Title:

The endocrine effects of bitter tastant administration in the gastrointestinal system – Intragastric versus intraduodenal administration

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Bitter infusion IG vs ID

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Abstract:

Bitter tastants are recently introduced as potential hunger-suppressive compounds, the so-called "Bitter pill". However, the literature about bitter administration lacks consistency in methods and findings. We want to test whether hunger ratings and hormone plasma levels are affected by: 1) the site of administration: intragastrically (IG) or intraduodenally (ID), 2) the bitter tastant itself, quinine hydrochloride (QHCl) or denatonium benzoate (DB), and 3) the timing of infusion. Therefore, 14 healthy, female volunteers participated in a randomized, placebo-controlled six-visit crossover study. After an overnight fast, DB (1 μ mol/kg), QHCl (10 μ mol/kg) or placebo were given IG or ID via a nasogastric feeding tube. Blood samples were taken 10 min prior to administration and every 10 min after administration for a period of 2 hours. Hunger was rated at the same timepoints on a visual analogue scale (VAS). ID bitter administration did not affect hunger sensations, motilin or acyl-ghrelin release compared with its PLC infusion. IG QHCl infusion tended to suppress hunger increase, especially between 50-70 minutes after infusion, simultaneously with reduced motilin values. Here, acyl-ghrelin was not affected. IG DB did not affect hunger or motilin, however acyl-ghrelin levels were reduced 50-70 minutes after infusion. Plasma values of glucagon-like peptide 1 and cholecystokinin were too low to be properly detected or to have any physiological relevance. In conclusion, bitter tastants should be infused into the stomach to reduce hunger sensations and orexigenic gut peptides. QHCl has the best potential to reduce hunger sensations, and it should be infused 60 minutes before food intake.

New & Noteworthy:

Bitter tastants are a potential new weight-loss treatment. This is a non-invasive, easy approach, which should be received with considerable enthusiasm by the public. However, literature about bitter administration lacks consistency in methods and findings. We summarize how the compound should be given based on: the site of administration; the best bitter compound to use; and at what timing in respect to the meal. This paper is therefore a fundamental step to continue research towards the further development of the "bitter pill".

Introduction

Lingual detection of bitter tastants is often associated with a repellent reaction, a likely evolutionary necessity to recognize noxious or spoiled food items before digestion. Nowadays, the bitter flavour gains more appreciation and is part of our daily diet, being present in cabbages, coffee, tea, and as food additives. A first taste distinction is made by specialized G-protein-coupled receptors from the taste receptor 2 (TAS2R) family on the tongue (1, 2). Humans are able to detect over thousands of bitter compounds, although only 25 active human TAS2R subtypes have been identified (3). Interestingly, women have a higher chance of detecting these compounds at lower concentrations than men (4). A second taste distinction in food bitterness is made in the upper gastrointestinal (GI) tract by TAS2Rs expressed on enteroendocrine cells (EECs) (5, 6). Subsequently, the release of appetite regulating GI hormones such as acyl-ghrelin, motilin, cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1) is affected and may serve to inhibit further food intake of toxic bitter compounds (7).

In vivo experiments in rodents support the hypothesised repulsive nature of denatonium benzoate (DB), the bitterest tasting compound, as gastric emptying is inhibited, gastric accommodation is reduced and food intake decreased (8-10). Changes in (an)orexigenic hormone levels after bitter

43 exposure might be the underlying mechanism (5, 11). In addition, TAS2Rs are also expressed on
44 smooth muscle cells and may have direct effects on contractility (9). It is no surprise that several
45 research groups show interest in bitter tastants as a potential new type of weight-loss treatment.
46 Unfortunately, the findings on hunger sensations and food intake in humans are inconsistent, due to
47 heterogeneity of study design and agents used. Two major theories have been tested: suppression of
48 orexigenic gut peptides motilin and acyl-ghrelin by intragastric (IG) administration and the
49 stimulation of satiety-related gut peptide release CCK and GLP-1 by intraduodenal (ID)
50 administration. Based on the current literature, hunger sensations may be suppressed when bitter
51 compounds are infused into the stomach (12-14), but not into the duodenum (15-17). The IG effect is
52 associated with a reduction of release of the orexigenic hormones motilin and/or acyl-ghrelin (12,
53 13).

54 Acyl-ghrelin is released from the P/D1 cells in the stomach, according to the metabolic status of the
55 body. It reaches a peak level right before a spontaneous meal initiation and drops to a nadir after
56 meal consumption (18). Motilin is released from the M cells in the proximal part of the duodenum
57 and is generally accepted as the major initiator of strong antral contractions which recur in a two-
58 hour cyclic manner during the interdigestive period, named the migrating motor complex (MMC)
59 (19). Moreover, the MMC's active phase is synchronized with intense hunger sensations (20-22),
60 which are suppressed by the IG infusion of a bitter food additive, quinine hydrochloride (QHCl), and
61 are accompanied by a reduction in motilin plasma levels (13).

62 It is rather unexpected to find that the infusion of a small bitter tasting load (< 10 mL) into the
63 stomach has a significant effect on motilin, the release of which is limited to the small bowel (23).
64 Hence, we hypothesize for the presence of a 'gastric inhibitory factor'. A potential candidate is
65 somatostatin (SST), released from the antral D-cells that contact the gastric lumen, and that inhibits a
66 range of different gut functions, including release of gut hormones (24).

67 The overall aim of this placebo-controlled, single-blind, randomized, crossover study is to obtain a
68 prototype manual for bitter tastant administration with hunger suppression as outcome. The
69 following specific questions will be addressed: 1) At which site should bitter agonists be administered
70 in the gut, IG or ID? 2) Are the target gut peptides the orexigenic peptides motilin and acyl-ghrelin or
71 the satiety-related hormones CCK and GLP-1, and is there a 'gastric inhibitory factor' namely SST? 3)
72 Which bitter tastant, QHCL or DB, has the best potential in reducing hunger sensations? 4) How long
73 after infusion is hunger and food intake affected? This new information is a *conditio sine qua non* to
74 be able to effectively work towards the 'bitter pill' as a new type of weight-loss treatment.

