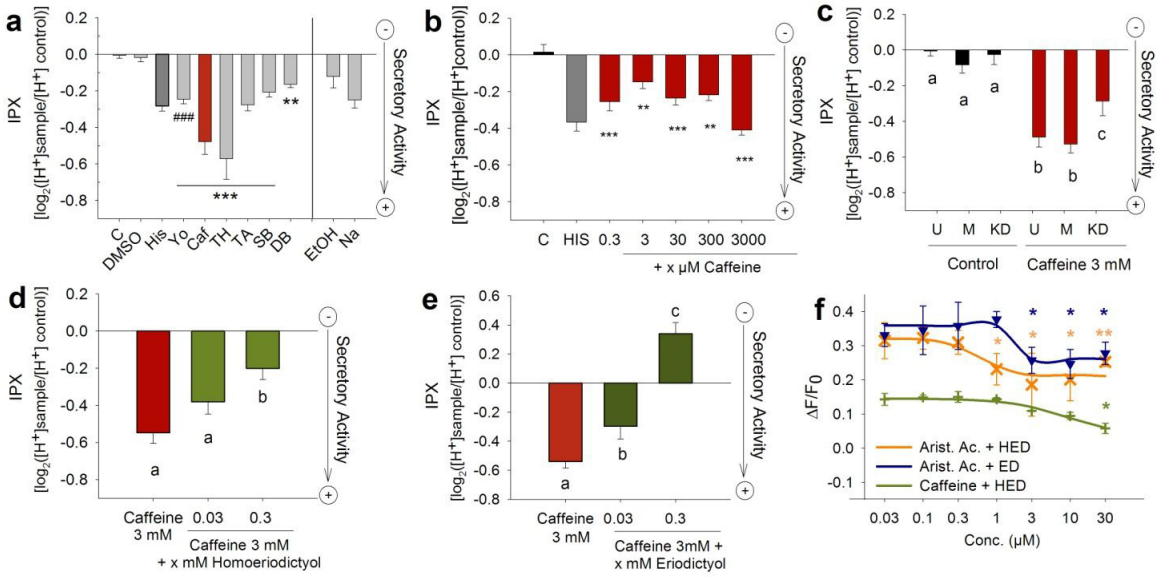


**Figure 2**. **Bitter tastants evoke increases in proton secretion in human gastric tumour cells.** Studies were performed with cultured HGT-1 cells loaded with the pH sensitive fluorescent dye SNARF-1 AM and treated with test compounds for 10 min (**a-d**). Results are presented as the intracellular proton index (IPX). A lower IPX value indicates increased proton secretion. Data displayed as mean IPX±SEM. (**a**) IPX of HGT-1 cells after treatment with histamine (His, 1 mM), yohimbine (Yo, 30 µM), denatonium benzoate (DB, 30 µM), caffeine (Caf, 3.0 mM), theobromine (TH, 0.3 mM), tannic acid (TA, 3 µM) and sodium benzoate (SB, 3.0 mM) in comparison to untreated cells (Control, C) or 0.1% DMSO treated cells (DMSO, solvent control for Yohimbine). Naringin (Na, 30 µM) was compared to its solvent control ethanol (EtOH, 1%) (*n*=3-16, *tr*=6,). (**b**) Caffeine stimulates proton secretion (*n*=5, *tr*=6). (**c**) IPX of HGT-1 cells treated with caffeine (3.0 mM) in comparison to untreated cells (U), mock transfected cells (M), siRNA targeted against TAS2R10 transfected cells (KD) (*n*=3, *tr*=6). Co-administration of (**d**) homoeriodictyol (HED) and (**e**) eriodictyol (ED) reduces the stimulating effect of caffeine on proton secretion (*n*=4-37, *tr*=6). (**f**) Inhibition curves of TAS2R43 assessed through calcium imaging experiments[16] in transfected HEK293T cells. Cells were costimulated with 0.03 µM aristolochic acid (Arist. Ac.) and increasing concentrations of the inhibitors HED and ED. Cells were also costimulated with caffeine 1 mM and increasing concentrations of HED. Caffeine and aristolochic response amplitudes were $\Delta F/F_0$ 0.14 and 0.39, respectively, data: mean±SD. (**a**,**b**,**c**,**d**) data: mean±SEM. (**a**,**b**) statistics: one-way ANOVA with Holm-Sidak *post hoc* test. (**a**,**f**) statistics: Student's *t*-test. ***, *p*<0.001 or **, *p*<0.01, * *p*<0.05 *vs.* control. (**a**) statistics: ###, *p*<0.001 *vs*. DMSO 0.1%. (**c,d,e**) one-way ANOVA with Holm-Sidak *post hoc* test significant (*p*<0.05) differences are indicated by letters.

Since iso-alpha acids in beer[18] and caffeine in coffee[19] are potent stimulants of gastric acid secretion, we tested additional bitter compounds: theobromine, tannic acid, yohimbine, denatonium benzoate and sodium benzoate. These compounds increased proton secretion in HGT-1 cells (**Fig. 2a**). The responses were similar, or even more pronounced, compared to treatment with histamine, a major activator of proton secretion in parietal cells[10]. The bitter substance naringin, which is not a ligand for TAS2Rs[16], did not significantly stimulate proton secretion compared to the solvent control (1% EtOH) (**Fig. 2a**). The concentration-dependent effect of caffeine on proton secretion in HGT-1 cells is shown in **Fig. 2b.** HED and ED which reduce the bitter taste of caffeine in human sensory panels[9,20], also reduced proton secretion in HGT-1 cells exposed to bitter compounds, indicating the involvement of bitter taste receptors (**Fig. 2d, e**). Theobromine, an analog of caffeine with comparable bitterness, also promotes mechanisms of gastric acid secretion in HGT-1 cells, and the secretion is similarly inhibited by HED and ED (**Supplementary Fig. 2**). To determine whether TAS2R10 is involved in the mechanism of caffeine-promoted acid secretion, HGT-1 cells were transfected with siRNA targeted against *TAS2R10*. mRNA levels of *TAS2R10* decreased by 25 ± 2.1%, compared to untreated cells. The stimulating effect of caffeine on proton secretion in HGT-1 cells was reduced in *TAS2R10* knockdown cells (**Fig. 2c**) compared to untreated and mock transfected cells. HED is a known agonist for TAS2R14 and TAS2R39[21], and may also function as an antagonist for some caffeine-sensitive TAS2Rs. To determine if TAS2R43, which is highly expressed in HGT-1 cells and has been linked to coffee liking[22], is also involved, we performed siRNA knockdown of *TAS2R43* in HGT-1 cells. However, since the mock transfection protocol significantly reduced *TAS2R43* expression in this cell line (**Supplementary Fig. 3**), *TAS2R43* was instead transiently transfected into HEK293T cells, which do not normally express any TAS2Rs. TAS2R43 in these cells was then stimulated with

the strong activator aristolochic acid[23] or with caffeine[16], in calcium imaging experiments at increasing concentrations (0.03−30 µM) of HED and ED. Both compounds reduced TAS2R43 response to aristolochic acid, but the inhibition by HED was statistically significant at 1 µM, *vs*. 3 µM for ED (**Fig. 2f**). Therefore, HED is optimal to demonstrate the involvement of TAS2R43 on bitter-induced proton secretion.

To verify that caffeine bitterness response in the stomach regulates gastric acid secretion, a human intervention study was performed using the Heidelberg pH diagnostic system[24-27]. Heidelberg capsules, swallowed by volunteers, measured gastric pH in real time[24-27]. Since pH in the fasted stomach is very low, it was challenged with 5 mL of saturated $NaHCO_3$, and reacidification time was measured for three distinct TAS2Rs activation protocols. Each protocol ensured different activation sites for TAS2Rs (**Fig. 3a**): (1) a dose of 150 mg of encapsulated caffeine was swallowed along with 125 mL water to stimulate gastric receptors while bypassing oral receptors, (2) volunteers drank a caffeine solution (150 mg caffeine in 125 mL water) stimulating both oral and gastric receptors, and (3) sip-and-spit, where only oral taste receptors were stimulated by the caffeine solution followed by swallowing of 125 mL water. Oral taste receptor stimulation, both by sip-and-spit or by drinking, led to a prolonged ($p<0.05$) reacidification time, indicating an inhibition of gastric acid secretion. Stimulation of oral taste receptors only (sip-and-spit) resulted in the longest reacidification time (**Fig. 3b**). The gastrograms were quantified by determining the slope after the onset of reacidification. A higher slope indicates that, once reacidification has started, the gastric pH returns to its initial pH faster. The slope of the gastrogram from encapsulated caffeine administration was much higher compared to that of the sip-and-spit and drinking gastrograms, where an oral stimulation of receptors was allowed (**Fig. 3c**). Since previous research on the effect of caffeine on gastric acid secretion used gavage to bypass oral cavity receptors[12,13,15], it has been

thought ever since that caffeine promotes acid secretion and acid-related diseases, in spite of epidemiological data to the contrary[28]. Our results demonstrate that, while caffeine-sensitive TAS2Rs in the stomach stimulate gastric acid secretion, oral receptors counteract this effect. In the reacidification time window captured in this experiment, encapsulated caffeine appears to weakly influence stomach reacidification relative to the control. This is in accordance with previous studies showing that caffeine administered directly into the stomach stimulates acid production following only after ~30 min[12,14]. In order to access this later time period, we repeated the Heidelberg experiments with encapsulated caffeine administered with 125 mL water 25 min before the alkaline solution was swallowed instead of 5 min after the alkaline solution. This allows the effect of caffeine on acid secretion to be observed over the time period from 25 to about 55 minutes after caffeine administration (**Fig. 3e**). With this delivery, caffeine greatly reduced reacidification time compared to the control (empty capsule plus 125 mL water), demonstrating a strong stimulation of gastric acid secretion.

To confirm that bitter taste receptors in the human stomach are directly involved in regulating gastric acid secretion, 30 mg of the bitter masker HED were administered with the caffeine. Again, each protocol ensured different activation sites for TAS2Rs: (i) 30 mg HED and 150 mg caffeine in 125 mL water drunk by straw 5 min after challenging the gastric pH (oral and gastric activation), and (ii) 30 mg HED and 150 mg caffeine were encapsulated and administered with 125 mL water 25 min before challenging the gastric pH (gastric activation). In both cases, HED largely eliminated the effect of caffeine on reacidification time (**Fig. 3d, e**). Thus, HED acts as an antagonist for TAS2Rs in both the mouth and the stomach. HED itself (21 ± 2 min, $n$=8) showed no influence on reacidification time in comparison to the water control (24 ± 1 min, $n$=10) when administered by drinking by volunteers.

