ORIGINAL ARTICLE

Glycemic response to low sugar apple juice treated with invertase, glucose oxidase and catalase

Running title: Glycemic response to low sugar apple juice

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Abstract

Objectives:

Investigating the effect on post-prandial glycemic and venous serum insulin response of an apple drink following conversion of its glucose to gluconate.

Volunteers/Methods:

In a double-blind randomized placebo-controlled clinical trial with cross-over design 30 male adults with impaired fasting glucose (IFG) received a drink of 500 ml: *1. Verum*: Apple juice treated with invertase, glucose oxidase/catalase (glucose 0.05 g; gluconate 18.2 g); *2. Control*: Untreated apple juice (free glucose 8.5 g; bound glucose 6.7 g; gluconate below detection limit). Postprandial fingerprick capillary blood glucose and venous serum insulin were measured twice at baseline and at times 0 (start of drink), 15, 30, 45, 60, 90 and 120 min. Gastrointestinal symptoms, stool consistency and satiety were also assessed.

Results:

The incremental area under the curve ($iAUC_{120}$) of glucose levels (primary parameter) was significantly lower after verum (mean±SD: 63.6±46.7 min x mmol/l) compared to control (mean±SD: 198±80.9 min x mmol/l) (ANOVA $F=137.4$, $p<0.001$; $\alpha=0.05$). Also $iAUC_{120}$ of venous serum insulin levels (secondary parameter) was significantly lower after verum (mean±SD: 2045±991 min x mmol/l) compared to control (3864.3±1941 min x mmol/l), (ANOVA $F=52.94$, $p<0.001$; $\alpha=0.025$). Further parameters of glucose metabolism and ISI=2/[AUC venous serum insulin x AUC glucose+1] were also improved after verum compared to control. Verum increased stool frequency and decreased stool consistency, as assessed by Bristol stool form scale.

Conclusions:

By enzymatic treatment of apple juice its sugar content could be reduced by 21% and postprandial glycemic and venous serum insulin response by 68% and 47%, respectively resulting in a reduction of glycemic load by 74.6% without any adverse gastrointestinal side-effects.

Introduction

A process was developed by which palatable, sugar and energy reduced juices were produced by conversion of free glucose and glucose bound in sucrose to gluconate/D-gluconolactone with the aid of invertase, glucose oxidase and catalase (1).

Blood glucose elevations after ingestion of a food item where gluconate replaces glucose are expected to be lower. In rats absorption of gluconate from the upper small intestine was only 20%, whereas glucose was completely absorbed (2). Most orally ingested gluconate thus reaches the large intestine. Absorbed gluconate is metabolized to glucose only to a minor extent as has been demonstrated by unchanged urinary excretion of a significant portion (60-85%) of parenterally administered gluconate (3).

The study aimed to provide evidence for a beneficial effect of conversion of glucose to gluconate in apple juice on postprandial glycemic and venous serum insulin response in men with impaired fasting glucose. According to EFSA the reduction of postprandial blood glucose responses (PBGR) may be considered a beneficial physiological effect (e.g. for volunteers with impaired glucose tolerance) if venous serum insulin responses are not disproportionally increased (4) and, according to WHO, low postprandial glycemia is given priority in food choice (5). Apple juice was selected as an example of juice based on the fact that it is one of the two most popular juices based on consumption data.

Gluconate and its derivatives are considered safe and permitted as food additives (E 575). Gluconate is also a metabolite of glucose oxidation. The daily production of gluconate from endogenous sources is about 450 mg/kg for a 60 kg person (3). The NOAEL of sodium gluconate determined from a 28 day study on rats was equal to 1000 mg/kg Wt for males and 2000 mg/kg Wt for females (3). However, it has been noted that when gluconate is orally consumed in large single doses exceeding 20 g, a laxative effect is observed (6). Yet daily intakes up to 20 g gluconate for supplementation of 2 g potassium are within the limits of recommended daily allowances (7). Although safety issues were not expected for this reason, safety parameters were assessed in these tests. To detect potential gastrointestinal side effects (osmotic diarrhoea due to non-absorbed gluconate), gastrointestinal symptoms

and stool consistency as well as scores of fullness and satiety were assessed before and after ingestion of the juices.

Since pH of treated apple juice was adjusted for palatability reasons by adding calcium and potassium hydroxides, plasma electrolytes beside other safety parameters were assessed before and 120 min after ingestion of the juices.

Methods

Design

The study was a cross-over, mono-centric, double-blind, randomised, placebo-controlled trial (Figure 1). The study was registered at ClinicalTrials.gov (identifier: NCT02542033). The study received approval (AZ 046/15) from an independent ethics committee (The Ethical Committee of the Medical Council of Schleswig-Holstein, Bad Segeberg, Germany)

Volunteers

Volunteers were recruited from the database of the study site and from advertisements. Written informed consent was obtained from all participants before any study specific procedure was performed. 30 male volunteers were included fulfilling the following eligibility criteria: age ≥ 18 y, diagnosed impaired fasting plasma glucose (5.6-6.9 mmol/l) (8). Main *exclusion criteria* were: food allergy, acute or chronic infections, renal insufficiency, gastrointestinal illness or surgery, fructose intolerance, diabetes mellitus, a disease or condition which might compromise significantly any body system except for a condition defined by the inclusion criteria. Individuals withdrawing or discontinuing prematurely were replaced.

Random Sequence Generation and Allocation Concealment

Volunteers were randomly assigned to either verum first, and then control product, or *vice versa*. To avoid selection bias, randomisation was generated independently according to the Cochrane guidelines (9). The randomisation list was kept confidential apart from those involved in product production (Nofima AS).