Material and methods

Ethical approval

This study was approved by the Leuven University Hospital Medical Ethics Committee and performed in full accordance with the Declaration of Helsinki. This trial was registered at clinicaltrials.gov as NCT03985865.

Subjects

Sixteen healthy, female volunteers were recruited and fourteen finished the experiment (BMI 21.7 ± 1.2 kg/m²; age 22 ± 0.7 years). One participant dropped out due to personal reasons and one because of discomfort while placing the feeding tube. Before participation, all volunteers signed the informed consent and confirmed that they did not meet the exclusion criteria. These criteria were age limits (<18 and >65 years), potential threatening diseases (e.g. heart failure), clinical history of abdominal surgery, gastrointestinal complaints, pregnancy and breastfeeding. Only women were recruited as they generally have a lower bitter detection threshold than men (4). In addition, intragastric bitter infusion affects antral motility in females, but not in men (12). Assuming a similar bitter sensitivity of the volunteer group as in these previous studies, n=14 is sufficient to achieve at least 80% power to find a difference of 15% motilin plasma levels at $\alpha=0.05$ (14).

Test compounds

The synthetic bitter compound denatonium benzoate (DB) and the former anti-malarial drug which is now used as a food additive, quinine hydrochloride (QHCl) were purchased from Sigma-Aldrich (St Louis, MO, USA) and Fagron (Rotterdam, The Netherlands) respectively. Both solutions were prepared in Milli-Q water and the dose was determined based on similar studies (12, 13, 25). Product administration concentrations were 1 μ mol/kg body weight, 10 μ mol/kg body weight and 0.1 ml/kg body weight for DB, QHCl and placebo (PLC) (water) respectively.

Study design

This study was a placebo-controlled, single-blind, randomized, six-visit crossover study (Figure 1). After an overnight fast, the volunteers visited the endoscopy department of the UZ Leuven hospital. A nasogastric-duodenal feeding tube was positioned into the upper part of the stomach or proximal part of the duodenum, controlled with a pH strip or fluoroscopy respectively. A trained nurse placed an intravenous dwelling line for blood collection, followed by a 10 minutes recovery period. PLC, DB or QHCl were administered in a single-blinded fashion. A reference blood sample was collected, 10 minutes before PLC, DB or QHCl administration, continued by blood collections every 10 minutes for a period of two hours. Next, the feeding tube and dwelling line were removed and chocolate milkshake (1 kcal / 1 ml) was offered to the volunteers to drink ad libitum. The chocolate milkshake recipe was as follows: 710 mL of 2% milk (Lactel, Brussels, Belgium), 4 table spoons of Imperial's chocolate topping (Continental foods, Puurs, Belgium) and 8 scoops of vanilla ice-cream (Ijsboerke, Tienen, Belgium). Homeostatic hunger sensations were scored every 10 minutes during the whole experiment on a 100 mm visual analogue scale (VAS) and right before and after chocolate milkshake drinking. Taste of the chocolate milkshake was scored on a VAS.

113 **Blood collections**

114 Blood samples for motilin analysis were collected in lithium heparin tubes, and acyl-ghrelin, GLP-1,
115 SST and CCK samples were collected in EDTA-coated tubes all containing 500 kIU aprotinin/mL
116 (Roche Applied Science, Penzberg, Germany). Additional DPP IV inhibitor (0.1 mM; Millipore, The
117 Netherlands) was added for GLP-1 and phenylmethanesulfonyl fluoride solution (0.57 μ M; Sigma-
118 Aldrich, Steinheim, Germany) for acyl-ghrelin to reduce further degradation. Blood samples were
119 centrifuged at 4°C for 10 minutes at 1400 X g. Acyl-ghrelin plasma was further acidified to a final
120 concentration of 0.1 N HCl, extracted on a Sep-Pak C18 column (Waters Corporation, Milford, MA)
121 and vacuum-dried. All plasma samples were directly stored at -80°C until they were used for analysis.
122 Motilin, acyl-ghrelin and CCK levels were determined according to a radioimmunoassay as is
123 described in earlier studies (10, 26, 27). A commercial kit (EKE-060-03, Phoenix Pharmaceuticals,
124 Burlingame, USA) measured SST levels, and GLP-1 levels was measured by the Active GLP-1 (ver. 2)
125 Assay kit (Meso Scale Discovery, Maryland, USA).

126 **Statistical analysis**

127 SAS University Edition software (SAS institute, Cary, NC, USA) was used to analyze the data. To
128 ensure that all the assumptions were met, transformations were applied if data were not normally
129 distributed. These data will be presented as the transformed values. Significance was set at $P < 0.05$
130 and the data were presented as mean $\pm$ SEM.

131 First, baseline values (time = -10) were compared between the six treatments using a mixed model
132 One-Way ANOVA. Next, statistical analysis of all hormonal changes (motilin, acyl-ghrelin, CCK, GLP-1
133 and SST) or hunger sensations over time were calculated based on their delta value (absolute values
134 subtracted with the baseline value). A two-way mixed model analysis was used to detect a possible
135 main effect of condition (which product), time or the interaction effect for IG and ID administration
136 separately. The *a priori* hypothesis was to compare the effect of DB and QHCl on hunger and gut
137 peptide release separately with the PLC condition 50-70 minutes after infusion, based on a previous
138 study (14). Stepdown Bonferroni correction was applied for planned contrast analysis testing these
139 specific hypotheses.