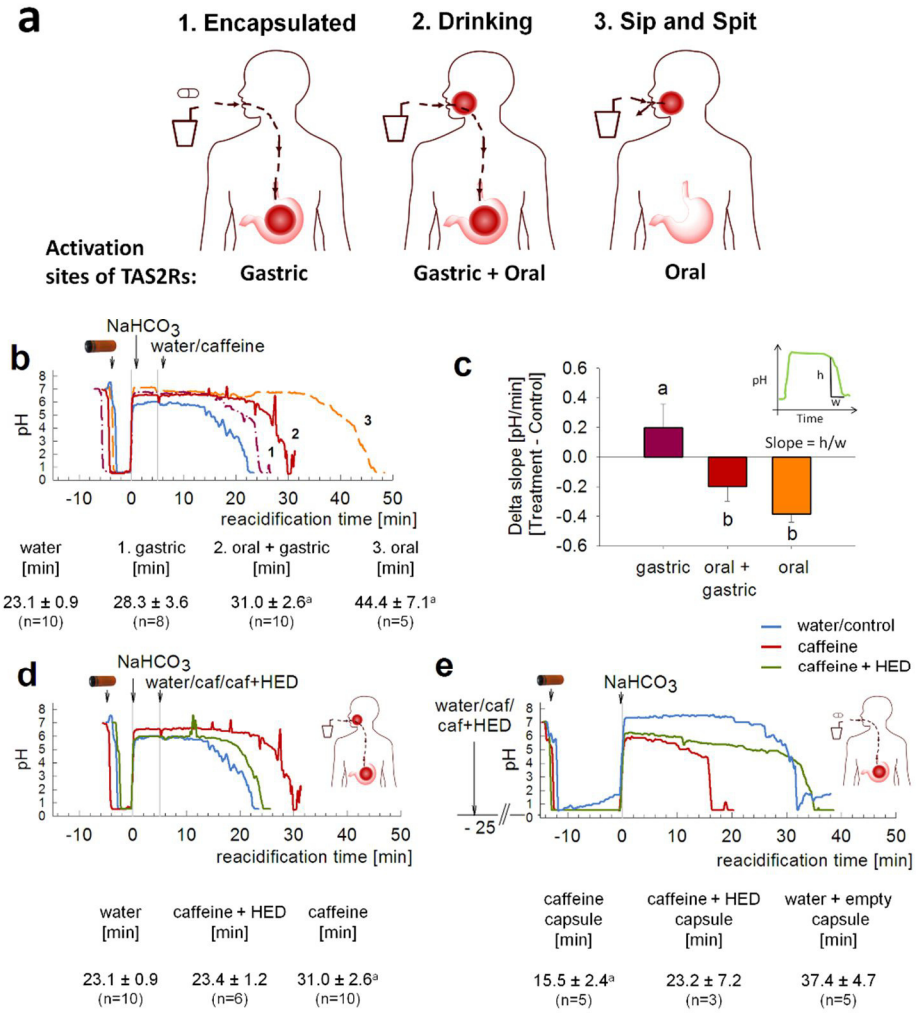


**Figure 3. Gastric pH measurements in human subjects using the Heidelberg detection system.** (**a**) Overview of the different administration types in the human intervention trial. (**b,e,d**) Gastrograms of different Heidelberg capsule measurements from one test subject combined in one graphic showing the time until the original pH is reached again (=reacidification time). At 0 min, the gastric pH was raised to about 6 by administration of 5 mL of saturated $NaHCO_3$. (**b,d**) Administration of test solutions or capsules were 5 min after or (**e**) 25 min before administration of the 5 mL $NaHCO_3$ solution. Prolonged reacidification time indicates an inhibiting effect on gastric acid secretion. (**b**) Either 125 mL water or 150 mg caffeine/125 mL water were administered by three different types of application to allow activation of bitter taste receptors on different activation sites. (**c**) Delta slope of the gastrograms. (**d**) Addition of 30 mg HED to 150 mg caffeine/125 mL water abolished the prolonging effect of 150 mg caffeine/125 mL water on reacidification time when administered by drinking. (**e**) Addition of 30 mg HED to 150 mg caffeine abolished the reducing effect of 150 mg caffeine on reacidification time when administered by swallowing the substances encapsulated with 125 mL water. Data is displayed as mean±SEM, Statistics: (**b**) Mann–Whitney *U* test or Student's *t*-test treatment *vs*. control (water). **, $p<0.01$, * $p<0.05$ *vs.* control (**c**,**d**,**e**) one-way ANOVA Holm-Sidak *post hoc* test, significant ($p < 0.05$) differences are indicated by distinct letters. The study has been registered at clinicaltrials.gov, registration number NCT02372188.

The same sensorically untrained test subjects who underwent the gastric pH measurements, rated the reduction of bitter sensation when 240 mg/L HED was added to 1200 mg/L caffeine in a blinded sensory test, identifying a significant reduction ($p$<0.01) of bitterness (-16±4% *vs*. caffeine alone, **Supplementary Fig. 4b**). Sensorically trained volunteers identified a bitter taste reduction of 43% in a similar experiment[9] using lower concentrations of caffeine and HED[9]. The caffeine bitterness sensed by our test subjects correlated with the effect of caffeine administered by drinking (oral+gastric stimulation of TAS2Rs) on reacidification time (correlation coefficient: 0.70, $p$=0.03, $n$=9) (**Supplementary Fig. 4c**). Their bitter recognition threshold was 119 ± 45 mg/L caffeine. One subject, unable to taste caffeine bitterness below 1200 mg/L, also did not exhibit changes in gastric acid secretion when administered caffeine, either by drinking or encapsulated.

Our findings indicate that activation of TAS2Rs, expressed in the stomach, results in the stimulation of gastric acid secretion while activation of oral TAS2R receptors suppresses gastric acid secretion. **Figure 4** outlines the proposed mechanism by which TAS2Rs on the tongue and in the stomach regulate gastric acid secretion. Bitter perception on the tongue results in brain-mediated regulation of gastric acid secretion, while bitter receptors in the fundus and corpus of the stomach likely regulate gastric acid secretion via local pathways in parietal cells. Activation of cell surface receptors by histamine or acetylcholine stimulates HCl secretion in HGT-1 cells, and TAS2Rs expressed in parietal cells may co-regulate the same intracellular signaling pathways without the involvement of the nervous system.

This suggests that bitter tastants and bitter masking compounds could be used therapeutically to regulate gastric pH, to make bitter medications more palatable, and to prevent bitter medications from triggering gastric acid secretion. Our results strengthen the hypothesis that taste receptors are widely expressed throughout the

body and are functional beyond the oral cavity. Accumulating molecular evidence indicates that tastants are broadly active in physiology and pathophysiology, with a particular role beyond nutrient sensing.

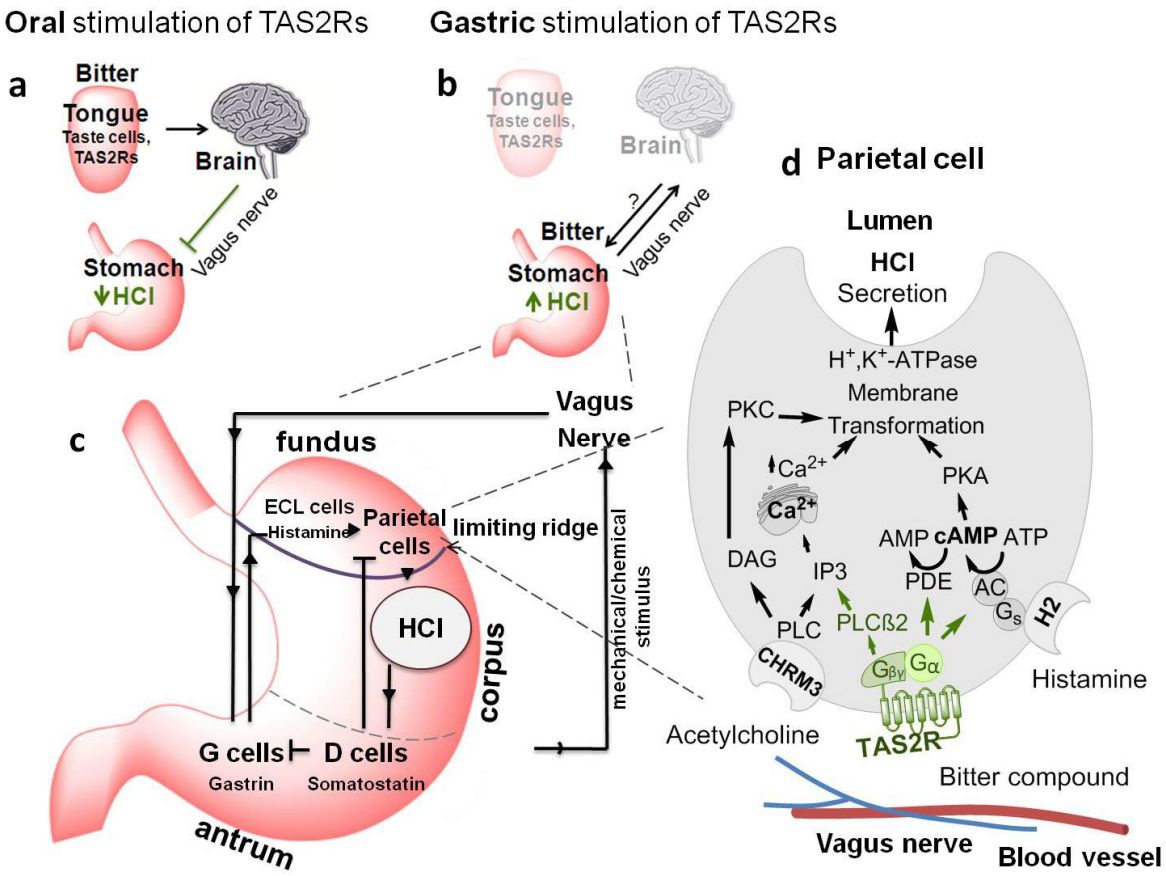


**Figure 4. Schematic representation of the apparent mechanism of regulation of gastric acid secretion by bitter receptors on the tongue and the stomach.** Additions to established pathways are indicated in green (**a**) Bitter perception on the tongue leads to an inhibition of brain (cephalic)-mediated gastric acid secretion. (**b**) Bitter receptors in the stomach may lead to a non-cephalic stimulation of gastric acid secretion. (**c**) Parietal cells in the fundus and corpus of the stomach produce HCl. Multiple regulation pathways are possible, e.g.: (i) gastrin produced in G cells in the antrum are transported in the blood to ECL cells, which stimulates histamine production and thus gastric acid secretion, (ii) somatostatin produced in D cells leads to an inhibition of gastric acid secretion. (**d**) Schematic HCl secretion from parietal cells. Histamine binding to the histaminic (H2) receptor leads to the activation of adenylate cyclase (AC) which catalyzes ATP conversion to cAMP and further activates cAMP dependent protein kinase (PKA). Phosphodiesterase (PDE) breaks down cAMP to AMP. Binding of acetylcholine to the cholinergic (M3) receptor activates the phospholipase C, which leads to the production of insositol triphosphate (IP3) and diacylglycerol (DAG). IP3 activates release of $Ca^{2+}$ from membrane stores. Release of PKA and further downstream signals by DAG and $Ca^{2+}$ activates recruitment of $H^+$,$K^+$-ATPase into the apical membrane for active HCl secretion[29]. Activation of TAS2Rs in parietal cells leads to the dissociation of heterotrimeric G proteins, which are known to activate the two major signal transduction pathways cAMP and PLCß2[30].