Test Products and Blinding of Participants and Personnel

Pasteurized conventional apple juice used as control was produced by Askim Frukt - og Bærpresseri AS, Askim, Norway. Composition of this juice is shown in Table 2. For verum sugar depleted apple juice was manufactured as follows with all enzymes and processing aids commercially available and EU approved as food grade. Control apple juice (95 l) was transferred to a kettle with lid, mixing, heating and cooling options (Proline Touch-Mix, Classic Gastro A/S, Denmark). The juice was warmed to 85°C and held there for 5 min and then cooled to 24°C. Invertase (Maxinvert L10000, DSM) was added (5 000 U/l) to split

sucrose overnight at room temperature (ca. 18-21°C). Next morning the content of sucrose was <0.01 g/l. To regulate pH prior to glucose oxidase/catalase treatment calcium hydroxide and potassium hydroxide were added. Glucose oxidase/catalase (Hyderase L, Amano/Mitsubishi, Japan) were added (3000 U/l) with simultaneous addition of molecular oxygen to maintain a constant supply into the reaction tank of 3 mg/l. pH was maintained at 3.6-4.6 by batch addition of solid calcium hydroxide and potassium hydroxide. An incubation of 12 h at room temperature was enough to convert almost all glucose to gluconic acid (glucose 0.1 g/l; Table 2). At the end of the incubation enzyme activity was terminated by stopping the oxygen supply. Glucose was monitored by a reflectometric kit (Reflectoquant, Merck) and HPLC. Sucrose content after invertase incubation was also measured in this way (<0.1 g/l; Table 2). Gluconate was determined via enzymatic assay (R-Biopharm). Prior to such analysis all enzymes were irreversibly denatured by boiling. The organoleptic properties were optimized by further addition of calcium and/or potassium hydroxide. The final pH of the mixture was approximately 4. It was pasteurised in a KTM-Troxler (Ettenheim, Germany) pasteur and bottled hot into identical 500 ml brown glass bottles, capped, cooled, labelled by coding with consecutive numbers according to the randomization protocol and stored in a fridge (1-4°C) prior to shipment to the study site. Verum and control were similar in flavour, color, texture, and appearance and identical in packaging throughout the study. The components of bottled juice (treated and untreated) were analysed by Eurofins Analytics, Nantes, France, a certified laboratory.

The study site ensured that the study products were stored according to the instructions given by the producer (Nofima AS, Ås, Norway) and kept in a secured location (fridge) to which only the investigator and designated study staff had access. Dispensing of study products was recorded in a product accountability log. Monitoring of product accountability was performed by the quality manager after the visits and at the end of the trial. Code-breaking systems were available in case of an adverse event.

Procedure/Conduct

After giving the informed consent, the medical history, concomitant medication and alimentary supplements were assessed at screening visit (V0) and fasting state was ascertained. Furthermore, vital signs and anthropometric data were collected. If all inclusion and no exclusion parameters were fulfilled the volunteer was enrolled into the study. Volunteers were requested to attend the next visit after an overnight fast of at least 12 h and provided with a diary for daily reply regarding adverse event and medication and with a questionnaire (EPIC FFQ) for recalling food frequency over the last 12 months, to be completed before the randomisation visit. Impaired fasting glucose was confirmed by two independent measurements (one from prior screenings during the preceding two years) and another at V0).

Interventional visit *1 (V1)* followed V0 within four weeks. Any adverse events since V0 were documented. If eligibility was confirmed the subject was randomised. Fasting for at least 12 h prior to this visit was checked and an intravenous catheter (Vasofix® Braunüle® 18G, Braun Melsungen, Germany) was inserted into a forearm vein for blood withdrawal at baseline, directly before ingestion of test product (time point 0). The bottle was shaken well, opened and its entire contents were ingested (500ml) by each volunteer within 5 min. The time point of ingestion was kept consistent between visit 1 and visit 2 in each individual and was in all cases between 8:00 and 8:40 a.m. At 15, 30, 45, 60, 90 and 120 min after starting the ingestion a venous blood sample was collected. Serum was separated within 60 min and stored at -20°C until venous serum insulin concentration was determined using a chemoluminescence immunoassay (Liaison®), DiaSorin S.p.A., Saluggia, Italy).

From the venous blood samples taken at baseline and 120 min after consumption of the test product safety parameters were determined on the day of blood withdrawal (serum Na, K, Ca, Mg, AST, ALT, γGT, CHE, AP, LDH, CK, bilirubin, creatinine, urea-N, uric acid, complete blood count, cholesterol, HDL-C, LDL-C, triglycerides, hsCRP). All laboratory parameters were determined in a certified laboratory (Laboratory Dr. Krause & Colleagues

MVZ GmbH, Kiel, Germany) using a Beckman Coulter AU analyser. Na and K were determined with selective electrodes. Ca was determined by photometry using arsenazo III as complexing agent and Mg using xylidylblue as complexing agent, AST by photometry measuring NADH after transamination of aspartate and 2-oxoglutarate to L-glutamate and oxaloacetate and reaction of the oxalacetate to L-malate catalysed by malate dehydrogenase, ALT by measuring NADH after transamination of alanine and 2-oxoglutarate to pyruvate and glutamate and reduction of pyruvate by LDH, yGT by photometric measurement of 5-amino-2-nitrobenzoate resulting from catalysis of gamma-glutamyl-3-carboxy-4-nitroanlide to glycylglycine, ALP by measuring p-nitrophenol at 410/480 nm resulting from the conversion of p-nitro-phenylphosphate, cholinesterase by detecting yellow hexacyanoferrate (III) us reduced by thiocholine to colourless hexacyanoferrate (II) after catalysis of the hydrolysis of butyrylthiocholine to butyrate and thiocholine, LDH by measuring NADH at 340nm resulting from oxidation of lactate to pyruvate and the reduction of NAD+ to NADH, CK by measuring NADPH resulting from the catalysis of CK, hexokinase and glucose-6-phosphate dehydrogenase, uric acid by detecting a blue dye resulting from the H_2O_2 reaction with N,N-bis(4-sulfobutyl)-3,5-dimethylaniline and 4-aminophenazone under catalysis by uricase and peroxidase, Urea by detecting NAD^+ resulting from the catalysis of urease and the GLDH catalyzed reaction of 2-Oxoglutarate + 2 $NH4^+$ + 2 NADH, creatinine by measuring a dye generated by catalysis through creatininase, creatinase, sarcosine oxidase and peroxidase, bilirubin by measuring azobilirubin after conjugation with 3,5-dichlorphenyl-diazonium-tetrafluorborate, HDL-C by quantification of cholesterol by an enzyme chromogen system after blocking enzymatic reaction with lipoproteins other than HDL (LDL, VLDL and chylomicrons) through anti-human-β-lipoprotein antibody, LDL-C by a homogeneous assay using an enzymatic selective protection method, triglycerides by detecting a chromophore produced in reactions catalyzed by lipases, glycerol kinase, glycerol phosphate oxidase and peroxidase, and hsCRP by turbimetric quantification of CRP bound to rabbit anti-CRP-antibodies coated on latex particles.

Fingerprick capillary blood glucose was measured instantaneously using an HemoCue® 201 analyzer (Radiometer GmbH, Willich, Germany) at the same time frequency intervals as for venous serum insulin.