140 Milkshake intake, milkshake tasting and hunger scores right before and after milkshake were
141 analyzed with mixed model analysis to detect a possible main effect of condition, administration or
142 interaction effect.

Results

Effect on homeostatic hunger sensations

Absolute baseline hunger sensations did not differ between the six treatment arms ($P = 0.86$).

Both IG and ID PLC administration represented a steadily increase from the beginning until the end of the experiment (50 ± 6 mm to 68 ± 6 mm and from 52 ± 5 mm to 71 ± 5 mm, respectively). To test differences in hunger sensation changes between the two bitter compounds and PLC, a planned contrast analysis was performed for the time interval of 50-70 minutes after bitter infusion where DB and QHCl were compared individually with PLC. Hunger scores tended to increase less 50-70 minutes after IG DB infusion (6 ± 3 mm) in comparison to the PLC values (13 ± 2 mm), although the effect was not significantly different ($P_{\text{adj}} = 0.12$). QHCl significantly suppressed hunger sensations at this same time (3 ± 2 mm) in comparison with PLC, but the significance was lost after correction for multiple testing ($P = 0.049$; $P_{\text{adj}} = 0.10$). Hunger sensations were not affected after ID DB (8 ± 2 mm) or ID QHCl (10 ± 2 mm) infusion in comparison to ID PLC (11 ± 2 mm) ($P_{\text{adj}} = 0.71$ and $P_{\text{adj}} = 0.71$, respectively) (Figure 2).

Table 1

Site of infusion	Main effect	P-value	F-value	Num; Den Df
IG administration	Condition	0.18	1.83	2; 26
	Time	0.025	2.08	11; 143
	Interaction	0.18	1.29	22; 286
ID administration	Condition	0.61	0.50	2; 26
	Time	<0.001	4.35	11; 143
	Interaction	0.92	0.61	22; 286

Site of infusion	<i>A priori</i> hypothesis	P-value	P_{adj} -value	t-value	Df
IG administration	DB vs PLC	0.12	0.12	1.55	286
	QHCl vs PLC	0.049	0.10	1.98	286
ID administration	DB vs PLC	0.36	0.71	0.92	286
	QHCl vs PLC	0.68	0.71	0.41	286

Effect on motilin release

Absolute baseline for motilin plasma levels did not differ between the six treatment arms (Supplemental Table S1: <https://doi.org/10.6084/m9.figshare.14529261>; $P = 0.30$).

During both PLC infusions, motilin plasma levels fluctuated for every volunteer over the time period of two hours as was expected. IG QHCl significantly reduced motilin release 50-70 minutes after infusion in comparison to the PLC (-81 ± 20 pg/mL vs. 20 ± 26 pg/mL, respectively; $P_{\text{adj}} = 0.046$). No reduction was observed after IG DB infusion (5 ± 23 pg/mL; $P_{\text{adj}} = 0.76$). There was no difference in motilin release after ID infusion of QHCl in comparison with the control (-42 ± 21 vs. -53 ± 16 pg/mL, respectively; $P_{\text{adj}} = 1.00$), and a similar result was found after ID DB infusion (-26 ± 22 pg/mL; $P_{\text{adj}} = 1.00$) (Figure 3).

Table 2

Site of infusion	Main effect	P-value	F-value	Num; Den Df
IG administration	Condition	0.14	2.14	2; 25
	Time	0.26	1.26	11; 143
	Interaction	0.009	1.93	22; 261
ID administration	Condition	0.69	0.37	2; 24
	Time	0.002	2.83	11; 143
	Interaction	0.11	1.40	22; 256

Site of infusion	<i>A priori</i> hypothesis	P-value	<i>P</i> _{adj} -value	<i>t</i> -value	Df
IG administration	DB vs PLC	0.77	0.77	0.30	261
	QHCl vs PLC	0.023	0.046	2.29	261
ID administration	DB vs PLC	0.69	1.00	-0.39	256
	QHCl vs PLC	0.95	1.00	-0.07	256

167 Effect on acyl-ghrelin release

168 Absolute baseline plasma levels for acyl-ghrelin were not different between the six treatments
 169 (Supplemental Table S2: <https://doi.org/10.6084/m9.figshare.14529261>; *P* = 0.22).

170 Delta acyl-ghrelin plasma levels showed a small, non-significant reduction 50-70 min after IG QHCl
 171 infusion compared to the PLC (-13 ± 4 pg/mL vs. -2 ± 4 pg/mL, respectively; *P*_{adj} = 0.26). IG DB
 172 however significantly reduced acyl-ghrelin release (-31 ± 7 pg/mL; *P*_{adj} = 0.028). This strong DB effect
 173 was lost when it was ID administered (-1 ± 5 pg/mL vs. -12 ± 5 pg/mL, for DB and PLC respectively;
 174 *P*_{adj} = 0.26). Although the absolute reduction after ID QHCl infusion was similar with the IG DB
 175 infusion, its effect on acyl-ghrelin plasma levels was not significantly different from ID PLC infusion ($-$
 176 33 ± 8 vs. -12 ± 5 ; *P*_{adj} = 0.22) (Figure 4).

Table 3

Site of infusion	Main effect	P-value	F-value	Num; Den Df
IG administration	Condition	0.09	2.65	2; 25
	Time	0.001	3.06	11; 143
	Interaction	0.0001	2.67	22; 272
ID administration	Condition	0.17	1.89	2; 26
	Time	0.39	1.07	11; 143
	Interaction	0.55	0.93	22; 282

Site of infusion	<i>A priori</i> hypothesis	P-value	<i>P</i> _{adj} -value	<i>t</i> -value	Df
IG administration	DB vs PLC	0.014	0.028	2.47	272
	QHCl vs PLC	0.26	0.26	1.13	272
ID administration	DB vs PLC	0.26	0.26	-1.12	282
	QHCl vs PLC	0.11	0.22	1.61	282

Effect on anorexigenic GLP-1 and CCK gut peptide release

These plasma levels were measured during the fasted state. GLP-1 values ranged between 0 and 5.94 pg/mL. The commercial kit had a lower limit of detection of 0.12 pg/mL, resulting in a loss of more than 50% of the data, therefore no meaningful statistics could be performed.