## Methods

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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## Author Contributions:

K.I.L., J.L., V.So., V.S., B.L., C.M.H., M.B., A.M., A.R., J.H., W.M, S.W., G.K. designed the experiments, provided technical expertise and performed data analysis. K.I.L., E.K. performed measurements in HGT-1 cells. M.B., A.M. did experiments in HEK-cells. K.I.L., J.L., B.L., V.S., C.M.H., V.S. conducted the human intervention study. A.R. performed immunohistological staining. K.I.L., M.M.S., V.So. wrote the manuscript. All authors edited the manuscript and provided comments.

## Conflict in Interest

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**Supplemental Material**

# Bitter taste receptors in the stomach regulate gastric acid secretion

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## Methods

### Chemicals

The sodium salt of Homoeriodictyol (3`-methoxy-4`,5,7-trihydroxyflavanone) was used in all experiments and were provided by the company Symrise (Holzminden, Germany). All other chemicals were obtained from Sigma Aldrich.

### HGT-1 cell culture

The human gastric tumor cell line HGT-1 was obtained from Dr. C. Laboisse, Laboratory of Pathological Anatomy, Nantes, France. Cells were cultured under standard conditions at 37°C, 95% humidity, and 5% $CO_2$ in DMEM with 4 g/L glucose, 10% fetal bovine serum, 2% L-glutamine, and 1% penicillin/streptomycin. When 90% confluence was reached, cells were passaged or harvested using trypsin/EDTA. Cytotoxicity of the tested substances and treatment reagents was excluded by analyzing cell viability using trypan blue staining. Tested cells had at least 90% cell viability.

### Intracellular pH measurement in HGT-1 cells indicating proton secretion

Intracellular pH, as indicator for proton secretion in HGT-1 cells, was measured using the pH-sensitive fluorescence dye 1,5 carboxy-seminaphto-rhodafluor

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acetoxymethylester (SNARF-1-AM, Life Technologies). A total of 100 000 HGT-1 cells were seeded into a black 96-well plate and allowed to settle for 24 h at 37°C, 95% humidity, and 5% $CO_2$. Cells were washed once with Krebs-Ringer-HEPES buffer (KRHB), and incubated with the fluorescence dye SNARF-1-AM at a concentration of 3µM for 30 min. Afterward, cells were washed twice with KRHB and treated with 100 µL of the test substances in different concentrations diluted in phenol red-free media for 10 min or over a time period of 30 min. As positive control, the cells were treated with 1 mM histamine. After 10 min treatments with the test solutions, fluorescence was analyzed on an Infinite 200 Pro plate reader (Tecan, Switzerland) using an excitation wavelength of 488 nm and emission wavelengths of 580 nm and 640 nm. The intracellular pH was determined by analyzing the ratio of the fluorescence intensities and plotting them on a calibration curve. For each experiment, a calibration curve was generated by treating the cells in potassium buffer solutions of varying pH values, ranging from 7.2 to 8.2 adjusted with NaOH, using a pH-meter pH 211 (HANNA Instruments), in the presence of 2 µM nigericin to equilibrate intracellular and extracellular pH. The potassium buffer consisted of 20 mM NaCl, 110 mM KCl, 1 mM $CaCl_2$ , 1 mM $MgSO_4$, 18mM D-glucose, and 20 mM HEPES. Intracellular proton concentration was calculated from the pH. The intracellular proton index (IPX) in the cell was calculated by $\log_2$ transformation of the ratio between treated and untreated (control) cells. The lower the IPX, the fewer protons are in the cell, indicating a higher secretory activity in HGT-1 cells.

**mRNA expression of bitter taste receptors in HGT-1 cells and human biopsies using RT-qPCR**

Total RNA was extracted from HGT-1 cells and human biopsies using the peqGold Total RNA Kit (Peqlab). Quantity and quality were checked spectrophotometrically. Reverse transcription was carried out with 2 µg RNA and the High Capacity cDNA

Reverse Transcription Kit (Life Technologies). Real-time PCR was performed with an Applied Biosystems StepOneplus Real Time PCR system and using the Fast SYBR green master mix (Applied Biosystems). Primers were designed using the primer designing tool of NCBI (using Primer 3 and Blast) (**Supplementary Data Table 3**). Cycling conditions were: 20 s/95°C (activation), 3 s/95°C (denaturation), 30 s/60°C (annealing), 15 s/67°C (elongation with fluorescence measurement). The PCR products were verified by melting curve analysis and agarose gel electrophoresis. The subsequent sequence analysis of PCR products was carried out by eurofins genomics (Ebersberg, Germany) and sequences were checked using the NCBI Blastn tool. Primers showing no product in HGT-1 in at least one of the 3 replicates *TAS2R 8, 9, 45* and *60* were tested with cDNA derived from a human tongue biopsy kindly provided by J.-D. Raguse, Charite, Berlin, Germany. While primers for *TAS2R 8, 9* and *60* could be verified, *TAS2R45* was not detected. For TAS2R45, high-frequency copy-number variants are known, and some people do not possess the tested variant of the mRNA for this gene[1,2]. The open source software LinRegPCR was used for qPCR data analysis. This software enables the calculation of the starting concentration ($N_0$) of each sample, expressed in arbitrary fluorescence units. The calculated starting concentrations of the TAS2Rs were compared to the starting concentrations of the acetylcholine receptor (*CHRM3*, primers previously described[3,4]) which is typically expressed in parietal cells on a functional level.

**Immunohistochemical staining of gastric tissues**

Histological specimens were obtained from two patients. The gastric fundus derived from a sleeve gastrectomy of a 42 year old adipose, but otherwise healthy, patient. The gastric antrum derived from a 71 year old patient undergoing distal partial gastrectomy for a benign gastrointestinal stroma tumor.

Immunohistochemistry was performed on 5 µm thick paraffin-embedded whole tissue sections. Tissue fixation was performed with 7.5 % buffered formalin. Slides were processed in the fully automated staining instrument Benchmark ULTRA using ultraView Universal DAB Detection Kit (Ventana Medical Systems).

The following primary antibodies were applied: TAS2R10 (Thermo Scientific, OSR00158W), 1:750 for 28 minutes at 37°C after heat mediated antigen retrieval using EDTA-buffer, pH 8.0 at 95°C for 36 minutes (CC1 buffer, Ventana Medical Systems). GNAT2 (Transducin alpha-2 chain) (Abgent, AP11077c), 1:50 for 28 minutes at 37°C after heat mediated antigen retrieval using EDTA-buffer, pH 8.0 at 95°C for 64 minutes (CC1 buffer, Ventana Medical Systems) and amplification at 95°C (Amplification Kit, Ventana Medical Systems). All counterstaining was performed with hematoxylin. Blocking experiments in order to control for unspecific staining were performed using the TAS2R10 control peptide (Thermo Scientific, GST00040P) and GNAT2 antibody blocking peptides (Abgent, BP11077c). For the TAS2R10 taste receptor, the blocking experiment consisted of the control peptide, 1:200 incubated together with TAS2R10 antibody 1:750 for 120 minutes at 4°C and thereafter incubation of the slide at 37°C for 28 minutes. The GNAT2 antibody blocking peptide, 1:10 was incubated together with GNAT2 antibody 1:50 for 120 minutes at 4°C and thereafter incubation of the slide for 28 minutes at 37°C. All other steps were performed exactly similar to the staining procedure as described above.

**Immunocytochemical staining of HGT-1 cells and HEK 293T-Gα16gust44 cells**

Transiently transfected HEK 293T-Gα16gust44 cells were prepared as described previously[5] and HGT-1 cells were seeded on coverslips 24 h before the staining procedure. Cells were washed twice with PBS, incubated for 30 min on ice followed by 1 hour with 20 µg/mL biotin-labeled concanavalin A (Sigma) for plasma membrane staining on ice. After washing, the cells were fixed for two minutes on ice with methanol

and acetone (1:1), blocked for 45 min in a blocking solution consisting of 5% normal horse serum and 0.5 % Triton X-100 in PBS. Immunostaining with anti-HSV (Novagen) (1:15000), anti-TAS2R10 and anti-GNAT2 antibodies (see immunohistochemical staining) was performed for 1 h at room temperature. For labeling specificity, pre-absorption of the anti-TAS2R10 and anti-GNAT2 antibody, with the corresponding immunogenic peptide (see immunohistochemical staining), was performed. The HSV epitope was detected with anti-mouse antibodies conjugated with Cy3 (1:2000, Sigma), biotin labeled concanavalin A with Streptavidin Alexa Fluor 633 (1:1000, Molecular Probes) and either TAS2R10 or GNAT2 with Alexa Fluor 488 goat anti-rabbit IgG (1:1000, Molecular Probes). Detection was carried out as described before[5].

**siRNA knockdown of *TAS2R10* and *TAS2R43***

Cells were grown to 50 % confluence in serum containing DMEM and mRNA expression of *TAS2R10* or *TAS2R43* was reduced by transfection of small interfering RNA (final siRNA concentration 1 nM and 10 nM, respectively) targeting *TAS2R10* (5'-GACACAGUCUGGGAUCUCA-3'; Sigma-Aldrich) or targeting *TAS2R43* (5'-GAAUGAGAUUGUGCGGACA-3'; Sigma-Aldrich) into HGT-1 cells with the HiPerFect transfection reagent (Qiagen, 1 µL/6 pmol siRNA). Cells were incubated for 48 h with the transfection complex. As negative control, unrelated non-silencing siRNA (Qiagen) was used; as positive control, siRNA targeted against Mn/Hs_MAPK1 (Qiagen) was used. Cells were also incubated with the HiPerFect transfection reagent alone as mock transfection, to exclude the effects of the transfection reagent itself. RT-qPCR were used to check knockdown efficiency as explained before. mRNA expression of *TAS2R10 or TAS2R43* was not influenced by mock transfection and non-silencing siRNA transfection. MAPK1 mRNA levels decreased by 58 ± 4 % compared to non-transfected cells.