Arterial blood pressure, pulse and waist circumference was assessed before and 120 min after ingestion as marker of abdominal bloating after ingestion of the test product. Volunteers completed validated questionnaires on gastrointestinal symptoms, the Gastrointestinal Symptom Rating Scale (GSRS) (10-12), which allows scoring of symptoms in 5 dimensions depicting abdominal pain, reflux, indigestion, constipation and diarrhoea syndrome, as well as a total symptom score based on standardized questions. The GSRS was assessed at time point 0 with regard to the previous three days and for the last h before starting ingestion. It was also assessed at time points 60 and 120 min with respect to the period 0 to 60 min and 60 to 120 min respectively. Stool frequency and stool form (Bristol Stool Scale) over the previous 3 days and previous 2 h (13) was *self*-assessed by the subject (questionnaires) directly also at time point 0. At 120 min time point these parameters were assessed again with respect to the previous 2 h.

Satiety, hunger, fullness and prospective food consumption were monitored during the visit by subject self-assessment at time point 0 as well as at time points 30, 60, 90 and 120 min using validated questionnaires. (14, 15). In these questionnaires visual analogue scales (VAS) were used, each with 100 mm in length and with words anchored at each end, expressing the most positive and the most negative rating. Volunteers could walk around at the study site, sit or lay down, but asked to abstain from eating, drinking or exercising during the test phase. The volunteers were surveyed during the whole observation period at the test day and adverse events were monitored. After the 2 h test period volunteers were provided with a diary for daily assessment of adverse events and medication. GSRS, stool frequency and stool form were assessed during the three-day lasting observation period starting with ingestion of the test drink at visit day V1 and two subsequent days.

Interventional visit 2 (V2) was scheduled on the seventh day after V1 at the earliest. Volunteers were requested to return their diaries and questionnaires. Adverse events since

V1 were documented. Fasting for 12 h prior to V2 was checked and the test was conducted as described for V1. Again, volunteers were provided with a diary for daily assessment of the sample parameters as at V1. Volunteers received a stamped envelope and were requested to send back their diaries and completed questionnaires. The first volunteer was selected on 20.05.2015, the first subject inclusion was on 28.05.2015 and the last visit of the last randomised subject was on 30.07.2015.

Outcome measures

The incremental area under the curve ($iAUC_{120}$) of the fingerprick capillary blood glucose levels from baseline to 120 min after ingestion of the test drinks was defined as the primary outcome. Although fingerprick capillary and venous blood glucose values have been shown to be highly correlated, fingerprick capillary blood samples are regarded preferable for reliable GI testing (16, 17). Therefore, glucose was determined using the HemoCue® 201 analyzer), which had been tested for glycemic index (GI) assessment (18). The iAUC was calculated according to Wolever, 2006 (19) ignoring the area under the baseline. The iAUC of the venous serum insulin levels from baseline to 120 min after ingestion (iAUC120) of the test drinks was defined as the secondary parameter.

Exploratory outcome measures included the iAUC ($iAUC_{60}$) of glucose and venous serum insulin levels from baseline to 60 min after ingestion of the test drinks, the postprandial glucose peak (G_{max}), the amplitude between baseline and G_{max} ($G_{max}–G_{base}$), and the maximal amplitude of glucose excursions ($G_{max}–G_{min}$) were calculated for further characterization of postprandial glucose response (20). Proportional reduction in glucose load (21) was calculated by $100-100(iAUC_{120verum} \times CH_{verum})/(iAUC_{120control} \times CH_{control})$, whereby CH was carbohydrate (sugar) content of verum (86.4 g) and control (109 g), respectively. Postprandial venous serum insulin sensitivity was expressed by $ISI=2/[AUC_{120}$ venous serum insulin x AUC_{120}glucose+1] (22, 23). Satiety, hunger, fullness and prospective food uptake were assessed before and 30, 60, 90 and 120 min after ingestion of test drinks according to (14, 15). The score values of each of these four sensations as well as their $iAUC_{120}$ were evaluated by two-way analysis of variance with repeated measures (ANOVA RM).

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280 ***Statistical Analysis***

281 The results were expressed as mean±SD, whereas mean±SEM were shown in the figures, in
282 order to better fit to the format of figures. Since no previous iAUC data were available for
283 apple juice, sample size estimation was based on literature data (24). After a 25 g glucose
284 load the authors found an $iAUC_{30}$=55.6±20.4 (mean±SEM) of glycemic response (GR). We
285 expected a reduction in GR by at least 30% by enzymatic treatment of the juice resulting in a
286 reduction of iAUC by 16.7. Further assuming a standard deviation of 23.0, a power of 0.95
287 and α=0.05, a sample size of N=27 was calculated for paired t-test. A sample size of N=30
288 was therefore defined for the trial.

289 To meet the Cochrane Collaboration recommendations for preventing detection bias
290 (9) blinding of outcome assessment was ensured by a blind review of raw data and by un-
291 blinding only after the database was locked, and by conducting statistical analysis in
292 compliance with the statistical analysis plan. Reporting bias by selective outcome reporting
293 (9) was prevented by the availability of the study protocol and pre-specification of (primary
294 and secondary) outcomes and by adhering to these specifications.

295 The Intention-To-Treat (ITT) collective was defined to comprise all volunteers
296 randomized and having taken at least one dose of the test products (intervention 1 at V1).
297 The Per-protocol (PP) set comprised all volunteers randomized, who have no major protocol
298 deviation. The analysis included the Full Analysis Set (FAS).

299 The baseline and demographic characteristics of the two groups with different order
300 of intervention (verum-control versus control-verum) were compared using Student's t or
301 Mann-Whitney test as appropriate depending on distribution of data. The effect of
302 intervention (verum and control) was evaluated by two-way analysis of variance with
303 repeated measures (ANOVA RM), to take cross-over design and potential effects by the
304 order of intervention into account. The intervention was the factor with repetition. The order

of treatments was not repeated. Normality (Shapiro-Wilk) and equal variance (Levene) was tested within the two-way ANOVA RM and confirmed for the primary ($iAUC_{120}$ of glucose) and secondary ($iAUC_{120}$ of insulin) parameter. The significance level of the primary and secondary parameters was adjusted to multiple testing according to Bonferroni-Holm.