Baseline CCK values were logarithmically transformed for the statistics, and did not differ between the 6 treatment arms (Supplemental Table S3: <https://doi.org/10.6084/m9.figshare.14529261>; $P = 0.47$).

Delta CCK values were transformed according to the box-cox method. Data were presented as such for the statistics, though data in figure 5 were absolute changes from baseline without transformation. No significant difference was observed between PLC and DB or QHCl, 50-70 minutes after IG infusion (31.5 ± 0.4 pmol/L vs. 32.2 ± 0.7 pmol/L or 30.0 ± 0.4 pmol/L, respectively; $P_{adj} = 0.55$ and $P_{adj} = 0.38$). Similar results on CCK changes were seen after ID infusion, where both DB and QHCl lack an effect compared to the PLC condition (29.9 ± 1.2 pmol/L vs. 33.4 ± 1.8 pmol/L or 30.3 ± 0.6 pmol/L for PLC, DB and QHCl respectively; $P_{adj} = 0.54$ and $P_{adj} = 0.80$) (Figure 5).

Table 4

Site of infusion	Main effect	P-value	F-value	Num; Den Df
IG administration	Condition	0.56	0.59	2; 26
	Time	0.07	1.73	11; 143
	Interaction	0.002	2.22	22; 275
ID administration	Condition	0.42	0.89	2; 26
	Time	0.54	0.91	11; 143
	Interaction	0.14	1.35	22; 279

Site of infusion	<i>A priori</i> hypothesis	P-value	P_{adj} -value	t-value	Df
IG administration	DB vs PLC	0.55	0.55	-0.59	275
	QHCl vs PLC	0.19	0.37	1.32	275
ID administration	DB vs PLC	0.27	0.54	-1.10	279
	QHCl vs PLC	0.82	0.82	-0.23	279

Effect on the candidate gastric inhibitory factor, SST

One volunteer was excluded from these data as the values were 10-fold of the general group ($n = 13$). Absolute baseline SST plasma values did not differ between conditions (Supplemental Table S4: <https://doi.org/10.6084/m9.figshare.14529261>; $P = 0.88$).

Delta SST plasma levels were not affected 50-70 minutes after any IG bitter infusion compared to the PLC (-14 ± 12 pg/mL vs. -41 ± 10 pg/mL or -32 ± 13 pg/mL for PLC, DB and QHCl respectively; $P_{adj} = 0.48$ and $P_{adj} = 0.48$). ID PLC infusion, during the same time window, was not different from the ID DB infusion (-20 ± 9 pg/mL vs -2 ± 16 pg/mL; $P_{adj} = 0.90$), nor was ID QHCl infusion (-14 ± 7 ; $P_{adj} = 0.90$) (Figure 6).

Table 5

Site of infusion	Main effect	P-value	F-value	Num; Den Df
IG administration	Condition	0.59	0.54	2; 24
	Time	0.15	1.46	11; 132
	Interaction	0.22	1.23	22; 248
ID administration	Condition	0.70	0.37	2; 22
	Time	0.015	2.25	11; 132
	Interaction	0.003	2.13	22; 228

Site of infusion	<i>A priori</i> hypothesis	P-value	<i>P</i> _{adj} -value	<i>t</i> -value	Df
IG administration	DB vs PLC	0.17	0.48	-1.18	248
	QHCl vs PLC	0.35	0.48	-0.71	248
ID administration	DB vs PLC	0.49	0.90	0.76	228
	QHCl vs PLC	0.73	0.90	0.34	228

200 Chocolate milkshake volume intake

201 Chocolate milkshake was consumed 120 minutes after bitter or PLC infusion. The ingested volume of
 202 milkshake did not differ between treatment arms ($P = 0.70$). Hunger sensations were scored
 203 immediately prior to drinking the milkshake, showing that suppression of hunger, induced by bitter
 204 infusion, had already normalized 120 min after infusion ($P = 0.92$). Taste perception of the chocolate
 205 milkshake was also not affected by the different treatments ($P = 0.14$).

Table 6

	Hunger before milkshake	Milkshake volume	Taste milkshake
	Mean $\pm$ sem (mm)	Mean $\pm$ sem (g)	Mean $\pm$ sem (mm)
IG_PLC	67 $\pm$ 6	384 $\pm$ 25	71 $\pm$ 4
IG_DB	67 $\pm$ 7	356 $\pm$ 28	73 $\pm$ 4
IG_QHCl	71 $\pm$ 5	403 $\pm$ 37	77 $\pm$ 4
ID_PLC	69 $\pm$ 6	419 $\pm$ 28	77 $\pm$ 4
ID_DB	71 $\pm$ 5	398 $\pm$ 31	73 $\pm$ 5
ID_QHCl	71 $\pm$ 5	398 $\pm$ 28	76 $\pm$ 3

Discussion:

This study shows that the stomach is the site of bitter administration to suppress appetite. The flavor additive QHCl but not DB suppresses fasted hunger sensations one hour after infusion, and simultaneously reduces motilin plasma levels. DB reduces acyl-ghrelin, but not motilin plasma levels. Two hours after infusion, the hunger scores normalize at the level of the placebo for all treatment arms and hence no difference in milkshake volume intake at this time point is found.