Intracellular pH measurements to evaluate the proton inducing effect of 3 mM caffeine in non-transfected, mock transfected and siRNA "knockdown" HGT-1 cells over a time course of 30 minutes with an interval of 5 minutes were carried out as described under "intracellular pH measurements".

**Calcium Imaging Experiments.** Calcium imaging experiments were essentially done as described previously[6]. Briefly, a TAS2R43 construct (Meyerhof et al., 2010) was transiently transfected with Lipofectamine2000 in HEK 293T cells stably expressing the chimeric G protein subunit Gα16gust44. 24 hours after transfection, cells were loaded with Fluo4-AM, washed 3 times in C1 buffer and changes in intracellular $Ca^{++}$ concentration upon agonists solution application were recorded, at least 3 times independently for each agonist-antagonist combination, using a fluorometric imaging plate reader FLIPR$^{TETRA}$ (Molecular Devices). Aristolochic acid was dissolved in C1 buffer at 0.03 µM concentration and caffeine at 1 mM concentration. Cells were exclusively stimulated with agonists and increasing concentrations of the antagonists in the concentration range of 0.03 - 30 µM. Inhibition curves were calculated with SigmaPlot 11 software, after signal responses were corrected and normalized to background fluorescence.

**Identification of the influence of bitter taste on gastric acid secretion *in vivo***

The 7 female and 4 male healthy test subjects had no gastrointestinal complaints were non-smokers, did not take antibiotics for 2 months prior to the test, and were between 21 and 32 years of age with a body mass index between 19 and 25 kg/m$^2$. Helicobacter pylori infection was excluded by an immunochromatographic rapid capillary blood test (Diagnostik Nord, Schwerin, Germany). Average caffeine consumption was 125 mg/day and determined by a food frequency questionnaire of caffeine containing food and beverages. Each volunteer was fully informed about the test, gave written consent, and was treated following the ethical principles of the declaration of Helsinki. The

experimental protocol was reviewed by the ethics committee of the city of Vienna (registration no. EK 13–180–VK_NZ). Prior to the intervention, the trial subjects had to fast from food and liquid for 10 h, except for 200 mL of tap water that were allowed during this time period. To make the gastric pH measurements as comfortable as possible for the test subjects, the capsule was not tethered, but the test subject had to be in a left sided supine position. For the non-invasive measurement of the gastric pH, the Heidelberg Detection System (Heidelberg Medical Inc., USA) has been applied, which has been successfully used in our group[21,22]. This system consists of a pH-sensitive capsule (called a Heidelberg capsule), with a length of 2 cm, that has to be swallowed and contains a miniature radio transmitter. This system allows the detection of the actual gastric pH of the volunteer over a specific time period.

Prior to each test, Heidelberg capsules were placed for 5 min in a 0.9% sterile NaCl solution and calibrated using two calibration points, pH 1 and 7. Afterward, the subjects swallowed the capsule and laid down on the left side. The capsule sends a signal to a transceiver, which has to be placed on the abdomen of the volunteer. The transceiver sends the signal to the recorder connected to a computer. Data is collected and shown as a gastrogram on the computer screen, where the pH is recorded as a function of time. When the intragastric pH was constant between pH 0.5 and 2.5 for at least 3 min, the capsule was considered to be in the stomach. Afterward, each trial started with the administration of 5 mL of a saturated sodium bicarbonate solution ($NaHCO_3$). This alkaline challenge triggers a rise in gastric pH to between pH 6 to 7 and subsequently leads to the secretion of stomach acid by the parietal cells. 125 mL water (control) or 150 mg caffeine, either diluted in 125 L water or encapsulated in a gelatin capsule (Type: Coni-Snap, Size: 1, Capsugel, Belgium) with 125 mL water were administered 5 minutes after the alkaline challenge. For exclusive activation of TAS2Rs in the mouth, 5 min after swallowing the alkaline solution, the volunteers swallowed 125 mL

water and then rinsed their mouth with 150 mg caffeine diluted in 125 mL water before spitting it out. A total of 150 mg caffeine in combination with 30 mg HED or 30 mg HED, itself diluted in 125 mL, were administered by drinking 5 min after the alkaline solution. In the second approach, an empty gelatin capsule (Type: Coni-Snap, Size: 1, Capsugel, Belgium), or 150 mg caffeine encapsulated, or 150 mg caffeine encapsulated with 30 mg HED were administered with 125 mL water 25 min before the alkaline solution. When HED was added, in three of ten subjects when oral and gastric stimulation where allowed, and in two of five participants when caffeine was encapsulated administered, the capsule entered the duodenum before the original pH was reached again. Reacidification time as well as the slope of the gastrogram was analyzed using the Heidelberg Detection System software and ImageJ software. Reacidification time is defined as the time starting when 5 mL saturated $NaHCO_3$ solution was administered until the original pH is reached again. The slope was calculated between the point when pH falls and the point where the original pH is reached again.

**Sensory study**

Taste sessions were carried out in the morning hours and the untrained panel volunteers (3 male and 7 female) were asked not to consume anything besides water 30 min prior to the sensoric test. The bitter recognition threshold of the volunteers was determined using a standardized test system starting with water and followed by 9 solutions with increasing concentration of caffeine, from 25 to 225 mg/L. Furthermore, the volunteers had to rank the bitterness of a caffeine solution (150 mg/125 mL) and a caffeine (150 mg/125 mL) + HED (30 mg/125 mL) solution by sip and spit on a scale of 1 (nothing) to 10 (extremely strong). This dual test was repeated 4 times in randomized order and under colored light. The mean bitterness rating for the caffeine solution was 7.3 ± 1.9 (mean ± SD), and for the caffeine + HED solution 6.1 ± 1.7

(mean ± SD). Statistical significance was calculated using Student's *t* test (double-sided, paired).

**Statistical analysis**

Data shown are representative of at least 3 biological replicates. All data is expressed as mean ± SEM unless stated otherwise. All data has been verified for normality distribution and statistical significant differences were considered if the *p* value was less than 0.05, determined by one-way ANOVA with Dunn's or Holm-Sidak *post hoc* test using SigmaPlot 11.0 software. Correlation analysis after Spearman was calculated by SigmaPlot 11.0 software.

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## Supplementary Data

**Supplementary Data Table 1. mRNA expression of TAS2Rs in HGT-1 cells normalized to the expression of the acetylcholine receptor (*CHRM3*).** Data is shown as mean ± SEM; *n*=3-4 (*n*, biological replicates), *tr*=3 (*tr*, technical replicates). The mRNA of *TAS2Rs* is similarly or even more highly expressed as compared with the mRNA of *CHRM3* in HGT-1 cells.

| | HGT-1 | |
|---|---|---|
| Receptor/ Gene | mean | SEM |
| *CHRM3* | 1.00 | 0.035 |
| *TAS2R1* | 0.20 | 0.050 |
| *TAS2R3* | 9.87 | 0.848 |
| *TAS2R4* | 5.66 | 0.765 |
| *TAS2R5* | 12.08 | 0.822 |
| *TAS2R7* | 0.32 | 0.073 |
| *TAS2R8* | no specific product | |
| *TAS2R9** | 0.12 | 0.019 |
| *TAS2R10* | 0.97 | 0.100 |
| *TAS2R13* | 1.69 | 0.144 |
| *TAS2R14* | 12.39 | 1.347 |
| *TAS2R16* | 0.71 | 0.239 |
| *TAS2R19* | 4.40 | 0.678 |
| *TAS2R20* | 9.09 | 1.139 |
| *TAS2R30* | 8.02 | 0.717 |
| *TAS2R31* | 4.00 | 1.767 |
| *TAS2R38* | 0.14 | 0.045 |
| *TAS2R39* | 3.64 | 0.807 |
| *TAS2R40* | 0.51 | 0.052 |
| *TAS2R41* | 0.66 | 0.143 |
| *TAS2R42* | 2.24 | 0.444 |
| *TAS2R43* | 6.47 | 0.316 |
| *TAS2R45* | not detected | |
| *TAS2R46* | 2.59 | 0.421 |
| *TAS2R50* | 2.91 | 0.290 |
| *TAS2R60* | no specific product | |
| *PLCB2* | 2.47 | 0.110 |
| *GNAT2* | 7.16 | 0.557 |
| *GNAT3* | 0.04 | 0.014 |

* in one of 3 replicates no product was detected.

**Supplementary Data Table 2. mRNA expression of TAS2Rs in human biopsies normalized to the expression of the acetylcholine receptor (*CHRM3*).** Data is shown as mean ± SEM; *n*=1-2 (*n* biological replicates), *tr*=3 (*tr*, technical replicates). mRNA of *TAS2Rs* is similarly expressed as mRNA of *CHRM3* in human epithelial cells.