Results

The distribution of volunteers through the study is shown in Figure 2. Volunteers (N=51) having had IFG in previous studies at the study site were screened for inclusion and exclusion criteria. In N=19 IFG was not verified and in N=1 an allergy was reported. Thus N=20 volunteers were excluded at screening and N=31 were enrolled. Between screening visit (V0) and randomization (V1) an unrelated erysipela occurred in N=1 individual. Consequently n=30 volunteers were randomized. There were neither drop-outs, missing data of main outcomes, nor major deviations from study protocol. Intention to treat (ITT), PP and FAS populations were therefore identical.

Population characteristics at baseline are shown in Table 1. The total population (ITT and PP) showed features of the metabolic syndrome suffering from overweight as indicated by elevated mean waist, blood pressure, fasting plasma glucose and triglycerides (Table 1). The baseline characteristics in the group with the order verum-control (VC) did not differ from those in the group with the order control-verum (CV).

By enzymatic treatment glucose and sucrose were mostly removed from apple juice (Table 2); Free fructose increased after cleavage of sucrose by invertase whereby total bioavailable fructose remained constant (Table 2). The sugar content was reduced by 21% with a 500 ml serving containing 18.2 g gluconate. The pH-value was similar between verum and control after addition of potassium and calcium hydroxides to the enzymatically treated verum juice. Potassium and calcium content accordingly differed between verum and control (Table 2).

The curves of fingerprick capillary blood glucose levels and venous serum insulin after ingestion of the test drinks differed considerably between verum and control (Figure 3). The $iAUC_{120}$ of glucose and venous serum insulin differed significantly ($p<0.001$; $\alpha=0.05$ and ($p<0.001$; $\alpha=0.025$ respectively) between verum and control. Similar differences were seen for $iAUC_{60}$, glucose maxima, the postprandial increase from baseline and the maximal glucose excursion and venous serum insulin sensitivity (Table 3). The order of intervention had no impact indicating that there were no significant carry-over effects (Table 3). By enzymatic treatment of apple juice GR to its oral ingestion was significantly reduced by 68%

resulting in a reduction of glycemic load (GL) by 74.6%. Concomitantly venous serum insulin response was also reduced by 47%.

Similar differences between verum and control were seen for $iAUC_{60}$ (Table 3). None of the assessed postprandial safety parameters showed any clinically relevant changes and remained within the normal range 120 min after ingestion of test drinks. Similarly, for satiety, hunger and prospective food uptake did not differ, neither in the fasting state nor post-prandially. Fullness ratings differed in the fasting state (time=0) between verum (12.9 mm ±6) and control (24.6±28.4 mm, p=0.04), but no longer in the following, postprandial assessments. Postprandial values of satiety, hunger, fullness and prospective food consumption did not differ between verum and control (Supplementary Figure 1). Fullness, however, differed before ingestion of the drinks (Supplementary Figure 1). The incremental area of satiety, hunger, fullness and prospective food consumption (expressed as iAUC) did not significantly differ (Supplementary Table 1).

Gastrointestinal symptoms did not differ at baseline 1 h before ingestion of the drinks, neither the total score, nor any of the dimensions pain, reflux, indigestion, diarrhoea or constipation. Within the first h after ingestion the total score was significantly higher in case of verum (1.14±0.22; mean±SD) compared to control (1.05±0.12, p=0.028) and the indigestion score was also higher (1.275±0.39) versus 1.117±0.313, p=0.008) (Supplementary Figure 2). During the second h after ingestion no differences between verum and control were seen (Supplementary Figure 2). This held true within the 3 days period before and beginning with ingestion of the test drinks (Supplementary Figure 2). The incremental area of gastrointestinal symptoms, as assessed by GSRS total score, pain, reflux, indigestion, constipation and diarrhoea score (expressed as iAUC, each) did not significantly differ (Supplementary Table 2).

Stool frequency did not differ between verum and control within the 2 h before ingestion but was significantly higher during the 2 h after consumption of the verum juice (0.567±0.935; mean±SD) compared to control (0.067±0.254), p=0.009). Accordingly stool form, as assessed by a Bristol Stool Form Scale, was looser during the 2 h after ingestion of verum (3.467±5.296) than after ingestion of control (0.333 ±1.295 p=0.004). Within the 3

days period beginning with ingestion of the test drinks no differences were reported, neither in stool frequency nor in stool form.

Four adverse events were observed. Three were assessed as ‘not related‘ to the study product (two respiratory tract infections, one case of accidental fall during the control visit). One case of diarrhoea (defined according to WHO) during the day of the verum visit was assessed as ‘probably related’ to the study product.

Discussion

The pronounced respective 68 and 74.6% reduction of GR and GL by only 21% reduction of sugar content is explained by the removed bioavailable glucose. This has a glycemic index (GI) of 100 whereas the remaining bioavailable fructose has a GI of only 19 (18). The choice of food with high GI and GL were shown to be associated with the risk for type 2 diabetes, coronary heart disease, stroke, gallbladder disease and breast cancer (25, 26). The significant reduction of venous serum insulin response to oral ingestion by 47% and the 7.39-fold increase in postprandial venous serum insulin sensitivity index underlines the beneficial effect in individuals with signs of the metabolic syndrome and impaired glucose metabolism in whom a reduction of venous serum insulin sensitivity is a key feature and islet cell function is becoming limited (27).

The effects on postprandial glycemia found after ingestion of the apple drink together with a mixed meal will depend on the type and amount of meal/apple drink macronutrients, such as available carbohydrate, protein, fat and dietary fibre (28). One might have expected some loss of sweetness of the juice by enzymatic removal of bioavailable glucose. Yet taste differences were not conspicuous. This is because of the release of fructose from sucrose. In comparative studies of sweetness, in which sucrose was set at 100, fructose had a sweetness of 173 and glucose a sweetness of 74 (29).

The conversion of bioavailable glucose to gluconate may have resulted in a reduction of caloric value of the juice. In pigs and humans gluconate was primarily fermented by microbiota in the large intestine (2, 30). After oral administration of 10 to 30g gluconate human volunteers excreted an amount varying from 7.7 to 15.0% of the dose in the succeeding 24 h (31). Unaltered satiety outcomes despite lower caloric intake may be considered promising for the dietary management of overweight. These findings, however, require confirmation by clinical trials with this focus.