Already several years, bitter tastants are speculated to be involved in gut peptide release and to influence hunger sensations via the gut-brain axis (28). EECs express TAS2Rs and based on extensive *in vitro* research, bitter tastants are able to affect gut peptide release (5, 11, 29-32). Although not all bitter compounds are poisonous, some have the characteristics to evoke a repellent reaction (33, 34), including hunger suppression and early termination of food intake (9, 12-14). Conflicting results have been reported with respect to the effect of bitter tastants in appetite and meal volume control in clinical studies in humans (12-14, 35) (15-17, 36). The contradiction derives from differences in study design: the site of infusion, use of different bitter tastants and thus another range of TAS2R targets, timing of infusion in respect to the meal and sensitivity of the volunteer population. Adverse reactions such as nausea or vomiting were not elicited in any of the studies.

In one of the earliest human studies, bitter tastants were infused into the small bowel with the aim to stimulate release of anorexigenic peptides CCK and GLP-1 (16). Both are released from enteroendocrine open-cell types dispersed over the duodenum-jejunum and ileum-colon respectively (37, 38). These hormones induce satiation via the vagus in the brain and indirectly by the delay of gastric emptying (39). During the fasted state however, plasma concentrations of both peptides verge on the detection limit (40, 41). Intragastric, nor intraduodenal bitter infusion evoked their release into the plasma. Postprandial gut peptide release is not considered in this study design. Bitarafan *et al.* reported a lack of effect on CCK release after an ID quinine infusion (15). IG quinine infusion does not affect total GLP-1 plasma levels before a meal, but postprandial levels are higher than the control (42). Another paper observed elevated CCK plasma levels, although these results are doubtful given the extremely high values and the use of a kit that is not specific for CCK, and also measures gastrin (43). The need for vigilance with regard to insensitivity of commercial peptide kits and their interpretation is recently discussed by Rehfeld (27, 44). Moreover, CCK might be less attractive as a weight-loss target, as it has a short half-life time and its satiation-inducing effect disappears between 15 to 30 minutes after meal termination (45). GLP-1 might be more interesting as it maintains its effect between 60 to 120 minutes (46). GLP-1 agonists with an even longer half-life, such as liraglutide, are currently one of the few effective pharmacological weight-loss treatments (47).

In comparison to the duodenum, the effect of IG infusion on appetite is remarkable. One hour after IG bitter administration, specifically for QHCl, hunger is lower than the placebo infusion. After, this effect disappears gradually and hunger feelings restore to placebo treatment levels 120 minutes after infusion. The mechanism underlying this site of administration effect is presumed to be the reduction of orexigenic peptide release. Motilin, although even discovered before ghrelin (48), is an insufficiently researched gut peptide, mainly due to its absence in rodents (49) and lack of a readily available peptide assay. It is the most important initiator of the active phase of the antral MMC, which is associated with hunger pangs (19). In addition, intravenous infusion of motilin or its agonist erythromycin stimulates these contractions and induces a hunger peak (20). Obese patients, who lack the physiological motilin peak, restore their antral activity and appetite after a low dose of erythromycin (50). Our results show that one hour after IG QHCl infusion hunger sensations and motilin release are decreased, and after two hours these are both restored. In this study, motilin

plasma levels are not affected by IG DB administration, which is in contrast with previous results of our research group (12). One explanation might be that DB affects an upstream pathway of motilin release (e.g. stomach pH, bile acid release), which is MMC dependent. Availability of a motilin antagonist would provide us the tools to define the exact role of motilin as a weight-loss target.

Plasma acyl-ghrelin levels reflect the metabolic state of the body. When energy sources are low, ghrelin stimulates hunger sensations steadily until it reaches its peak value associated with a spontaneous meal initiation (18). Unlike motilin, it is not associated with the MMC phases, although ghrelin infusion also evokes a phase III (26, 51). Two previous studies measured a reduction on acyl-ghrelin release by QHCl infusion (13, 14), which is in contrast with the present study. It is possible that the timing of the *a priori* hypothesis is chosen less ideally to find changes on ghrelin release, considering that our colleagues found an effect 40 and 90 minutes after infusion. IG DB reduces acyl-ghrelin, even for a period of two hours. We have shown with this treatment that hunger sensations are slightly, but not significantly, reduced in comparison to the PLC treatment one hour after infusion. Similar results have been found in previous studies, where the link between interdigestive hunger and acyl-ghrelin is less dominant compared to motilin (13, 52). Here, we observe a 23% acyl-ghrelin plasma reduction and unchanged motilin plasma levels, leading to a truncated hunger reduction. IG DB infusion is known to delay the return of hunger after a meal, and therefore its effect might be more pronounced at a later time (12). QHCl does not significantly change acyl-ghrelin plasma levels, although there is a reduction of almost 20%. There might be a slight effect as QHCl is easily absorbed in the small bowel, and P/D1 cells in the stomach are mainly closed-cell types and can be exposed by nutrients from the basal side (53, 54).

It is surprising that IG, and not ID infusion, of a bitter flavour additive, reduces motilin release from cells only present in the duodenum and not in the stomach (23). Therefore, we hypothesize that a gastric inhibitory factor in the stomach is stimulated by the IG bitter infusion, and that reduces in its turn the release of motilin. SST is a peptide with an inhibitory, regulating function released from D-cells with high densities in the antral part of the stomach, although it is also expressed more distally in the small bowel and pancreas (55, 56). Antral located D-cells release SST after triggers such as acid exposure or different nutrients. The inhibitory peptide is involved in the control of several gastric and endocrine processes (57). Intravenous SST administration inhibits motilin release in dogs (58) and ghrelin release in rats (59). Based on the results of this study, we do not confirm our hypothesis that bitter tastants affect SST plasma levels. However, it should be mentioned that two main forms of SST exist. SST14 has a paracrine function and SST28 is proportional the most present in the plasma and doubles in release as a response to a meal (60). The ELISA used for this study measures SST-14, SST-28, SST-25 and [Des-Ala1]-SST. We do believe that the hormonal SST-28 would be responsible for changes in motilin and ghrelin plasma levels, and this SST-form has the biggest proportional representation in the plasma. To distinguish between the different SST-form, a more specialized analysis is required (61). It must be mentioned that this kit has not yet been validated for human plasma samples by the literature, but has been used for human *in vitro* work and rodent *in vivo* studies (62-64).