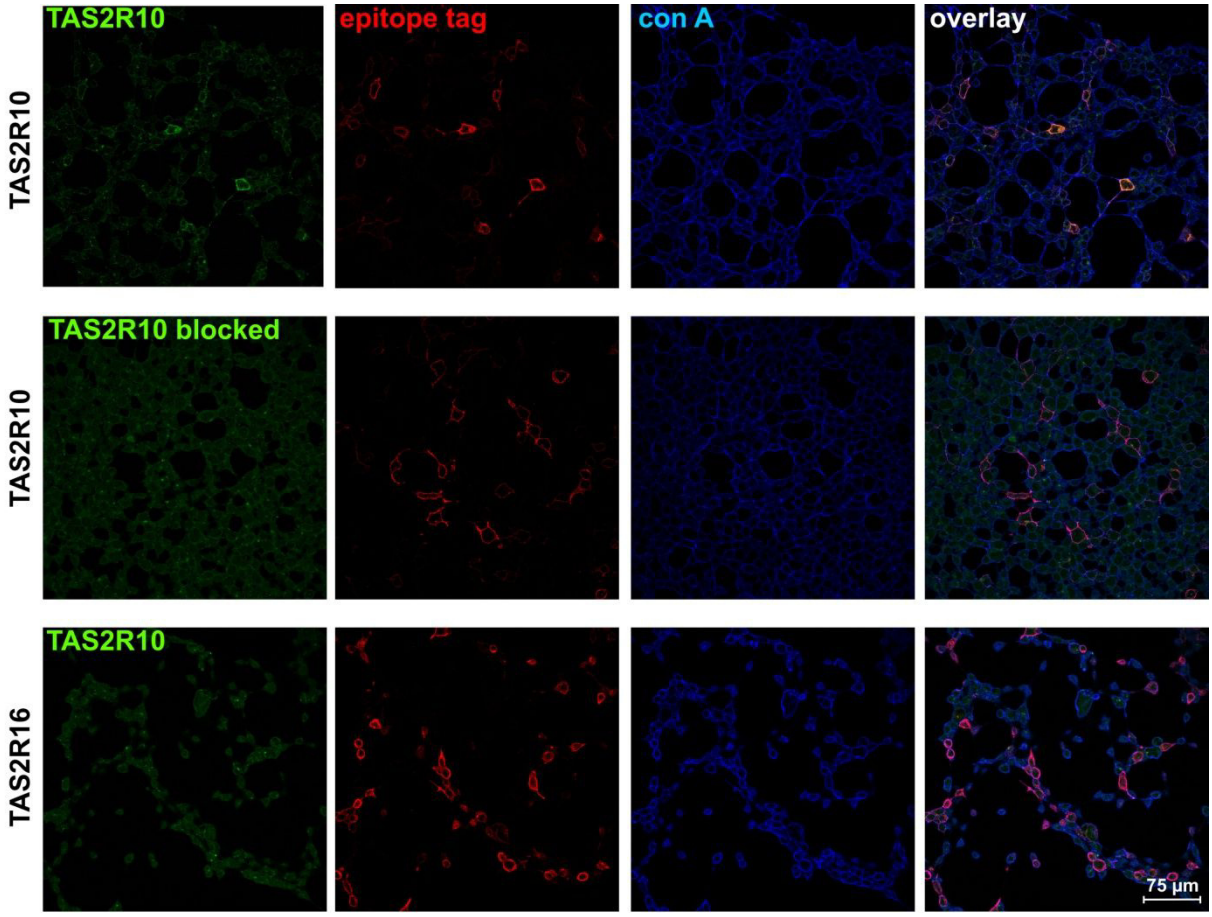
| **Human stomach biopsy** | | |
|---|---|---|
| Receptor | mean | SEM |
| *CHRM3* | 1.00 | 0.015 |
| *TAS2R7* | 0.76 | 0.039 |
| *TAS2R10* | 0.97 | 0.190 |
| *TAS2R14* | 1.16 | 0.025 |
| *TAS2R43* | 0.62 | 0.017 |
| *TAS2R46* | 0.83 | 0.071 |

**Supplementary Data Table 3**. **Primers used in this study.**

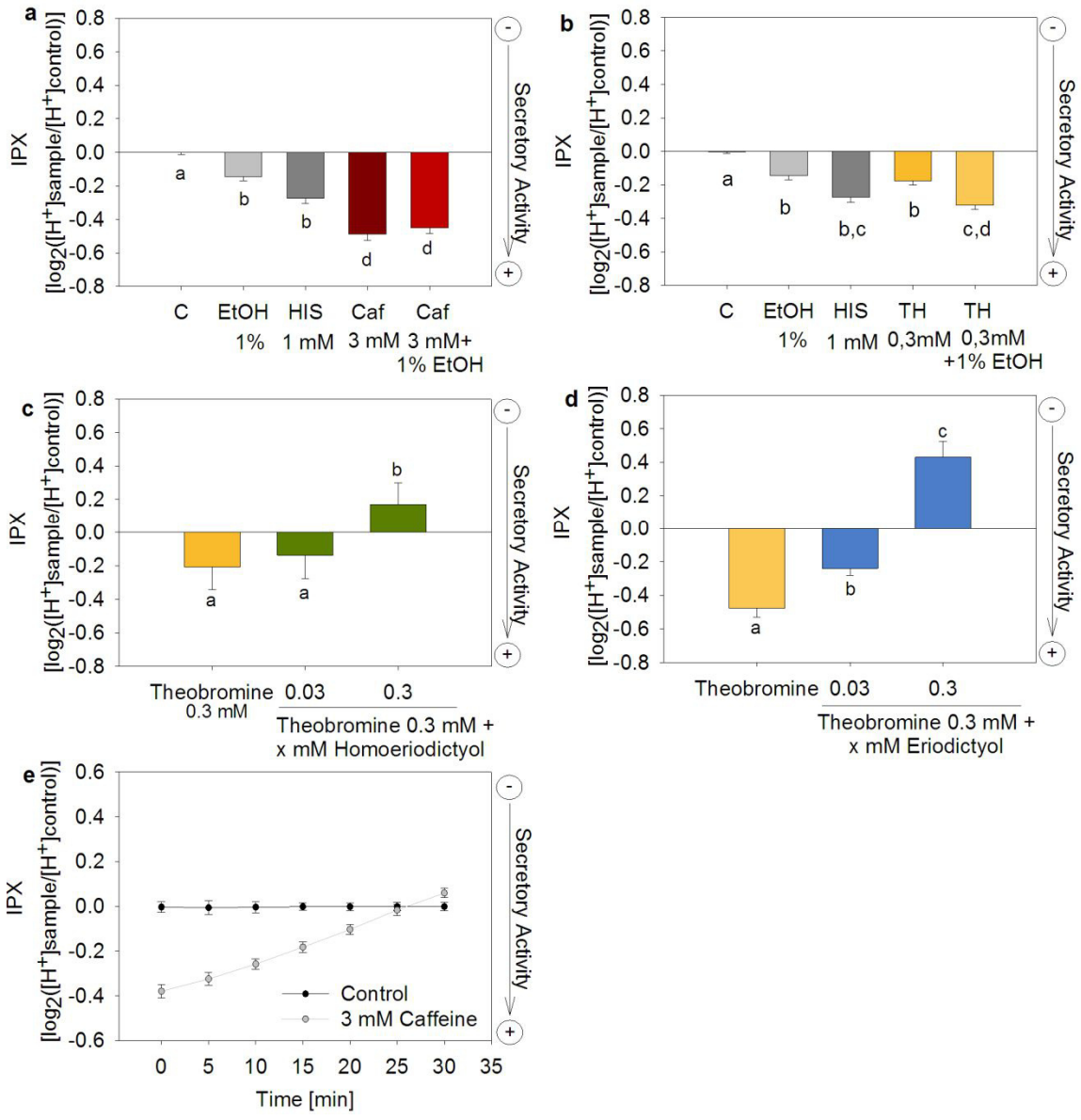
| Gene | | Sequence (5' to 3') | Amplicon length |
|---|---|---|---|
| *TAS2R1* | Forward | AAATGGCTCCGCTGGATCTC | 172 |
| | Reverse | GTGGCAAGCCAAAGTTCCAA | |
| *TAS2R3* | Forward | GGGACTCACCGAGGGGGTGT | 160 |
| | Reverse | CCTCAAGAGTGCCAGGGTGGTG | |
| *TAS2R4* | Forward | GCAGTGTCTGGTTTGTGACC | 168 |
| | Reverse | GCGTGATGTACAGGCAAGTG | |
| *TAS2R5* | Forward | ACACTCATGGCAGCCTATCC | 107 |
| | Reverse | CGAGCACACACTGTCTTCCA | |
| *TAS2R7* | Forward | GCAGGTGTGGATGTCAAACTC | 167 |
| | Reverse | TCTTGACCCAGTCCATGCAG | |
| *TAS2R10* | Forward | GCTACGTGTAGTGGAAGGCA | 73 |
| | Reverse | TCCATTCCCCAAAACCCCAA | |
| *TAS2R13* | Forward | GAAAGTGCCCTGCCGAGTAT | 177 |
| | Reverse | CCAGATCAGCCCAATTCTGGA | |
| *TAS2R14* | Forward | CCAGGTGATGGGAATGGCTTA | 128 |
| | Reverse | AGGGCTCCCCATCTTTGAAC | |
| *TAS2R19* | Forward | TCTTAGGACACAGCAGAGCA | 146 |
| | Reverse | AGCGTGTCATCTGCCACAAAA | |
| *TAS2R20* | Forward | ATTTGGGGGAACAAGACGCT | 183 |
| | Reverse | ACTACGGAAAAACTTGTGGGAA | |
| *TAS2R30* | Forward | GGCTGGAAAAGCAACCTGTC | 191 |
| | Reverse | ACACAATGCCCCTCTTGTGA | |
| *TAS2R31* | Forward | TTGAGGAGTGCAGTGTACCTTTC | 218 |
| | Reverse | ACGGCACATAACAAGAGGAAAA | |
| *TAS2R38* | Forward | CCCAGCCTGGAGGCCCACATT | 216 |
| | Reverse | TCACAGCTCTCCTCAACTTGGCA | |
| *TAS2R39* | Forward | TTCTGTGGCTGTCCGTGTTTA | 207 |
| | Reverse | GGGTGGCTGTCAGGATGAAC | |
| *TAS2R40* | Forward | CGGTGAACACAGATGCCACAGATA | 150 |
| | Reverse | GTGTTTTGCCCCTGGCCCACT | |
| *TAS2R41* | Forward | GCAGCGAATGGCTTCATTGT | 223 |
| | Reverse | TGGCTGAGTTCAGGAAGTGC | |
| *TAS2R42* | Forward | TCCTCACCTGCTTGGCTATC | 161 |
| | Reverse | GGCAAGCCAGGTTGTCAAGT | |
| *TAS2R43* | Forward | ATATCTGGGCAGTGATCAACC | 148 |
| | Reverse | CCCAACAACATCACCAGAATGAC | |
| *TAS2R46* | Forward | ACATGACTTGGAAGATCAAACTGAG | 200 |
| | Reverse | AGCTTTTATGTGGACCTTCATGC | |
| *TAS2R50* | Forward | CGCAAGATCTCAGCACCAAGGTC | 151 |
| | Reverse | GCCTTGCTAACCATGACAACCGGG | |
| *TAS2R8* | Forward | ATGTGGATTACCACCTGCCT | 135 |
| | Reverse | GGAAATGGCAAAGCATCCCAG | |
| *TAS2R9* | Forward | GCAGATTCGACTGCATGCTAC | 70 |
| | Reverse | TGCCTTTATGGCCCTCATGT | |
| *TAS2R16* | Forward | ATGGCATCACTGACCAAGCA | 255 |
| | Reverse | TTTCAACGTAGGGCTGCTCA | |
| *TAS2R45* | Forward | AGTACCCTTTACTGTAACCC | 170 |
| | Reverse | AGTAAATGGCACGTAACAAG | |
| *TAS2R60* | Forward | GGTGTTCAGTGCTGCAGGTA | 156 |
| | Reverse | CACCTTGAGGAACGACGACT | |

PCR-products were verified by sequencing. Primers for *TAS2R 45* showed no product in human tongue and HGT-1 cells.