Consistent with incomplete absorption of gluconate and potential osmotic effects, stool frequency transiently increased, and stool form became looser compared to control during the first hour after ingestion of the verum juice. This, however, was not accompanied by a statistically significant difference in diarrhea between the groups, neither as defined by

403 WHO (one event after verum compared to no event in control) nor as assessed by the
404 GSRS. According to EFSA, “maintenance of normal defecation by increasing stool frequency
405 (provided that it does not result in diarrhoea) is a beneficial physiological effect (31). Total
406 symptom score and the “indigestion” domaine, as assessed by GSRS, were transiently
407 higher 1 h after ingestion of verum compared to control (Supplementary Figure 2), but did not
408 differ when the complete 3 days observation period after ingestion of the juices was taken
409 into account. In this context one has to bear in mind that the evaluation of these parameters
410 had only exploratory character and therefore adjustment for multiple testing was not done.
411 Whether the symptom level will follow a dose-effect relation, remains to be clarified by dose-
412 effect studies. From the present study we can only say that 500 ml of apple drink (equivalent
413 to 2.5 servings) containing 18.2 g gluconate had no significant adverse effect in terms of
414 diarrhea. Which dose will be tolerated after ingestion of a drink has to be studied in a trial
415 dedicated to this goal. To clarify which dose will be tolerated with regular consumption and
416 what are the effects of the conversion of glucose to gluconate on intestinal microbiota in man
417 has to be investigated in long-term studies. According to the study by Asano et al., 1999,
418 gluconate is fermented selectively by the *Bifidohacterium adolescentis* group and some
419 species of other genera, including *Clostridium clostridiiforme, C. innocuum,*
420 *Propionibacterium acnes, Megasphaera elsdenii, Enterococcus faecium* and Klebsiella
421 pneumoniae; it, however, was not utilised by most other bacteria including *Bacteroidaceae*
422 (2). In 10 healthy volunteers the 9 g/d ingestion of glucono-6-lactone resulted in an increase
423 of the number of bifidobacteria, whereas *C. perfringens* decreased and *Enterobacteriaceae*
424 remained constant (2).

425 Calcium and potassium, which had been added to the enzymatically treated juice to
426 adjust pH for palatability reasons and to maintain activity of glucose oxidase during
427 production, did not show clinically relevant changes after ingestion of the test drinks.

428 In conclusion the enzymatic conversion of bioavailable glucose to gluconate
429 significantly reduced glycemic and venous serum insulin response to apple juice and its GL
430 and induced a similar satiety profile despite a lower caloric value which may be assumed
431 based on lower absorption and metabolism of gluconate compared to glucose.

Acknowledgements

We thank Andrea Liesegang, Maria Gatzmange, Tara Dezhahang and Hanne Zobel for their excellent support and for technical assistance. This research was financed by the "HealthBoost" project in the FORNY2020 verification program of the Research Council of Norway (Grant no: 243871). Additional financial support to SB and SHK was provided by a grant from the Norwegian Fund for Research Fees for Agricultural Products (Norwegian Research Council Grant no. 262300).

Conflict of interest

SB, SHK, JS: Are inventors of a patent on this matter (1) and hold shares (as does CL) in Glucozero GmBH. This company is currently licensing the patent from Nofima AS (full-time employer of SB and SHK).

Other information

The study was conducted in line with the principles of the Declaration of Helsinki (32), the guidelines for Good Clinical Practice (ICH E6) (33), and in accordance with European and National regulatory requirements. All clinical data were collected at the study site of the Clinical Research Center Kiel GmbH. Supplementary information is available at EJCN's website.

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Figure legends

Figure 1. Study design

Figure 2. Distribution of volunteers through the study.

Figure 3. Amplitude between baseline and peak fingerpick capillary blood glucose (**A**) and venous serum insulin (**B**) concentrations (mean ± SEM) before and after oral ingestion of 500 test product without (control) and with prior enzymatic treatment with invertase, glucose oxidase and catalase (verum) in 30 men with impaired fasting glucose.

Table 1 Population characteristics at baseline (mean ± SD)

(Mean ± SEM)	***Total Group*** (n=30)	***Group Order VC*** (n=15)	***Group Order CV*** (n=15)	***t-Test/ Mann-Whitney Test****
Age [years]	68.0 ± 6.5	68.7 ± 5.5	67.4 ± 7.5	p = 0.602
Body Height [m]	178.0 ± 6.9	177.9 ± 7.9	178.1 ± 5.9	p = 0.911
Body Weight [kg]	100.2 ± 16.4	98.3 ± 12.9	102.0 ± 19.6	p = 0.542
BMI [kg/m^2]	31.6 ± 4.8	31.1 ± 3.8	32.1 ± 5.6	p = 0.581
Waist [cm]	110.9 ± 12.6	110.3 ± 10.5	111.8 ± 14.4	p = 0.699
Syst. Blood Pressure [mmHg]	131.7 ± 15.0	129.3 ± 15.3	134.0 ± 14.8	p = 0.403
Diastol. Blood Pressure [mmHg]	81.8 ± 7.0	81.0 ± 7.8	82.7 ± 6.2	p = 0.524
Fasting Plasma Glucose [mmol/L]	6.04 ± 0.4	6.0 ± 0.3	6.08 ± 4.2	*p = 0.818
Fasting Plasma Triglycerides [mmol/L]	1.90 ± 1.12	1.87 ± 0.97	1.94 ± 1.28	*p = 0.648
Fasting Plasma HDL-C [mmol/L]	1.28 ± 0.25	1.28 ± 0.24	1.27 ± 0.27	p = 0.927
Total Energy [kJ/day]	10322 ± 3336	10451 ± 3526	10193 ± 3254	p = 0.836
Carbohydrates [g/day]	219.7 ± 72.6	222.9 ± 83.2	216.5 ± 63.0	p = 0.815
Proteins [g/day]	91.6 ± 31.1	88.7 ± 28.3	94.6 ± 34.5	p = 0.612
Fats [g/day]	118.1 ± 35.7	117.4 ± 32.4	118.7 ± 39.8	*p = 0.967
Cholesterol [g/day]	0.432 ± 0.13	0.433 ± 0.10	0.432 ± 0.15	p = 0.991
Calcium [g/day]	0.958 ± 0.36	0.923 ± 0.34	0.992 ± 0.39	p = 0.610
Iron [mg/day]	14.2 ± 5.0	13.9 ± 5.3	14.4 ± 4.9	*p = 0.648
Vitamin B12 [mg/day]	0.0080 ± 0.0030	0.0077 ± 0.0026	0.0083 ± 0.0035	p = 0.637
Fibre [g/day]	18.2 ± 6.1	17.6 ± 6.4	18.8 ± 6.0	p = 0.595

Mann-Whitney test and t-test, respectively, were used depending on the distribution of data.