The chocolate milkshake is meant to be a palatable food item, from which the volunteers are craving to drink. In spite of changes in gut peptide release and even suppressed hunger sensations, none of the treatments influence the volume milkshake consumption. The absence of an effect is probably explained by the meal timing. The timespan of two hours is essential to understand how the peptide release is governed, but our effect on hunger and gut peptides is situated 60 minutes after infusion and these values normalized right before drinking the milkshake. IG QHCl infusion significantly reduces milkshake volume intake in a previous study with the 60 minutes timing (14).

As a last issue, the proportion of bitter sensitive volunteers in the study population is often overestimated. QHCl activates 9 TAS2Rs and DB 8 TAS2Rs of which 5 are in common with QHCl. One of these receptors, TAS2R43, activated by both compounds shows a deletion polymorphism in 33% of the Western population which may result in a reduced bitter taste sensitivity. TAS2R46 also contains several haplotypes, each with a different affinity to bind quinine. Genotyping can reveal if the target TAS2Rs are functionally present or which type of phenotype is involved. Evaluating for smaller populations will probably be a case-by-case interpretation whether an effect is expected or not (65-67). In the present study, we do not screen the volunteers for their sensitivity and therefore we might lose significance of several tests due to this heterogeneity. It has not yet been elucidated if lingual sensitivity is correlated with TAS2R expression of specific gut regions, but women who are generally better at tasting experience reduced antral contractions after DB infusion in comparison to men (12). If similar studies are performed in the overweight or obese population, it needs to be taken into account that the TAS2R expression pattern in the gut is affected, and may affect the outcome of bitter infusion (32). Indeed, *in vitro* studies have shown that the effect of DB on ghrelin release in isolated mucosal segments was selectively blunted in the human small intestine but not in the fundus. (62).

Conclusion:

In future studies, the quinine food additive should be administered one hour before a meal into the stomach with a similar dose as given in this study to reduce motilin plasma values and suppress fasted hunger pangs. DB has an interesting potential to reduce acyl-ghrelin plasma levels. TAS2Rs should be standardized in future studies, to give more insights in the volunteer pool sensitivity.

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Figure Captions

Figure 1 - Schematic overview of the study design. The timeline starts with a 10 minutes adaptation period after the placement of both an intragastric feeding tube and an intravenous dwelling line. A first blood sample is taken at time point -10. At time point 0: DB (1 $\mu\text{mol/kg}$), QHCl (10 $\mu\text{mol/kg}$) or placebo (0.1 ml/kg) are administered into the stomach or duodenum. For 2 hours, every 10 minutes blood samples are taken. When both catheters are removed 2 hours after the administration, the volunteers drink chocolate milkshake ad libitum until fully satisfied. At every time point presented in the schematic overview (from -20 until 120), the volunteer scores hunger on a VAS scale (100 mm).

Figure 2 - Effect of placebo (PLC), denatonium benzoate (DB) and quinine hydrochloride (QHCl) on hunger sensations, scored by a 100 mm visual analogue scale ($n = 14$). Data were presented as delta hunger sensations adjusted by baseline (time = -10) and presented as mean $\pm$ SEM. A) Intragastric (IG) infusion of the different conditions. B) Intraduodenal (ID) infusion of the different conditions. An *a priori* condition effect was expected 50 to 70 minutes after infusion, which is analyzed with a two-way mixed model analysis and *P*-values were adjusted for multiple testing by stepdown Bonferroni; #*P*_{adj} < 0.1.

Figure 3 - Effect of placebo (PLC), denatonium benzoate (DB) and quinine hydrochloride (QHCl) on motilin release ($n = 14$). Data were presented as delta motilin values adjusted by baseline (time = -10) and presented as mean $\pm$ SEM. A) Intragastric (IG) infusion of the different conditions. B) Intraduodenal (ID) infusion of the different conditions. An *a priori* condition effect was expected 50 to 70 minutes after infusion, which is analyzed with a two-way mixed model analysis and *P*-values were adjusted for multiple testing by stepdown Bonferroni; **P*_{adj} < 0.05.

Figure 4 - Effect of placebo (PLC), denatonium benzoate (DB) and quinine hydrochloride (QHCl) on acyl-ghrelin release ($n = 14$). Data were presented as delta acyl-ghrelin values adjusted by baseline (time = -10) and presented as mean $\pm$ SEM. A) Intragastric (IG) infusion of the different conditions. B) Intraduodenal (ID) infusion of the different conditions. An *a priori* condition effect was expected 50 to 70 minutes after infusion, which is analyzed with a two-way mixed model analysis and *P*-values were adjusted for multiple testing by stepdown Bonferroni; **P*_{adj} < 0.05.

Figure 5 - Effect of placebo (PLC), denatonium benzoate (DB) and quinine hydrochloride (QHCl) on cholecystokinin release (CCK) ($n = 14$). Data were presented as delta CCK values adjusted by baseline (time = -10) and presented as mean $\pm$ SEM. A) Intragastric (IG) infusion of the different conditions. B) Intraduodenal (ID) infusion of the different conditions.

Figure 6 - Effect of placebo (PLC), denatonium benzoate (DB) and quinine hydrochloride (QHCl) on somatostatin release (SST) ($n = 13$). Data were presented as delta somatostatin values adjusted by baseline (time = -10) and presented as mean $\pm$ SEM. A) Intragastric (IG) infusion of the different conditions. B) Intraduodenal (ID) infusion of the different conditions.