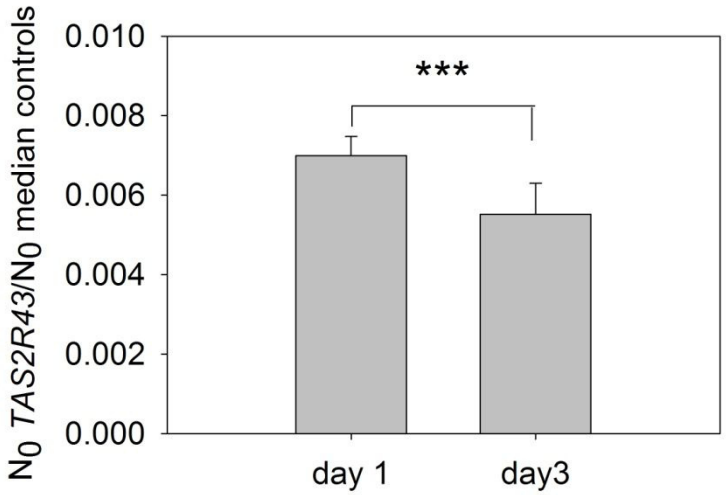
**Supplementary Figure 1**. **Immunocytochemical co-staining patterns of anti-TAS2R10 and epitope tag-specific antibodies in HEK 293T-Gα16gust44 cells.** Specific staining of HEK 293T-Gα16gust44 cells expressing TAS2R10 is demonstrated by the TAS2R10 antibody (green). TAS2R10 antibody blocked with specific blocking peptide showed no staining of cells expressing TAS2R10 as well as in cells expressing irrelevant target TAS2R16. The epitope-tagged receptor proteins were detected using an hsv-specific antiserum (red). Cell surface labeling (blue) was achieved using concanavalin A (con A).



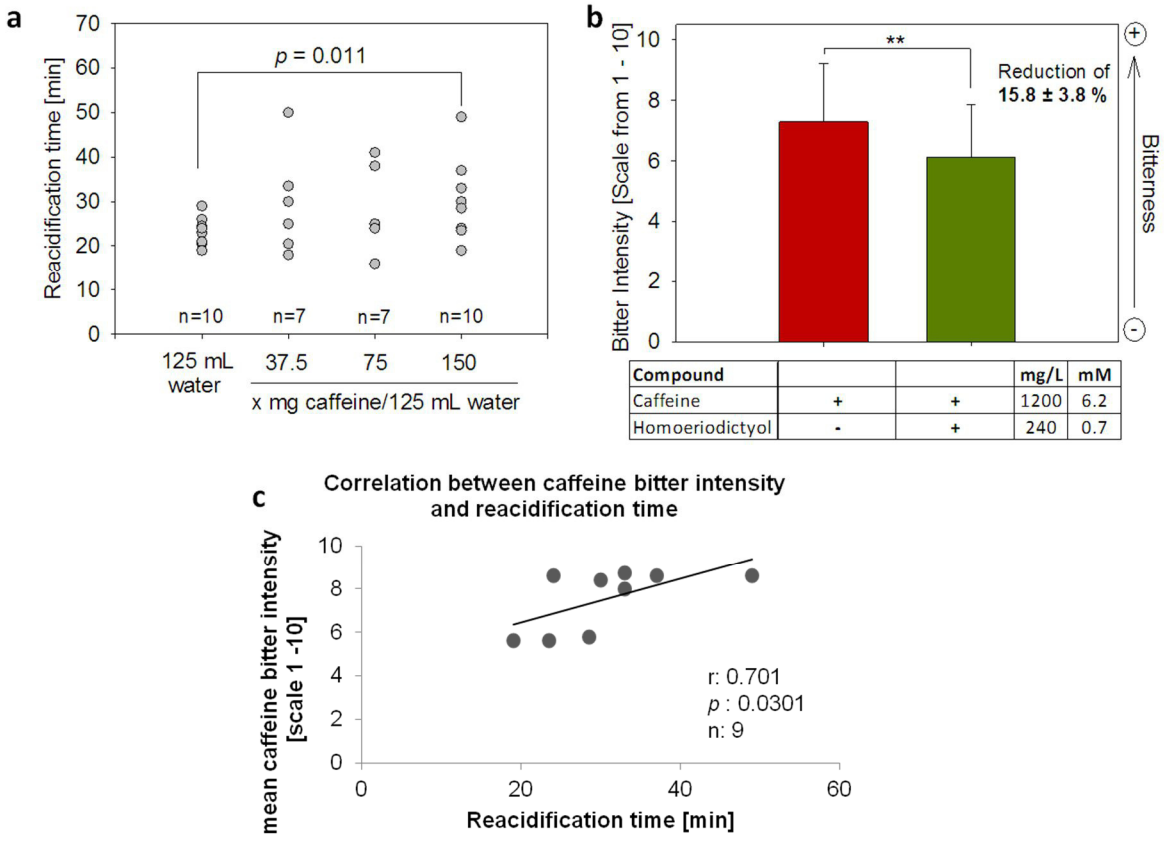
**Supplementary Data Figure 2. Homoeriodictyol and Eriodictyol reduce the theobromine-evoked effect on proton secretion in HGT-1 cells.** Intracellular proton Index (IPX) of HGT-1 cells treated for 10 min with: (**a**) caffeine or (**b**) theobromine alone and in combination with the diluent for eriodictyol 1 % EtOH; Data displayed as mean ± SEM, *n*= 4-37, *tr*=6, Statistics: one-way Anova on ranks with Dunn's *post hoc* test. (**c**) theobromine alone and in combination with two concentrations of homoeriodictyol (**d**) or eriodictyol (**c,d**); Data displayed as mean ± SEM, *n*= 4-37, *tr*=6, Statistics: one-way Anova on ranks with Dunn's *post hoc* test. (**e**) IPX of HGT-1 cells treated with 3.0 mM caffeine over a time period of 30 min, showing a time-dependent stimulation of proton secretion, *n*=2-3, tr=6. The lower the IPX, the stronger the proton secretion.



**Supplementary Data Figure 3. mRNA expression of *TAS2R43* in HGT-1 cells treated following the standard culture protocol (one day of culture) in comparison to treatment according to the transfection protocol (three days of culture).** Calculation of the starting concentration ($N_0$) of the samples, expressed in arbitrary fluorescence units was determined using the software LinReg and normalized to $N_0$ of peptidylprolyl isomerase A (*PPIA*) and TATA box binding protein (*TBP*) (=median controls). Statistics: Student's *t*- test, data shown as mean ± SD, *n*=3, *tr*=3, significant differences ($p < 0.05$) are indicated by letters.



**Supplementary Data Figure 4. Results of the gastric pH measurements and the sensoric tests in the human intervention study.** (**a**) Reacidification time, measured by the Heidelberg detection system, of different concentrations of caffeine and 125 mL water administered by drinking, allowing activation of oral and gastric TAS2Rs, in comparison to 125 mL water alone. Statistics: Students' *t*-test 150 mg caffeine vs. water. (**b**) Bitter intensity of 1200 mg/L caffeine and 1200 mg/L caffeine in combination with 240 mg/L HED were assessed in 10 sensorically untrained test subjects under colored light, repeated 4 times. Statistics: Student's *t*-test, **; $p < 0.01$ (**c**) Correlation analysis after Spearman between caffeine bitter intensity and reacidification time after administration of 150 mg caffeine by swallowing.



# IV. Conclusion and Perspectives

Dysregulation of gastric acid secretion can cause gastric discomfort and is associated with chronic diseases such as gastro-esophageal-reflux disease (GERD) [4], gastritis or ulcer [5], which might, in the course of time, result in carcinogenesis in the stomach and lower esophagus [2,6]. Especially patients suffering from reflux disease or peptic ulcer are advised to avoid certain beverages such as wine, beer and coffee.

The research group of V. Somoza extensively studied the effects of coffee and coffee components on gastric acid secretion [40,47,48,76,78,79]. As a result, the bitter tasting caffeine has been identified as the most potent pro-secretory coffee component. However, the impact of other bitter tasting compounds from beverages like beer and wine and the role of the bitter taste receptors TAS2Rs have not been addressed so far. Therefore, the present cumulative thesis focused on the identification of wine and beer constituents, which contribute to the pro-secretory activity of these beverages. Furthermore, the underlying mechanisms with focus on the role of bitter taste receptors were identified.

The first study ((1) *"Identification of organic acids in wine that stimulate mechanisms of gastric acid secretion"*), demonstrates by means of *in vitro* and *in vivo* experiments that the studied red wine sample stimulated gastric acid secretion to a higher extend than the white wine sample. Furthermore, red wine was shown to have a more pronounced effect on genes regulating gastric acid secretion than white wine or ethanol in HGT-1 cells. Since the organic acids succinic and maleic acid have been shown to stimulate acid secretion in a previous study [21], the most abundant organic acids in wine were quantified and tested in the HGT-1 cell line. The organic acids tartaric, citric, malic, succinic and lactic acid stimulated proton secretion in wine representative concentrations in HGT-1 cells. Malic acid, the most abundant organic acid in white wine, stimulated proton secretion to the highest extend compared to the other organic acids. These fruit derived organic acids in wine contribute to the typical lightly acidic taste and flavor of wine. Since the organic acids solely or combined and in concentrations determined in the respective wine samples had more pronounced effects than the wine samples themselves, the question arose, whether there are compounds present in wine which counteract the effect of the tested compounds.

Ethanol itself showed a stimulatory effect in this study. However, when ethanol was added to red wine and to tartaric acid, their pro-secretory effect was reduced. This indicates that matrix effects, especially the influence of ethanol, have to be considered as well.