Table 2 Composition of apple juice with (verum) and without (control) enzymatic treatment using invertase, glucose oxidase and catalase and pH adjustment using calcium and potassium hydroxides. Numbers in brackets are measurement uncertainty.

	Apple Juice	
g/L	**untreated**	**treated**
Glucose	17 (1.2)	[a]0.1 (0.05)
Fructose	65.2 (2.8)	86.3 (3.5)
Sucrose	26.7 (3.2)	[a]0.01 (0.005)
Sugar*	109 (7.2)	86.4 (3.5)
Gluconate	**- <0.0005	36.4 (0.55)
Calcium	0.032 (0.0096)	1.5 (0.45)
Potassium	0.960 (0.14)	3.1 (0.47)
Sodium	<0.001 (0.0002)	<0.0023 (0.0003)
pH	3.1	3.7

*Sugar: Total content of glucose, fructose and sucrose
**below limit of quantitation

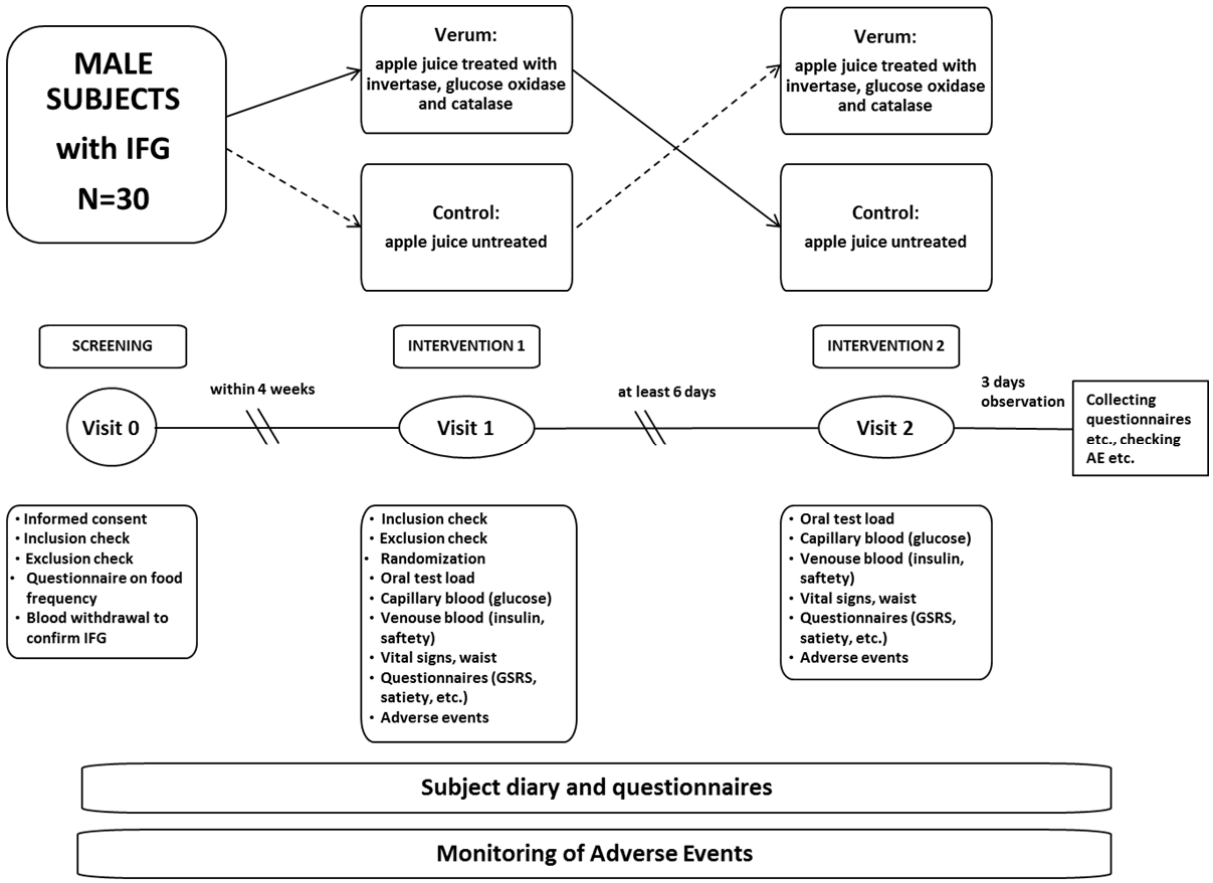
[a]Determined by Merck reflectoquant and HPLC

Numbers in brackets are measurement uncertainty

Table 3. Glycemic and insulin response to apple juice *without* (control) and *with* (verum) enzymatic treatment

Parameter (mean±SD)	***Verum*** (N=30)	***Control*** (N=30)	***F***	***V versus C*** *p*	**Carry-Over** *p*	**Normality failed**	**Equal Var. Failed**
$iAUC_{120}$ Glucose [min x mmol/L]	63.6 ± 46.73	198.0 ± 80.92	137.4	< 0.001	0.866		
$iAUC_{60}$ Glucose [min x mmol/L]	29.7 ± 17.57	108.0 ± 36.89	217.1	< 0.001	0.945		
Gmax [mmol/L]	6.97 ± 0.88	8.77 ± 1.39	138.3	< 0.001	0.876		
Gmax–G base [mmol/L]	0.984 ± 0.55	2.796 ± 0.92	156.8	< 0.001	0.701		
Gmax–Gmin [mmol/L]	1.157 ± 0.50	3.026 ± 0.96	150.6	< 0.001	0.579		
$iAUC_{120}$ Insulin [min x mU/L]	2045 ± 991	3864 ± 1941	52.94	< 0.001	0.608		
$iAUC_{60}$ Insulin [min x mU/L]	739 ± 369	1603 ± 890	46.2	< 0.001	0.401	*	

Figure 1


MALE SUBJECTS with IFG N=30
Verum: apple juice treated with invertase, glucose oxidase and catalase
Control: apple juice untreated
Verum: apple juice treated with invertase, glucose oxidase and catalase
Control: apple juice untreated
SCREENING
INTERVENTION 1
INTERVENTION 2
Visit 0
within 4 weeks
Visit 1
at least 6 days
Visit 2
3 days observation
Collecting questionnaires etc., checking AE etc.
• Informed consent
• Inclusion check
• Exclusion check
• Questionnaire on food frequency
• Blood withdrawal to confirm IFG
• Inclusion check
• Exclusion check
• Randomization
• Oral test load
• Capillary blood (glucose)
• Venouse blood (insulin, saftety)
• Vital signs, waist
• Questionnaires (GSRS, satiety, etc.)
• Adverse events
• Oral test load
• Capillary blood (glucose)
• Venouse blood (insulin, saftety)
• Vital signs, waist
• Questionnaires (GSRS, satiety, etc.)
• Adverse events
Subject diary and questionnaires
Monitoring of Adverse Events