Table Captions

Table 1 - Overview of the two-way mixed model analysis results for each site of administration. Condition, time and condition-by-time are the main effects. Significance of the *P*-value was set at 0.05. The *a priori* hypothesis was to compare the effect of denatonium benzoate (DB) and quinine hydrochloride (QHCl) on hunger scores separately with the placebo (PLC) condition 50-70 minutes after infusion. The multiple testing was adjusted with step-down Bonferroni (*P*_{adj}-value). IG = intragastric; ID = intraduodenal; Df = Degrees of Freedom.

Table 2 - Overview of the two-way mixed model analysis results for each site of administration. Condition, time and condition-by-time are the main effects. Significance of the *P*-value was set at

0.05. The a priori hypothesis was to compare the effect of denatonium benzoate (DB) and quinine hydrochloride (QHCl) on motilin release separately with the placebo (PLC) condition 50-70 minutes after infusion. The multiple testing was adjusted with step-down Bonferroni (Padj-value). IG = intragastric; ID = intraduodenal; Df = Degrees of Freedom.

Table 3 - Overview of the two-way mixed model analysis results for each site of administration. Condition, time and condition-by-time are the main effects. Significance of the P-value was set at 0.05. The a priori hypothesis was to compare the effect of denatonium benzoate (DB) and quinine hydrochloride (QHCl) on acyl-ghrelin release separately with the placebo (PLC) condition 50-70 minutes after infusion. The multiple testing was adjusted with step-down Bonferroni (Padj-value). IG = intragastric; ID = intraduodenal; Df = Degrees of Freedom.

Table 4 - Overview of the two-way mixed model analysis results for each site of administration. Condition, time and condition-by-time are the main effects. Significance of the P-value was set at 0.05. The a priori hypothesis was to compare the effect of denatonium benzoate (DB) and quinine hydrochloride (QHCl) on cholecystokinin release separately with the placebo (PLC) condition 50-70 minutes after infusion. The multiple testing was adjusted with step-down Bonferroni (Padj-value). IG = intragastric; ID = intraduodenal; Df = Degrees of Freedom.

Table 5 - Overview of the two-way mixed model analysis results for each site of administration. Condition, time and condition-by-time are the main effects. Significance of the P-value was set at 0.05. The a priori hypothesis was to compare the effect of denatonium benzoate (DB) and quinine hydrochloride (QHCl) on somatostatin release separately with the placebo (PLC) condition 50-70 minutes after infusion. The multiple testing was adjusted with step-down Bonferroni (Padj-value). IG = intragastric; ID = intraduodenal; Df = Degrees of Freedom.

Table 6 - Values of milkshake volume consumption, taste and hunger before milkshake. Values are presented as mean $\pm$ SEM. IG_PLC = intragastric placebo; IG_DB = intragastric denatonium benzoate; IG_QHCl = intragastric quinine hydrochloride; ID_PLC = intraduodenal placebo; ID_DB = intraduodenal denatonium benzoate; ID_QHCl = intraduodenal quinine hydrochloride.

A IG bitter tastant infusion on hunger sensations

B ID bitter tastant infusion on hunger sensations

A**IG bitter tastant infusion on motilin release** **B****ID bitter tastant infusion on motilin release**

A IG bitter tastant infusion on acyl-ghrelin release

B ID bitter tastant infusion on acyl-ghrelin release

A**IG bitter tastant infusion on CCK release** **B****ID bitter tastant infusion on CCK release**

A**IG bitter tastant infusion on SST release** **B****ID bitter tastant infusion on SST release**

Table 1 - Overview of the two-way mixed model analysis results for each site of administration. Condition, time and condition-by-time are the main effects. Significance of the P-value was set at 0.05. The a priori hypothesis was to compare the effect of denatonium benzoate (DB) and quinine hydrochloride (QHCl) on hunger scores separately with the placebo (PLC) condition 50-70 minutes after infusion. The multiple testing was adjusted with step-down Bonferroni (P_{adj} -value). IG = intragastric; ID = intraduodenal; Df = Degrees of Freedom.

Site of infusion	Main effect	P-value	F-value	Num; Den Df
IG administration	Condition	0.18	1.83	2; 26
	Time	0.025	2.08	11; 143
	Interaction	0.18	1.29	22; 286
ID administration	Condition	0.61	0.50	2; 26
	Time	<0.001	4.35	11; 143
	Interaction	0.92	0.61	22; 286

Site of infusion	A priori hypothesis	P-value	P_{adj} -value	t-value	Df
IG administration	DB vs PLC	0.12	0.12	1.55	286
	QHCl vs PLC	0.049	0.10	1.98	286
ID administration	DB vs PLC	0.36	0.71	0.92	286
	QHCl vs PLC	0.68	0.71	0.41	286

Table 2 - Overview of the two-way mixed model analysis results for each site of administration. Condition, time and condition-by-time are the main effects. Significance of the P-value was set at 0.05. The a priori hypothesis was to compare the effect of denatonium benzoate (DB) and quinine hydrochloride (QHCl) on motilin release separately with the placebo (PLC) condition 50-70 minutes after infusion. The multiple testing was adjusted with step-down Bonferroni (P_{adj} -value). IG = intragastric; ID = intraduodenal; Df = Degrees of Freedom.

Site of infusion	Main effect	P-value	F-value	Num; Den Df
IG administration	Condition	0.14	2.14	2; 25
	Time	0.26	1.26	11; 143
	Interaction	0.009	1.93	22; 261
ID administration	Condition	0.69	0.37	2; 24
	Time	0.002	2.83	11; 143
	Interaction	0.11	1.40	22; 256

Site of infusion	A priori hypothesis	P-value	P_{adj} -value	t-value	Df
IG administration	DB vs PLC	0.77	0.77	0.30	261
	QHCl vs PLC	0.023	0.046	2.29	261
ID administration	DB vs PLC	0.69	1.00	-0.39	256
	QHCl vs PLC	0.95	1.00	-0.07	256

Table 3 - Overview of the two-way mixed model analysis results for each site of administration. Condition, time and condition-by-time are the main effects. Significance of the P-value was set at 0.05. The a priori hypothesis was to compare the effect of denatonium benzoate (DB) and quinine hydrochloride (QHCl) on acyl-ghrelin release separately with the placebo (PLC) condition 50-70 minutes after infusion. The multiple testing was adjusted with step-down Bonferroni (*P*_{adj}-value). IG = intragastric; ID = intraduodenal; Df = Degrees of Freedom.