To address the impact of the complex food matrix a chemical characterization of four red wine and six white wine samples was conducted and their content in various organic acids, several amines and phenolic compounds was determined, in the second study ((2) *"Identification of phenolic compounds catechin, syringic acid and procyanidin B2 in wine that stimulate mechanisms of gastric acid secretion"*). About 40 wine parameters were correlated to the intracellular proton index, the IPX, of the wine as a factor for the wine`s effect on proton secretion from HGT-1 cells. Red wines stimulated proton secretion to higher extends than white wine samples, confirming the result of the first study [28]. The major differences between the red and white wine samples were their content in malic acid, lactic acid and phenolic compounds. While malic acid was only detected in white wines, lactic acid was only found in red wines. Furthermore, red wines contained much higher amounts of phenolic compounds. The IPX values of the wine samples were correlated to the 40 wine parameters determined in those samples. The highest pro-secretory significant correlation coefficient was detected for lactic acid, procyanidin B2, syringic acid and catechin. Malic and lactic acid stimulated proton secretion in the HGT-1 cells, and affected regulation of genes relevant for gastric acid secretion. In red wines malic acid is fermented to lactic acid during malolactic fermentation [27]. From the presented results, it is very likely that lactic acid replace the effect of malic acid on gastric acid secretion. Therefore, these acids probably cannot be accounted for the different effects of red wines compared to white wines. Instead, it seems more plausible that the content of phenolic compounds as syringic acid, procyanidin B2 and catechin, contribute to the different effect of red wine and white wine. These phenolic compounds were shown to stimulate proton secretion in HGT-1 cells in wine representative concentrations. In addition, when these three compounds were added to the least effective white wine, its pro-secretory potential was enhanced. Interestingly, these substances were described to contribute to the bitter and astringent taste of red wine [81] a first indication for involvement of bitter and astringent taste perception in the pro-secretory effect of wines.

Beer is known for its typical bitter taste originating from hop-derived bitter acids. In the third publication ((3)*„Identification of beer bitter acids regulating mechanisms of gastric*

*acid secretion"*) several beer types and components thereof were investigated regarding their effect on proton secretion in HGT-1 cells. All tested beer types stimulated proton secretion. However, the effect of non-alcoholic beer was less pronounced compared to alcoholic varieties. Organic acids and hop-derived bitter acids (*α*-, *β*-, and iso-*α*-acids) stimulated proton secretion in HGT-1 cells in beer representative concentrations. Interestingly, the pro-secretory efficacy of the bitter acids corresponded to their contribution to bitter taste in beer. Iso-*α*-acids comprise the greatest contribution followed by *β*-acids, which account for a long-lasting bitterness, whereas the $\alpha$-acids contribute only little to the bitter taste of beer. Furthermore, the bitter acids were identified to up-regulate the expression of the acetylcholine receptor encoding gene (*CHRM3*). Activation of the acetylcholine receptor M3 leads to a pro-secretory signaling cascade. However in beer represantitive concnetrations, the $\alpha$-acids had also the lowest effect in the gene expression analysis. These results indicated that HGT-1 cells are sensitive to the bitter taste of the bitter acids. The common characteristic of the phenolic compounds in wine and the beer bitter acids is their bitter taste and contribution to bitter taste in wine or beer. In addition, previous studies of Rubach et al. [40] identified the bitter tasting compound caffeine as the most potent pro-secretory ingredient from coffee in HGT-1 cells. To summarize, bitter substances contribute to the pro-secretory effect of the beverages wine, beer and coffee on gastric acid secretion. In the present studies, the pro-secretory activity was measured using human gastric tumor cells. This cell model shows the characteristics of parietal cells but excludes oral taste perception in comparison to the *in vivo* situation. Besides in the mouth, taste receptors have also been found in non-gustatory tissues including airway epithelia [65], intestinal cells [66,67] of humans and rodents, and in gastric epithelia of rats and mice [68,69]. In the present thesis, the hypothesis that bitter taste receptors are expressed in parietal cells of the stomach and regulate mechanisms of gastric acid secretion was investigated.

To study a possible interaction between gastric taste receptors and gastric acid secretion, we first had to determine whether taste receptors are expressed in the human stomach, especially in the parietal cells and in our in vitro model the HGT-1 cell line. So far, taste receptors have been identified in the stomach mucosa of rats and mice [82], but not in humans. The fourth study ((4) *"Activation of gastric bitter taste receptors stimulates gastric acid secretion and counteracts oral bitter taste receptors"*) presents for the first time that TAS2R10 and GNAT2 are expressed in the gastric

epithelium by immunohistochemical staining of stomach surgical specimens from the gastric antrum and fundus. Localization of TAS2R10 and GNAT2 was confined to parietal cells and chief cells in the gastric fundus, showing a strong cytoplasmic granular reactivity. In contrast, only faint staining of glandular cells was detected in the gastric antrum. In addition, foveolar cells, the mucus producing cells in the fundus and antrum, were not demonstrated to express TAS2R10, but membranous GNAT2. In HGT-1 cells, expression of GNAT2 and TAS2R10 was visible although it was weak for TAS2R10. Nevertheless, mRNA expression of 22 TAS2Rs out of 25 human TAS2Rs and genes coding for the downstream signaling proteins, *PLCß2* and *GNAT2,* were demonstrated in HGT-1 cells to similar or even higher extend as the expression of *CHRM3*. In addition, a pro-secretory activity for several bitter compounds, such as denatonium benzoate, sodium benzoate, yohimbine, caffeine and theobromine, was demonstrated in HGT-1 cells. The standard bitter compound in sensory panels, caffeine, was very potent in stimulating proton secretion and has also been shown to activate the bitter taste receptors TAS2Rs 7, 10, 14, 43 and 46 [55]. In a sensory panel [57] it has been evaluated that several bitter masking compounds extracted from *Herba santa*,especially homoeriodictyol (HED) and eriodictyol (ED), reduce the bitterness of caffeine by 43 %. In addition, in several human studies, caffeine was associated with increased gastric acid secretion [41-44,50] when oral taste perception was bypassed. Caffeine was, thus, chosen to investigate the hypothesis that bitter taste receptors in the stomach are involved in the regulation of gastric acid secretion.

In HGT-1 cells, the caffeine- and theobromine-evoked effects on proton secretion were reduced by concomitant administration of HED or ED and the bitter compound. Furthermore, the involvement of *TAS2R10* in the caffeine-evoked effect on proton secretion was proven by means of a targeted knock-down approach.

In a human intervention trial, caffeine was administered via three administration types to distinguish whether oral or gastric or both types of bitter taste receptors are involved in the regulation of gastric pH.

Activation of oral TAS2Rs was demonstrated to inhibit gastric acid secretion by prolonging the reacidification time. However, when caffeine was released only in the stomach by administering encapsulated caffeine, a time-dependent effect on gastric acid secretion occurred. The time-dependency of the caffeine-effect was investigated in follow-up experiments, allowing gastric activation of TAS2Rs only by preponing the administration time by 25 min. In this experiment, a clear stimulating effect of caffeine

on gastric acid secretion was demonstrated. Addition of the bitter masking compound HED inhibited the caffeine-evoked effect when either gastric or gastric and oral TAS2Rs were activated. In addition, the volunteers rated caffeine less bitter when HED was added in a blinded sensory duo-comparison test. A positive correlation was detected between the rated caffeine bitter intensity and the influence of caffeine administered by drinking (oral + gastric stimulation of TAS2Rs) on the gastric pH output measure reacidification time. In one test subject, gastric acid secretion did not respond to caffeine, independent of the administration type. Interestingly, the same test subject was not sensitive to caffeine-evoked bitterness in low doses in the sensory evaluation. To summarize, the last study comprises three major findings: first we revealed for the first time that TAS2Rs and a sensory G protein are expressed in the human gastric epithelia, especially in parietal cells. Second, caffeine, does not necessarily stimulate gastric acid secretion as previously assumed, it rather inhibits gastric acid secretion when intense oral perception was realized. Finally, the third major finding was that, when the bitterness of caffeine was reduced by HED either in the mouth or in the stomach, the different caffeine-evoked effects were reduced. This is another major indication for an involvement of bitter taste receptors in the mouth and the stomach in the regulation of gastric acid secretion.

Bitter perception in the mouth was or is necessary for the detection of potentially unhealthy or even toxic food. Activation of oral taste receptors by caffeine might therefore be interpreted as a signal of aversion, which initially leads, via vagal withdrawal, to an inhibition of gastric acid secretion to stop the urge of eating this potentially unhealthy food. In contrast, activation of gastric taste receptors induces a stimulation of gastric acid secretion, which could be physiologically explained by an attempt to detoxify the already consumed potentially poisonous food.

In the first study, red wine was also tested in comparison to white wine and ethanol in a human intervention trial allowing oral and gastric activation of TAS2Rs. Red wine stimulated gastric acid secretion more pronounced than white wine by oral administration. Taking into account the newly acquired results of the fourth study, which show that bitter taste in the oral cavity inhibits gastric acid secretion, this finding seems contradictive. However, the taste of the red wine was less repellent than that of the caffeine solution and the red wine had also high contents in the strong pro-

secretory organic acids. Thus, it is assumed that the pro-secretory effect of organic acids prevail the probable repellent bitter taste of wine on the tongue.

To summarize, the present thesis demonstrates the importance of bitter taste perception in the regulation of the digestive process, as shown for gastric acid secretion by oral and extra oral taste receptors.

Further studies need to address the pharmacological potential of an inhibiting effect of bitter taste sensation in the mouth on gastric acid secretion and the potential of bitter-masking compounds to reduce overshooting gastric acid secretion.
The addition of bitter-masking compounds to bitter medicines could have two advantages: first, bitter masking compounds can prevent the perceived bitterness on the tongue and make the medicines more palatable, which is an important point especially when medicines are administered to children. Second, bitter masking compounds could inhibit overshooting gastric acid secretion. Therefore, bitter masking compounds presumably prevent stomach discomfort after consumption of medicines and the common additional administration of gastric protective drugs could be avoided. However, future studies need to evaluate the pharmacological use of bitter-masking compounds.

Besides a pharmacological use, this knowledge can be used to optimize food production, making food products on one hand more tasteful and on the other hand more stomach friendly.

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# VI. Abstract

Overshooting gastric acid secretion can cause gastric discomfort and contribute to the symptoms of reflux disease. Consumption of wine, beer and coffee is supposed to promote gastric acid secretion. This thesis aimed to identify the effect of different wine and beer samples and constituents thereof on gastric acid secretion. Furthermore, the involvement of bitter taste receptors (TAS2Rs) on the regulation of gastric acid secretion was investigated in the HGT-1 cell line (human gastric tumor cells) and in healthy volunteers.