Flow Diagram

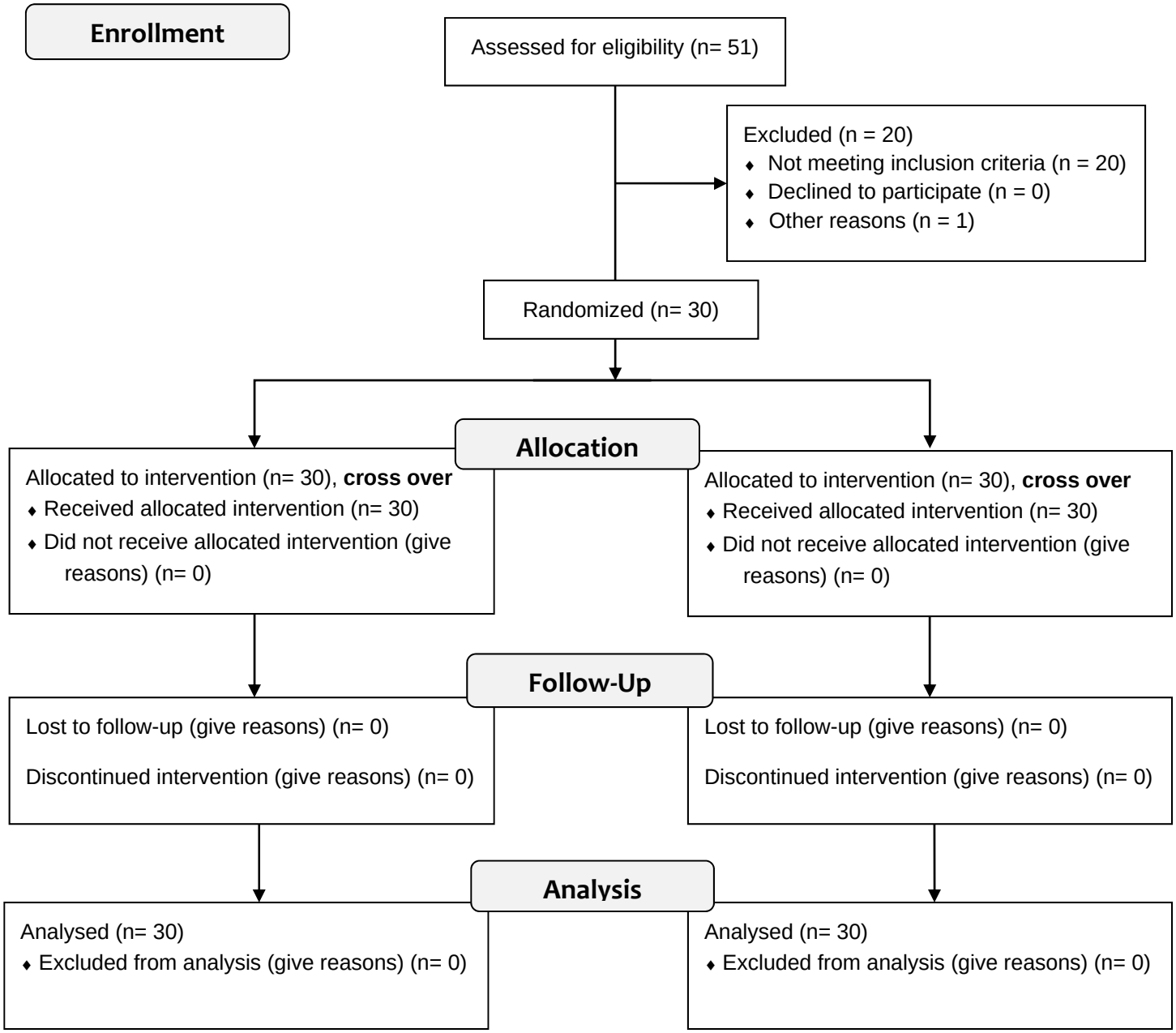
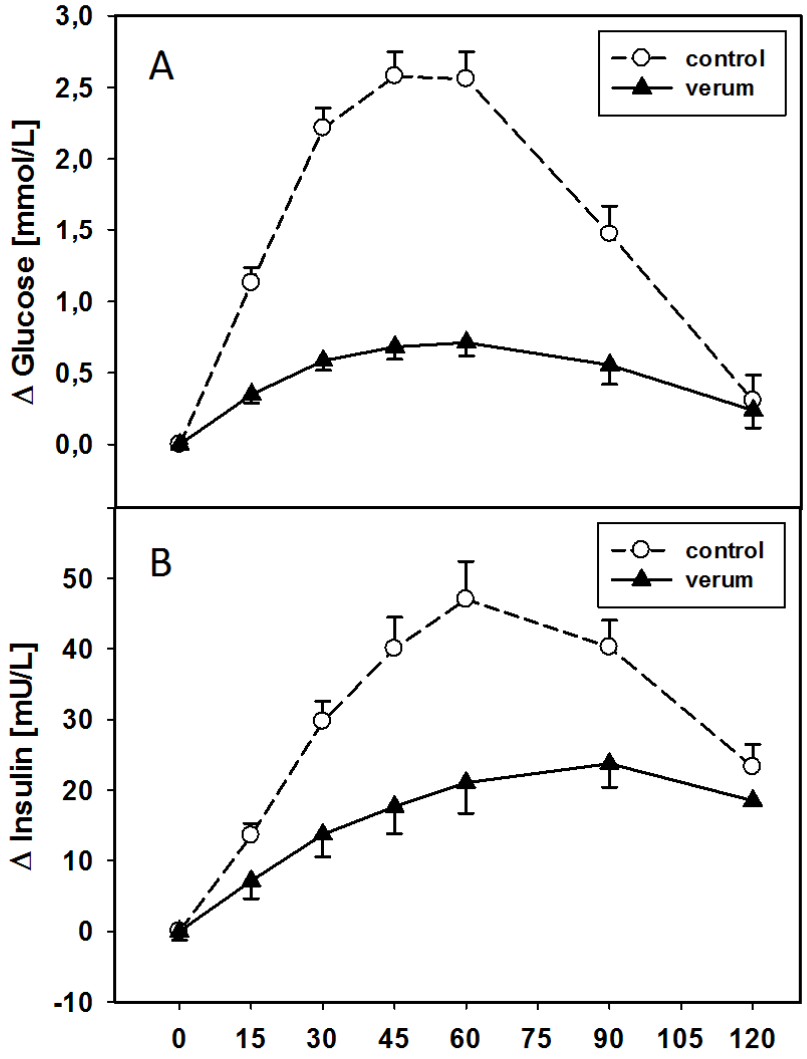