Site of infusion	Main effect	P-value	F-value	Num; Den Df
IG administration	Condition	0.09	2.65	2; 25
	Time	0.001	3.06	11; 143
	Interaction	0.0001	2.67	22; 272
ID administration	Condition	0.17	1.89	2; 26
	Time	0.39	1.07	11; 143
	Interaction	0.55	0.93	22; 282

Site of infusion	A priori hypothesis	P-value	P _{adj} -value	t-value	Df
IG administration	DB vs PLC	0.014	0.028	2.47	272
	QHCl vs PLC	0.26	0.26	1.13	272
ID administration	DB vs PLC	0.26	0.26	-1.12	282
	QHCl vs PLC	0.11	0.22	1.61	282

Table 4 - Overview of the two-way mixed model analysis results for each site of administration. Condition, time and condition-by-time are the main effects. Significance of the P-value was set at 0.05. The a priori hypothesis was to compare the effect of denatonium benzoate (DB) and quinine hydrochloride (QHCl) on cholecystokinin release separately with the placebo (PLC) condition 50-70 minutes after infusion. The multiple testing was adjusted with step-down Bonferroni (*P*_{adj}-value). IG = intragastric; ID = intraduodenal; Df = Degrees of Freedom.

Site of infusion	Main effect	P-value	F-value	Num; Den Df
IG administration	Condition	0.56	0.59	2; 26
	Time	0.07	1.73	11; 143
	Interaction	0.002	2.22	22; 275
ID administration	Condition	0.42	0.89	2; 26
	Time	0.54	0.91	11; 143
	Interaction	0.14	1.35	22; 279

Site of infusion	A priori hypothesis	P-value	P _{adj} -value	t-value	Df
IG administration	DB vs PLC	0.55	0.55	-0.59	275
	QHCl vs PLC	0.19	0.37	1.32	275
ID administration	DB vs PLC	0.27	0.54	-1.10	279
	QHCl vs PLC	0.82	0.82	-0.23	279

Table 5 - Overview of the two-way mixed model analysis results for each site of administration. Condition, time and condition-by-time are the main effects. Significance of the P-value was set at 0.05. The a priori hypothesis was to compare the effect of denatonium benzoate (DB) and quinine hydrochloride (QHCl) on somatostatin release separately with the placebo (PLC) condition 50-70 minutes after infusion. The multiple testing was adjusted with step-down Bonferroni (*P*_{adj}-value). IG = intragastric; ID = intraduodenal; Df = Degrees of Freedom.

Site of infusion	Main effect	P-value	F-value	Num; Den Df
IG administration	Condition	0.59	0.54	2; 24
	Time	0.15	1.46	11; 132
	Interaction	0.22	1.23	22; 248
ID administration	Condition	0.70	0.37	2; 22
	Time	0.015	2.25	11; 132
	Interaction	0.003	2.13	22; 228

Site of infusion	A priori hypothesis	P-value	P _{adj} -value	t-value	Df
IG administration	DB vs PLC	0.17	0.48	-1.18	248
	QHCl vs PLC	0.35	0.48	-0.71	248
ID administration	DB vs PLC	0.49	0.90	0.76	228
	QHCl vs PLC	0.73	0.90	0.34	228

Table 6 - Values of milkshake volume consumption, taste and hunger before milkshake. Values are presented as mean $\pm$ SEM. IG_PLC = intragastric placebo; IG_DB = intragastric denatonium benzoate; IG_QHCl = intragastric quinine hydrochloride; ID_PLC = intraduodenal placebo; ID_DB = intraduodenal denatonium benzoate; ID_QHCl = intraduodenal quinine hydrochloride.

	Hunger before milkshake	Milkshake volume	Taste milkshake
	Mean $\pm$ sem (mm)	Mean $\pm$ sem (g)	Mean $\pm$ sem (mm)
IG_PLC	67.1 $\pm$ 6.1	383.6 $\pm$ 25.0	71.4 $\pm$ 4.2
IG_DB	67.4 $\pm$ 6.5	356.2 $\pm$ 27.8	73.1 $\pm$ 3.7
IG_QHCl	70.5 $\pm$ 5.1	403.0 $\pm$ 36.5	77.4 $\pm$ 3.6
ID_PLC	68.5 $\pm$ 5.8	419.3 $\pm$ 27.8	77.0 $\pm$ 3.7
ID_DB	71.3 $\pm$ 5.3	397.7 $\pm$ 30.8	72.6 $\pm$ 4.6
ID_QHCl	71.1 $\pm$ 5.4	397.5 $\pm$ 28.2	76.4 $\pm$ 2.8

The endocrine effects of bitter tastant administration in the gastrointestinal system – Intragastric versus intraduodenal administration

METHODS

Six-visit crossover study (n = 14):

Infusion of placebo or bitter tastants:

Every 10 min:

Blood collections
Hunger VAS scales

During the fasted state, bitter tastants in the stomach suppress hunger hormone release, and hunger pangs. In the duodenum, no effect on satiety hormone release was found, nor on hunger scores.

CONCLUSION Bitter tastants should be infused into the stomach to reduce appetite and orexigenic peptide release. QHCl reduces appetite via motilin inhibition, one hour after infusion.