In HGT-1 cells, red and white wine samples, as well as beer samples stimulated proton secretion as a marker of gastric acid secretion. *In vivo*, one red wine sample stimulated gastric acid secretion more effectively than one white wine sample. A subsequent mechanistic approach identified, organic acids and the bitter compounds catechin, syringic acid, procyanidin B2, and the hop-derived *α*-, *β*-, and iso-*α*-acids as key compounds of beer and wine stimulating proton secretion in HGT-1 cells. These findings led to the hypothesis, that the HGT-1 cell line is sensitive to bitter tasting compounds. Further results demonstrated that several bitter compounds, e.g. methylxanthines, stimulated proton secretion in HGT-1 cells, and that the effects evoked by caffeine and theobromine were reduced by addition of the bitter masking compounds homoeriodictyol (HED) and eriodictyol. Furthermore, expression of TAS2R10 and GNAT2 was shown in HGT-1 cells and in human gastric biopsies by means of RT-qPCR and or immune histological staining. A knockdown experiment in HGT-1 cells using siRNA targeted against *TAS2R10* demonstrated the involvement of *TAS2R10* in the caffeine evoked stimulation on proton secretion. In healthy subjects, activation of gastric TAS2Rs stimulated gastric acid secretion, whereas activation of oral bitter taste receptors reduced gastric acid secretion. The bitter masking compound HED not only diminished the bitter sensation of caffeine, but also reduced the caffeine-evoked effects on gastric acid secretion in healthy subjects. These findings support the hypothesis that bitter taste receptors in the mouth and the stomach are involved in the regulation of gastric acid secretion and demonstrate a potential therapeutic application of bitter and bitter masking compounds.

# VII. Zusammenfassung

Übermäßige Magensäuresekretion kann zu Magenbeschwerden führen und ist eine der Ursachen für Sodbrennen und Reflux. Personen, die unter diesen Symptomen leiden, wird der Konsum von Kaffee, Wein und Bier abgeraten. Im Rahmen dieser Doktorarbeit wurde der Einfluss unterschiedlicher Geschmackstoffe aus Wein und Bier sowie einer Bittersubstanz des Kaffees, Koffein, auf die Magensäuresekretion untersucht. Zusätzlich sollte ein möglicher Einfluss von Bitter-Geschmacksrezeptoren auf die Regulation der Magensäuresekretion in der Zelllinie (HGT-1, human gastric tumor cell line) und in gesunden Probanden untersucht werden. Rot- und Weißweine sowie unterschiedliche Bierproben stimulierten die Protonensekretion in HGT-1 Zellen, als Parameter für die Magensäuresekretion. In gesunden Probanden wurde gezeigt, dass ein ausgewählter Rotwein die Magensäuresekretion stärker stimulierte als ein Weißwein. In weiteren mechanistischen Untersuchungen wurden die organischen Säuren und die bitter bzw. adstringierenden Substanzen Catechin, Syringasäure, Procyanidin B2 aus Wein und die bitteren *α*-, *β*-, und iso-*α*-Säuren des Bieres als Stimulanzien der Protonensekretion in der Zell-Linie HGT-1 identifiziert. Diese Ergebnisse führten zu der Hypothese, dass die HGT-1 Zell-Linie, die Charakteristiken einer Parietalzelle aufweist, sensitiv auf Bittersubstanzen reagiert. Weitere Ergebnisse zeigten, dass unterschiedliche Bittersubstanzen, wie zum Beispiel Methylxanthine die Protonensekretion förderten und dass dieser Effekt durch die Zugabe von bittermaskierenden Substanzen wie Homoeriodictyol (HED) und Eriodictyol reduziert wurde. Außerdem wurde die Expression von 22 der 25 Bitterrezeptoren des Menschen mittels RT-qPCR in HGT-1 Zellen gezeigt und die Expression des Rezeptors TAS2R10 und des G-Proteins Transducin (GNAT2) in HGT-1 Zellen und in humanen Biopsien mit Hilfe von immun-histologischer und –cytologischer Färbung nachgewiesen. Mittels siRNA-gerichteten Knockdowns in HGT-1 Zellen wurde belegt, dass eine Reduktion der mRNA Expression von *TAS2R10* die Wirkung von Koffein auf die Protonensekretion herabsetzt. In gesunden Probanden, wurde die Magensäuresekretion durch Aktivierung gastraler TAS2Rs mittels Koffein stimuliert. Hingegen wurde durch Aktivierung oraler TAS2Rs mittels Koffein die Magensäuresekretion gehemmt. Die gleichzeitige Gabe von HED reduzierte die Bitterwahrnehmung und zeigte auch eine Reduktion der konträren Effekte, die durch Koffein hervorgerufen wurden, auf die Magensäuresekretion. Diese Ergebnisse zeigen, dass sowohl Geschmacksrezeptoren im Mund, als auch jene im Magen in die Regulation der Magensäuresekretion involviert sind und demonstrieren eine mögliche therapeutische Nutzung von Bitter- und Bitter-maskierenden Stoffen.

# VII. Curriculum vitae

## Education

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| 2010 - 2015 | PhD research at the Department of Nutritional and Physiological Chemistry, University of Vienna, Austria<br><br>Research interest: Impact of flavoring food compounds on mechanisms regulating gastric acid secretion<br><br>Supervisor: Prof. Veronika Somoza |
| 10/2003 – 11/2009 | Academic studies of Nutritional Sciences at the University of Vienna, Austria, (completed with distinction)<br><br>Diploma thesis at the Department of Nutritional Sciences: Quantitative and qualitative analysis of *Clostridium cluster IV* in faeces with molecular methods<br><br>Supervisor: Doz. Dr. Alexander Haslberger |

## Submitted manuscripts

| | |
|---|---|
| 2015 | **Liszt KI**, Ley JP, Lieder B, Behrens M, Reiner A, Stöger V, Hochkogler CM, Köck E, Marchiori A, Hans J, Widder S, Krammer G, Meyerhof W, Somoza MM, Somoza V. (2015) Bitter taste receptors in the stomach stimulate gastric acid secretion. Nature Medicine, submitted |
| 2015 | **Liszt KI**, Eder R, Wendelin S, Somoza V. (2015) Identification of Catechin, Syringic acid and Procyanidin B2 in Wine as Stimulants of Gastric Acid Secretion. Journal of Agriculture and Food Chemistry, submitted |

## Publications

2012 **Liszt KI**, Walker J, Somoza V (2012) Identification of organic acids in wine that stimulate mechanisms of gastric acid secretion. J Agric Food Chem 60: 7022-7030.

2012 Walker J, Hell J, **Liszt KI**, Dresel M, Pignitter M, Hofmann T, Somoza V (2012) Identification of beer bitter acids regulating mechanisms of gastric acid secretion. J Agric Food Chem 60: 1405-1412.

2011 Hippe B, Zwielehner J, **Liszt KI**, Lassl C, Unger F, Haslberger AG (2011) Quantification of butyryl CoA:acetate CoA-transferase genes reveals different butyrate production capacity in individuals according to diet and age. FEMS Microbiol Lett. 316(2):130-5.

2009 **Liszt KI**, Zwielehner J, Handschur M, Hippe B, Thaler R, Haslberger AG (2011) Characterization of Bacteria, Clostridia, Bacteroides in faeces of vegetarians using qPCR and PCR-DGGE fingerprinting; Ann Nutr Metab. 27;54(4):253-257.

2009 Zwielehner J, **Liszt KI**, Handschur M, Lapin A, Haslberger AG (2009) Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of Bacteroides, bifidobacteria and Clostridium cluster IV in institutionalized elderly. Exp Gerontol. 44(6-7):440-6.

## Patent applications

2014 Hydroxyflavanone als Mittel zur Beeinflussung des Appetits und der Energieaufnahme, als Mittel zur Steigerung des Appetits sowie entsprechende Stoffmischungen, oral konsumierbare Produkte und Verfahren.
(EP 14179087.3) Ley J, Krammer G, Rohm B, **Liszt KI**, Pignitter M, Somoza V, Hochkogler CM

2013 Method for the identification of bitter tasting compounds and bitter taste modulating compounds. WO 2014 111546 (24.07.2014) Ley J, **Liszt KI**, Köck E, Somoza V

2013 Pharmaceutical compositions masking bitter taste and inhibiting food-induced gastric acid secretion. EP 2756765 (23.07.2014) Ley J, Widder S, **Liszt KI**, Köck E, Somoza V

## International Conferences

2015 Poster presentation: **Liszt KI**, Ley J.P, Lieder B., Stöger V, Köck E, Stübler A, Hochkogler CM, Somoza MM, Widder S, Hans J, Somoza V.: AChemS Meeting, April 22 – 25, 2015, Bonita Springs, Florida, USA. “Caffeine-induced activation of oral and gastric bitter taste receptors regulates gastric acid secretion.”

2013 Oral presentation: **Liszt KI**, Köck E, Widder S, Ley J.P, Somoza V.: ECRO Meeting, August 26-29, 2013, Leuven, Belgium. “The role of bitter taste receptors in activating mechanisms of human gastric acid secretion.”

2013 Poster presentation: **Liszt KI**, Köck E, Widder S, Ley JP, Somoza V: Blankenese Conference, 2013, Hamburg, Germany. “Bioactive Aroma Molecules: Bitter Masking Compounds as Inhibitors for Methylxanthine induced Proton Secretion.”

2012 Oral presentation: **Liszt KI**, Eder R, Marek M, Walker J, Somoza V: 244th American Chemical Society National Meeting & Exposition, August 22, 2012, Philadelphia, PA, USA. “The effects of red wine, white wine and wine constituents on gastric acid secretion in healthy subjects and in parietal cells in culture”

2012 Oral presentation + Poster presentation: **Liszt KI**, Eder R, Marek M und Somoza V: 49th scientific congress of German Nutrition Society (DGE), March 14, 2012, Freising, Germany: „Bestimmung des Magensäuresezernierenden Effekts von Rot-und Weißweinen unter Berücksichtigung des bioaktiven Potentials einzelner Weininhaltsstoffe.“

2011 Poster presentation: **Liszt KI**, Walker J, Somoza V: 48. Wissenschaftlicher Kongress der DGE, Griebnitzsee, Germany, March 15–16, 2011: „Einfluss von Rotwein, Weißwein und Ethanol auf die Magensäuresekretion“

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| 2008 | Poster presentation: **Liszt KI**, Hippe B, Zwielehner J, Haslberger AG 2008, Effects of ageing and vegetarism on diversity and population sizes of Clostridium cluster IV assessed with TaqMan-PCR and PCR-DGGE fingerprinting. International Probiotic Conference June 2008, Slovakia |

## Reviewing Activities

Journal of Agriculture and Food Chemistry