Figure 3


A
control
verum
Δ Glucose [mmol/L]
3,0
2,5
2,0
1,5
1,0
0,5
0,0
B
control
verum
Δ Insulin [mU/L]
50
40
30
20
10
0
-10
0
15
30
45
60
75
90
105
120

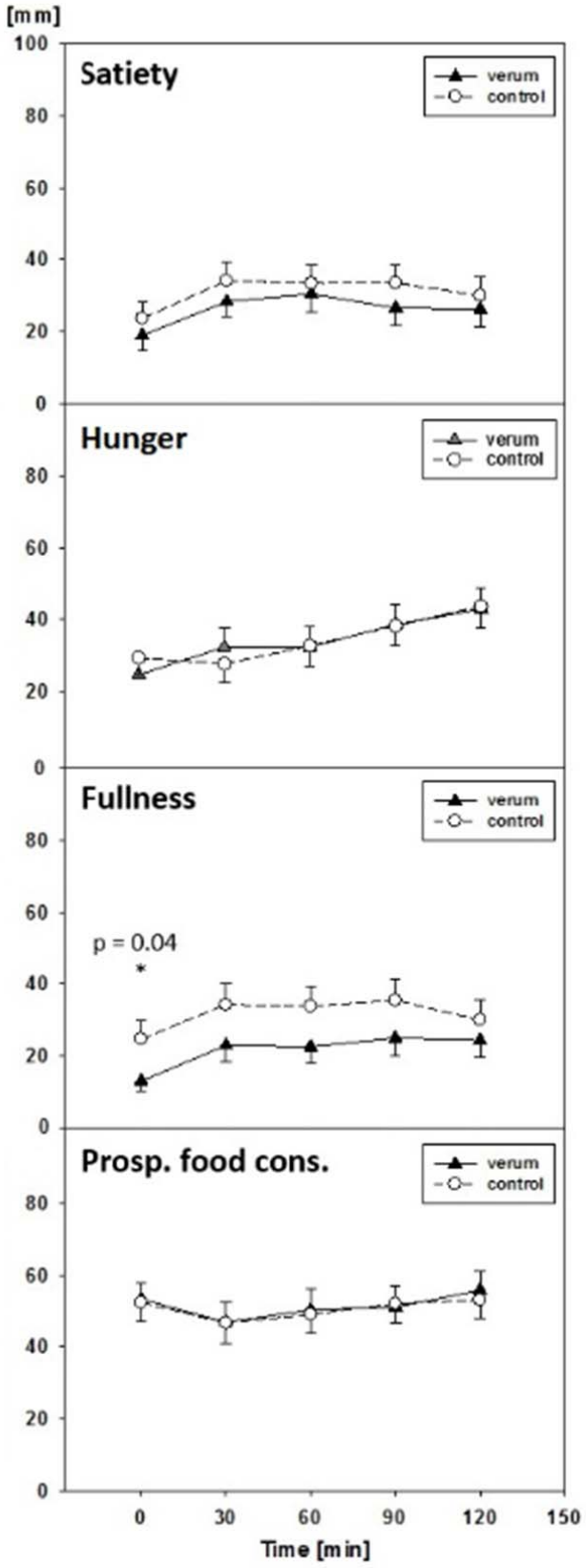
Supplementary Table 1 iAUC for Satiety, Hunger, Fullness and Prospective food consumption during intervention of 120 min (mean ± SD)

Parameter (mean ± SD)		*Verum* (N=30)	*Control* (N=30)	*F*	*V versus C* p	*Carry-Over* p	*Normality failed*	*Equal Var. failed*
iAUC	Satiety	1291 ± 1655	1371 ± 2198	0.03	0.86	0.06	*	
	Hunger	1640 ± 1810	1266 ± 1224	1.06	0.31	0.17	*	
	Fullness	1338 ± 1593	1547 ± 2528	0.25	0.62	0.11	*	
	Prosp. Food cons.	498 ± 641	761 ± 957	2.69	0.11	0.02		*

Supplementary Table 2 Gastrointestinal symptoms expressed as GSRS Scores (iAUC) 120 min after ingestion

Parameter (mean ± SD)		*Verum* (N=30)	*Control* (N=30)	*F*	*V vs C* *p*	*Carry-over* *p*	Normality failed	Equal Var. Failed
iAUC Scores	**Total**	12.07 ± 21.91	4.81± 10.70	4.13	0.052	0.37	*	
	Pain	5.67 ± 16.12	1.67 ± 5.31	1.95	0.17	0.70	*	
	Reflux	6.50 ± 19.96	5.50 ± 19.49	1.00	0.33	0.27	*	
	Indigestion	21.25 ± 39.64	11.75 ± 29.13	2.52	0.12	0.73	*	
	Constipation	1.33 ± 5.71	0.00 ± 0.00	1.67	0.21	0.21	*	
	Diarrhea	14.33 ± 30.81	3.00 ± 12.91	3.22	0.08	0.38	*	*

Supplementary Figure 1 Subjective scores for satiety, hunger, fullness, and prospective food consumption in N=30 individuals with IFG.



VAS ranged from 0 to 100 (mm), for satiety 0 = “I am completely empty” and 100 = “I cannot eat completely another bite”, for hunger 0 = “I am not hungry at all”, and 100 = “I have never been more hungry”, for fullness 0 = “not at all full” and 100 = “I am totally full”, and for prospective food consumption 0 = “I cannot eat anything at all” and 100 = “I can eat a lot”; (mean ± SEM).

Supplementary Figure 2 Gastrointestinal symptoms as assessed by GSRS-Scores during intervention (1 hour before t_0, t_{60} and t_{120}), left graph, and alteration of GSRS Δ(3 days after – 3 days before ingestion), right graph (mean ± SEM).